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Article

Property-Driven Design and Development of Lipids for Efficient Delivery of siRNA

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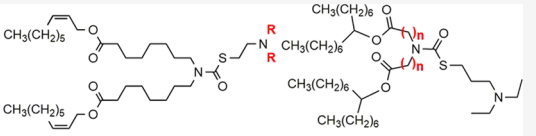
ABSTRACT: Ionizable cationic lipids are critical components involved in nanoparticle formulations, which are utilized in delivery platforms for RNA therapeutics. While general criteria regarding lipophilicity and measured pK_a in formulation are understood to have impacts on utility *in vivo*, greater granularity with respect to the impacts of the structure on calculated and measured physicochemical parameters and the subsequent performance of those ionizable cationic lipids in *in vivo* studies would be beneficial. Herein, we describe structural alterations made within a lipid class exemplified by **4**, which allow us to tune calculated and measured physicochemical parameters for improved performance, resulting in substantial improvements versus the state of the art at the outset of these studies, resulting in good *in vivo* activity within a range of measured basicity (pK_a = 6.0−6.6) and lipophilicity (cLog$D$ = 10−14).



10a R = CH_3; **10b** R = CH_2CH_3 **10q** n = 3; **10z** n = 1

Cmpd.	Calc. pKa	measured pKa	Δ-pKa	F-VII KD (%)
10a	8.68	6.03	2.65	63
10b	9.62	5.50	4.12	14
10q	10.20	6.31	3.89	97
10z	10.16	5.62	4.54	31

INTRODUCTION

The recent approval of the first ever lipid nanoparticle-encapsulated siRNA, patisiran (ONPATTRO), against transthyretin amyloidosis,[1,2] and continuing advances in RNA-based technologies such as CRISPR gene editing, mRNA therapeutics, and vaccines have generated intense interest in safe and effective nonviral vectors for drug delivery. Lipid-promoted nanoparticle formulations have shown great promise as a powerful delivery platform for small RNA (siRNA, miRNA, anti-miRNA, etc.) therapeutics[3−5] in general and mRNA medicines in particular.[6−8] While a small oligonucleotide of <30 nucleotides is amenable for and proven with alternate cell selective delivery approaches, such as through ligand-directed receptor-mediated endocytosis,[9−11] a large oligonucleotide with >1000 nucleotides in length such as an mRNA has limited delivery options. The stability of RNA outside its cellular environment is a major stumbling block in the development of RNA-based medicines. Hence, a primary goal of any delivery platform involving RNA is the protection of it from plasma nucleases while it is in circulation. An equally important consideration is the delivery of the RNA into the cytoplasm of a cell where the intended biological outcome must be realized. Both considerations can be satisfied, to some extent, if RNA can be encapsulated in a lipid nanoparticle (LNP). Currently, LNP is a clinically validated platform for the delivery of small RNAs and an emerging one for systemic delivery of mRNA therapeutics. Several LNPs with various siRNA cargoes have undergone clinical trials. Most notable ones are LNPs based on 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA)[12−14] **1**, DLin-MC3-DMA **2** (**MC3**),[5] and **3** (**L319**) (Table 1).[15] Lipids **1**−**3** have been reported to potently knock down the factor VII (FVII) expression, an siRNA paradigm; however, these lipids have not all been reported to demonstrate comparable activity in an mRNA setting. Lipid **2** (**MC3**) has been reported to encapsulate and deliver siRNA (patisiran, ONPATTRO) and has also been associated with the delivery of mRNA.[6] While the above lipids are considered to be ionizable, meaning that they are charge-neutral under plasma pH and they acquire progressively more cationic character in various endosomal compartments after the associated nanoparticles are endocytosed by low-density lipoprotein receptor (LDL-R), further improvements or chemical modifications are needed to have lipids that can be utilized for therapeutic applications of both small RNAs and mRNAs. Also, a major drawback of many lipids is their propensity for accumulation in tissues after repeated dosing due to insufficient biodegradability. Thus, apart from the safe and efficacious delivery of RNAs to the appropriate tissues, cells, and ultimately, intracellular compartments where either an RNAi may be effected, an mRNA may be introduced for translating into a therapeutic protein, or a gene may be edited,

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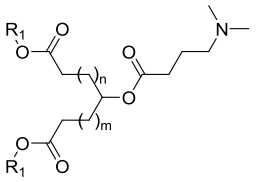
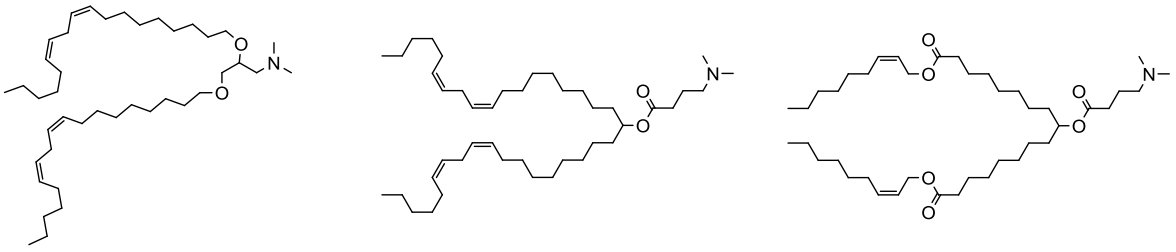
Table 1. Alnylam Lipid Acid Chain Length Study



Cmpd.	m	n	R_1O-	pKa	FVII ED_{50} (mg/kg)	FVII Knockdown (%) 0.1mg/kg [0.3mg/kg]
L354	2	2		7.0	>0.3	- -
L356	4	4		6.7	0.12	- -
L319	6	6		6.38	<0.01	90 [97]
L357	8	8		6.25	0.022	- -

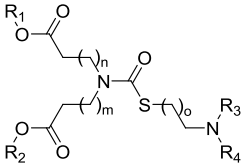


1 **2 (MC3)** **3 (L319)**

Figure 1. While many of the lipids used for RNA applications are ionizable in nature, further improvements or chemical modifications are warranted to create lipids that can be utilized for therapeutic applications of RNAs. It was our desire to initiate a systematic study of the impact of lipid structure on the calculated physicochemical properties (cLog*D* and c-pK_a) and measured (formulated) pK_a of the lipids designed for LNP-facilitated RNA therapeutic applications to design lipids that could function well in both the siRNA and mRNA therapeutic modalities. We planned to examine the impact of lipophilic chain length, number of chains and their relative orientations, and the nature of ester-forming alcohol to realize the desired/required LNP performance/biodegradability. Thus, we embarked upon lipid structure−activity relationship (SAR) studies to optimize performance in a biological system.

the terminal fate of the components of the LNP is an important consideration for successful therapeutic development. These considerations have prompted the incorporation of biodegradable chemical functionalities within the general chemical motif of traditional lipids of lipid nanoparticle formulations.[15] As shown in Figure 1, the lipids exhibit two basic structural motifs: DLin-DMA **1**[12−14] has the lipophilic moiety connectivity *via* stable ether linkages and DLin-MC3-DMA **2**[5] exhibits a basic 4-dimethylaminobutyrate head group annealed to a large lipophilic tail *via* a secondary ester linkage, while **L319** (Table 1)[15] has an identical (see **2**) basic, 4-dimethylaminobutyrate head group attached *via* a secondary ester linkage to a lipophilic 9-hydroxyheptadecanedioc acid tail, which also presents cleavable primary-ester linkages in the form of (*Z*)-2-nonenol esters. The ether linkages of **1** are not readily cleaved hydrolytically; thus, oxidative/hepatic metabolism is likely needed to degrade **1**. By comparison, the secondary ester moiety of **2** could undergo hydrolysis through various esterases, leaving a large lipophilic core to be degraded oxidatively. **L319** (Table 1) presents two sets of ester functions as esterase targets with the largest residual entity to be cleared in the form of 9-hydroxyheptadecanedioc acid.

FVII siRNA knockdown was selected as an *in vivo* primary screen to determine the appropriateness of lipid design and to serve as a proxy for mRNA delivery to hepatocytes. Lipid design notions were initiated using a central nitrogen as a lynchpin for the attachment of two lipophilic moieties and a single entity bearing the basic nitrogen of the ionizable lipid. Ester linkages were selected as the potential biodegradable entities that were to be resident on the lipophilic moieties and the basic nitrogen was to be introduced onto the lynchpin-N through a readily assembled, robust, thiocarbamate unit, as shown in **4**. The assembly of a structure such as **4** would allow diversity in acid chain length ($m = n$ or $m \neq n$), ready alteration of the ester-forming alcohol ($R_1 = R_2$ or $R_1 \neq R_2$), the introduction of linear, primary alcohols and branched, secondary alcohols during ester formation, and alteration of the basicity of the nitrogen head group by changing the distance between S and N as well as changing nitrogen substitution (R_3/R_4). Further, we anticipated that the use of a *lynchpin-N* would facilitate the syntheses of lipids **4**.



4

An early study of the impact of acid chain length on measured pK_a and FVII knockdown has been reported by Alnylam.[15a)] Table 1 presents the lipids in this study and the associated measured pK_a and FVII knockdown information. There is a progression in the decrease of measured pK_a from

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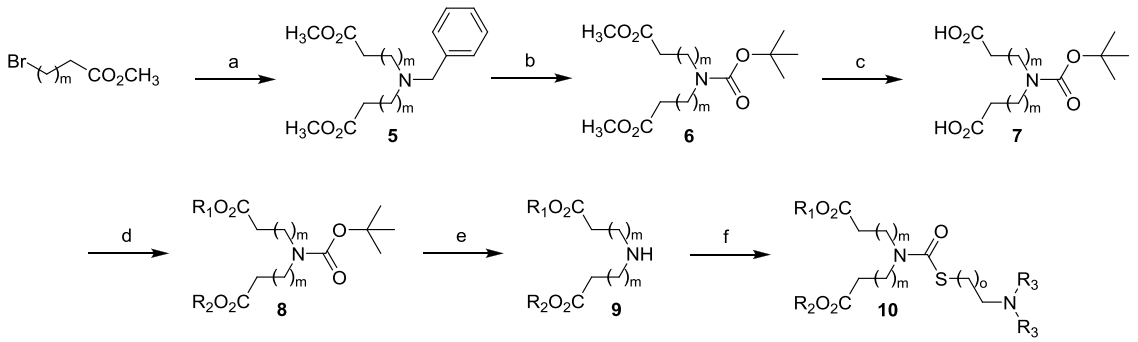

a) $PhCH_2NH_2$, K_2CO_3, CH_3CN, 80°C; b) H_2 (70psi), BOC_2O, 10% Pd/C, EtOH; c) NaOH, THF, MeOH, H_2O; d) R_1OH, EDC-HCl, DMAP, CH_2Cl_2; e) CF_3CO_2H, CH_2Cl_2, 0° to RT; f) i. triphosgene, CH_2Cl_2, pyridine; ii. amino-thiol, pyridine

Figure 2. Synthesis of symmetrically esterified lipids **10**. Conditions: (a) $PhCH_2NH_2$, K_2CO_3, CH_3CN, 80 °C; (b) H_2 (70 psi), BOC_2O, 10% Pd/C, EtOH; (c) NaOH, THF, MeOH, H_2O; (d) R_1OH, EDC-HCl, DMAP, CH_2Cl_2; (e) CF_3CO_2H, CH_2Cl_2, 0 °C to RT; (f) i. triphosgene, CH_2Cl_2, pyridine; ii. amino-thiol, pyridine.

the 7.0 reported for entry 1 where the acid chain length is 4 to pK_a = 6.26 for entry 4 with a C-10 acid chain length. The overall lipid tail length is kept roughly the same by decreasing the size of the *Z*-2-alkenol used to form the ester. The optimal pK_a with respect to the FVII ED_{50} (mg/kg) is found in the form of **L319** (Table 1) with a pK_a = 6.38 and a FVII ED_{50} < 0.01 mg/kg. These data have been reported[15b] to correspond to a 90% knockdown at 0.1 mg/kg and a 97% knockdown at 0.3 mg/kg. **L319** was found to be eliminated from plasma, falling to below the level of quantification (0.8 pmol/mL), at 8 h post dose.

For our planned study, we intended to examine the impact of acid chain length, starting from that exhibited by **L319**: octanoate ($m = n = 6$) decreasing systematically to butanoate ($m = n = 2$) and then to acetate ($m = n = 0$). The data of Table 1 (*viz* **L354** and **L356**) suggest some issues associated with chain shortening; however, the small data set argues for a more thorough examination. Toward that end, diversity in esterification was envisioned to utilize linear alcohols ((*Z*)-2-nonenol) as well as symmetrically branched alcohols (5-nonanol, 6-undecanol, 7-tridecanol, 8-pentadecanol, and 9-heptadecanol) for ester formation with $R_1 = R_2$ or $R_1 \neq R_2$. Nitrogen would be altered with respect to calculated basicity by restricting o = 1, 2 and $R_3 = R_4 = CH_3$, CH_2CH_3. Syntheses of the targeted entities were expected to be facilitated by the symmetrical chain lengths of the acid moieties with $R_1 = R_2$ for the esters being introduced *via* esterification of a diacid post-nitrogen alkylation or through nitrogen alkylation with an omega-bromo-ester. Differentially esterified entities ($R_1 \neq R_2$) could be pursued either through sequential esterification of a diacid with the selected alcohols or through nitrogen alkylation with differentially esterified omega-bromo-esters. We embarked upon this study with a poorly developed understanding of the interplay between lipid lipophilicity as measured by c-pK_a and desired measured pK_a; however, the measured pK_a range of 6.20−6.50 had been described as optimal.[5] Herein, we report the development of an SAR understanding that led to the evolution of more optimized lipid designs and a platform approach, which was called LUNAR, for encapsulation and delivery of small RNAs, with plans for future utilization with large RNAs such as mRNAs.

RESULTS AND DISCUSSION

The data of Table 1, for **L319**, prompted us to initiate our study utilizing the symmetrical octanoate chain length of **L319** (Table 1, $m = n = 6$) and a symmetrical linear diester ($R_1 = R_2$) where the alcohol fragment was the same (*Z*)-2-nonenol employed in **3**. The first construct plan was completed by selecting an S-to-N chain length of two carbons (o = 1) and a symmetrical $R_3 = R_4 = CH_3$. This will be followed by altering $R_3 = R_4$ to CH_2CH_3, and then S-to-N chain length could be altered to three carbons (o = 2) with the symmetrical $R_3 = R_4 = CH_3$ and $R_3 = R_4 = CH_2CH_3$. All subsequent lipid syntheses could be conducted as outlined in Figure 2.

In the event, commercially available methyl-8-bromo-octanoate and benzyl amine were combined to afford benzylic amine **5a**, which was utilized crude. Hydrogenolysis (10% Pd/C, 70 psi H_2) in the presence of BOC-anhydride led to protected diester **6a** in 81% yield after purification. Bis-ester hydrolysis (NaOH, THF, and aq. MeOH) provided crude di-acid **7a**, which underwent bis-esterification with (*Z*)-2-nonenol (EDC-HCl and DMAP) to give BOC-blocked diester **8a** in 72% yield. The synthesis was completed by BOC-removal (TFA and CH_2Cl_2) yielding crude amino-diester **9a** which was converted to an intermediate carbamyl chloride with triphosgene (CH_2Cl_2, pyridine), and reaction of the carbamyl chloride with commercially available 2-(dimethylamino)-ethanethiol-HCl in pyridine to yield target lipid **10a** in 79% yield over the last 2 steps.

Additional symmetrical octanoate lipids of Table 2 were prepared from **9a** by replacing 2-(dimethylamino)-ethanethiol-

Table 2. Data for Symmetrically Esterified Lipids 10 and Intermediates 5−9

compound	m	$R_1OH = R_2OH$	$(CH_2)_o$	R_3	yield (%)
5a	6				crude
6a	6				81
7a	6	H			crude
8a	6	(*Z*)-2-nonenol			72
9a	6	(*Z*)-2-nonenol			crude
10a	6	(*Z*)-2-nonenol	1	CH_3	79
10b	6	(*Z*)-2-nonenol	1	CH_2CH_3	71
10c	6	(*Z*)-2-nonenol	2	CH_3	74
10d	6	(*Z*)-2-nonenol	2	CH_2CH_3	78

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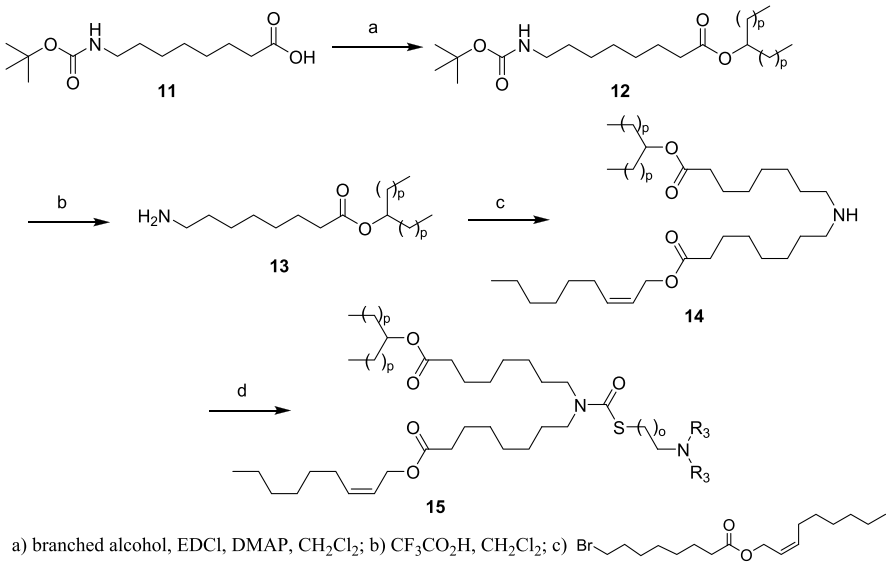

Figure 3. Synthesis of nonsymmetrically esterified octanoate-derived lipids **15**.

HCl with 2-(diethylamino)-ethanethiol-HCl to provide **10b** (71%), 3-(dimethylamino)-propanethiol-HCl[16] to yield **10c** (74%), and 3-(diethylamino)-propanethiol-HCl[17] to give **10d**.

With the octanoate-derived bis-esters, utilizing (*Z*)-2-nonenol as the alcohol, we turned our attention to the preparation of octanoate-derived lipids containing one linear alcohol-derived ester ((*Z*)-2-nonenol) and one branched chain alcohol-derived ester. As outlined above (*vide supra*), we planned to incorporate the symmetrically branched chain alcohols, 5-nonanol, 6-undecanol, 7-tridecanol, 8-pentadecanol, and 9-heptadecanol, for this effort. Two pathways of synthesis were considered: The first was the construction of differentially esterified versions of 8-bromo-octanoic acid and 8-BOC-amnio-octanoic acid and the alkylative addition of the bromoester to an 8-amino-octanoate ester. The second approach called for a selective mono-esterification of a symmetrical diacid[18] such as **8** ($m = n = 6$; $R_1 = R_2 = H$) followed by esterification with alcohol #2. The former of these approaches was investigated first as shown in Figure 3.

Commercially available BOC-blocked amino acid **11** was esterified with 5-nonanol to provide ester **12a** ($p = 3$, Table 3), which was utilized without purification. BOC removal (TFA and CH_2Cl_2) afforded amino ester **13a** ($p = 3$, 48% over two steps), which was then coupled with 8-bromo-octanoic acid *Z*-2-nonenylester,[19] leading to amine **14a** ($p = 3$) in a modest 28% yield. The target lipid was realized after **14a** was converted to an intermediate carbamyl chloride with triphosgene (CH_2Cl_2 and pyridine) and carbamyl chloride was reacted with commercially available 2-(dimethylamino)-ethanethiol-HCl in pyridine to yield target lipid **15a** in 46% yield. Replacing 5-nonanol with 6-undecanol in the esterification of **11** leads to **12b**, deprotection yields **13b** (56%), alkylation with 8-bromo-octanoic acid *Z*-2-nonenylester affords **14b** (22%), and conversion to the target thiocarbamate gives target lipid **15b** (70%, 8.6% overall). Similarly, the use of 7-tridecanol, 8-pentadecanol, and 9-heptadecanol leads to lipids **15c** (6.7% overall), **15d** (12% overall), and **15e** (6.7% overall). A consistent issue associated with the chemistry of Table 3 is the poor yield of the alkylative step, leading to **14a**–**14e**. Future endeavors will examine the selective mono-esterification of a diacid as a paradigm to avoid the poor alkylation yields.

Table 3. Synthesis of Nonsymmetrically Esterified Octanoate-Derived Lipids 15

compound	$(CH_2)_p$	$(CH_2)_o$	R_3	yield (%)
12a	3			crude
13a	3			49
14a	3			28
15a	3	1	CH_3	69
12b	4			crude
13b	4			56
14b	4			22
15b	4	1	CH_3	70
12c	5			crude
13c	5			51
14c	5			18
15c	5	1	CH_3	73
12d	6			crude
13d	6			49
14d	6			34
15d	6	1	CH_3	72
12e	7			crude
13e	7			51
14e	7			17
15e	7	1	CH_3	77

With the lipids of Table 2 (**10a**–**10d**) and Table 3 (**15a**–**15e**) available, we wished to examine the impact of the structural changes represented in the lipids on the calculated and measured physicochemical properties, the ability to formulate into a nanoparticle-encapsulating RNA, nanoparticle size, and the *in vivo* activity in the FVII knockdown assay.

The data of Table 4 include cLog*D*[20] and calculated pK_a,[20] as well as measured pK_a (in formulation), a Δ pK_a value that indicates the difference between calculated and measured pK_a values, measured nanoparticle size, and *in vivo* activity in the FVII knockdown assay. Calculated Log*D* values (cLog*D*) range from a low value of 11.01 for **10c** (calculated pK_a = 9.35) to a high value of 16.08, associated with **15e**, which exhibits the

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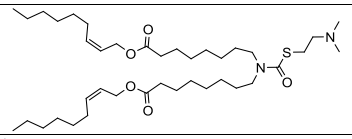
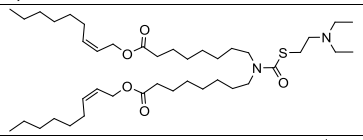
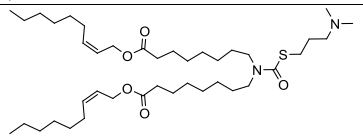
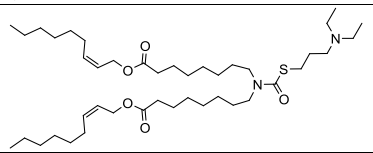
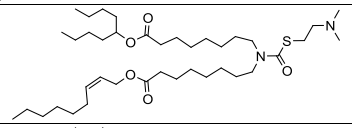
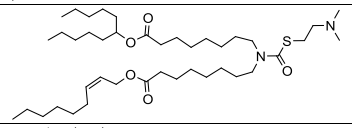
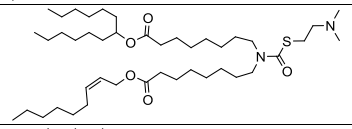
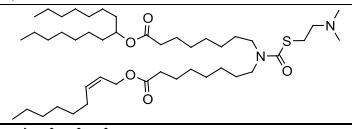
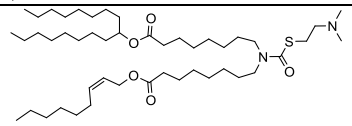
Table 4. Impact of Structure on Physicochemical Properties and *In Vivo* Activity in the FVII Knockdown Assay

#	Structure	cLogD[20]	c-pKa[20] (pKa)	Δ pKa	Particle Size (nm)	FVII Knockdown (%) 0.03mg/kg
10a		11.32	8.68 (6.03)	2.65	79	63
10b		11.49	9.62 (5.50)	4.12	77	14
10c		11.01	9.35 (6.77)	2.58	81	70
10d		11.34	10.20 (6.60)	3.60	84	57
15a		11.37	8.68 (5.81)	2.87	76	71
15b		12.38	8.68 (5.81)	2.87	73	68
15c		13.95	8.68 (5.79)	2.89	79	70
15d		15.02	8.68 (5.89)	2.79	79	71
15e		16.08	8.68 (5.59)	3.09	80	40

large branched heptadecanol moiety and a modestly basic (c-pK_a = 8.68) amine. All of the lipids of Table 4 formulated well-affording nanoparticle sizes ranging from 73 nm (**15b**) to 84 nm (**10d**), enabling us to measure basicity (pK_a) in formulation using the TNS assay and to test them *in vivo* in the FVII knockdown assay. The aforementioned paradigms to alter basicity (*vide supra*), increasing the distance between the heteroatoms of the head group and altering the nature of the nitrogen substituents, are apparent in the lipids of Table 4. Lipid **10a**, with a 2-C spacer between S and N, has a calculated pK_a (c-pK_a) of 8.68, while the 3-C spacer of lipid **10c** has a c-pK_a = 9.35. The measured pK_a values for lipids **10a** and **10c**, 6.03 and 6.77, respectively, then lead to a Δ pK_a value, the difference between the calculated and measured pK_a values of a consistent 2.65/2.58. These Δ values suggest that lipids afford an expected increase in basicity with the structural change, with the basic nitrogen in a similar environment. The anticipated change in basicity wrought by changing the substitution on nitrogen from methyl to ethyl results in a very different outcome/analysis. Lipid **10b** (2-C spacer, *N*-ethyl) has a c-pK_a = 9.62, a measured pK_a = 5.50 and a Δ pK_a = 4.12. Lipid **10d** (3-C spacer, *N*-ethyl) exhibits a c-pK_a = 10.20, a measured pK_a = 6.60, and a Δ pK_a = 3.60. These two lipids exhibit more than a 1 pK_a unit greater deviation between calculated and measured pK_a values when compared to lipids **10a** and **10c**, suggesting that the change from *N*-methyl to *N*-ethyl substitution, which increases lipophilicity at the basic terminus as well as impacting basicity with this substitution, has resulted in an environmental alteration, leading to a lower measured pK_a than might have been anticipated. The ability to manipulate the measured pK_a in this fashion allows us some latitude in design to alter the ultimate measured basicity in formulation to achieve a desired target value. The remaining lipids of Table 4 (**15a**−**15e**) restrict the S-to-N spacer to two atoms and the substitution of nitrogen to *N*-methyl while introducing a single branched chain alcohol to form an ester to

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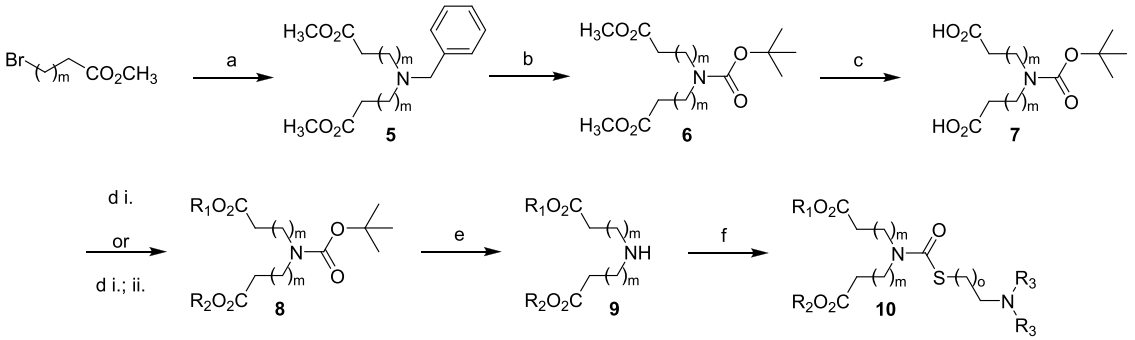

a) $PhCH_2NH_2$, K_2CO_3, CH_3CN, 80°C; b) H_2 (70psi), BOC_2O, 10% Pd/C, EtOH; c) NaOH, THF, MeOH, H_2O; d) i. R_1OH, EDC-HCl, *i*-Pr_2NEt, DMAP, CH_2Cl_2; ii. R_2OH, EDC-HCl, *i*-Pr_2NEt, DMAP, CH_2Cl_2; e) CF_3CO_2H, CH_2Cl_2; f) i. triphosgene, CH_2Cl_2, pyridine; ii. amino-thiol, pyridine

Figure 4. Syntheses of butanoate- and acetate-based lipids **10**. Conditions: (a) $PhCH_2NH_2$, K_2CO_3, CH_3CN, 80 °C; (b) H_2 (70 psi), BOC_2O, 10% Pd/C, EtOH; (c) NaOH, THF, MeOH, H_2O; (d) i. R_1OH, EDC-HCl, *i*-Pr_2NEt, DMAP, CH_2Cl_2; ii. R_2OH, EDC-HCl, *i*-Pr_2NEt, DMAP, CH_2Cl_2; (e) CF_3CO_2H, CH_2Cl_2; (f) i. triphosgene, CH_2Cl_2, pyridine; ii. amino-thiol, pyridine.

determine if there is an impact of increasing branched alcohol size on measured parameters. Given the general target measured pK_a in formulation of ca. 6.0 and the observed impact of measured pK_a on FVII knockdown, the data of Table 4 provide little granularity with respect to the impact of structural modification upon pK_a and FVII knockdown until one adds *N*-ethyl substituents to the 2-C S-to-N spacer of **10b** (measured pK_a = 5.50; FVII knockdown, 14%) and a large branched substituent (9-heptadecanol) to the C-8 acid in the form of **15e** (measured pK_a = 5.59; FVII knockdown, 40%). The remaining lipids of Table 4 exhibit measured pK_a values ranging from 6.77 (**10c**) to 5.79 (**15c**) with FVII knockdown observed in the 60−80% range.

As we move forward to the symmetrical butanoate versions of **10** ($m = n = 2$; $R_1 = R_2$) and acetate versions of **10** ($m = n = 0$; $R_1 = R_2$), as well as the nonsymmetrical butanoate **10** ($m = n = 2$; $R_1 \neq R_2$) and acetate versions of **10** ($m = n = 0$; $R_1 \neq R_2$), we will restrict our designs to entities wherein the ester moieties include R_1 = linear ((*Z*)-2-nonenol) and R_2 = branched (increasing in size from 7-tridecanol, 8-pentadecanol to 9-heptadecanol) and the situation wherein $R_1 = R_2$ = branched (increasing in size from 6-undecanol, 7-tridecanol, 8-pentadecanol to 9-heptadecanol). These choices were derived from the data of Table 1, wherein little physicochemical impact was observed for bis-linear esters regardless of acid chain length, and Table 4 where, in the bis-octanoate chain length, physicochemical impact was observed in the guise of **15e**, with a single large branched ester moiety held at the octanoate chain length. Based on the data of Table 4, it is likely that we will observe a large environmental impact on the measured pK_a at $m = n = 2$ as the size of the branch increases and an even greater impact in the shorter acetate series ($m = n = 0$).

While the syntheses of the symmetrically esterified butanoate and acetate congeners could be carried out as shown in Figure 2, the poor yields obtained for the syntheses of the differentially esterified lipids exemplified in Figure 2 and Table 3 caused us to adopt a modified synthetic protocol. Utilizing the same di-acid that would afford access to a symmetrically esterified target lipid, we envisioned a selective mono-esterification[18] followed by the addition of the second, different alcohol as a better route to the desired target nonsymmetrically esterified lipids. The syntheses of the aforementioned butanoate and acetate congeners are outlined in Figure 4. Methyl-4-bromo-butyrate was reacted with benzyl amine (K_2CO_3 and CH_3CN, 80 °C) to give **5b** (73%, Table 5). Hydrogenolysis (H_2 and Pd/C) in the presence of BOC-anhydride led to di-ester **6b** (95%), which then afforded the target butanoate precursor diacid **7b** (aq. NaOH, MeOH, and THF, 89%). The precursor to all butanoate-derived, non-symmetric diesters **8b** is then prepared by mono-esterification with (*Z*)-2-nonenol (EDC-HCl, *i*-Pr_2NEt, and DMAP, 53%). A second esterification with 7-tridecanol (EDC-HCl, *i*-Pr_2NEt, and DMAP) then converts **8b** to **8c** (71%), which yields amine **9b** (78%) after BOC removal (TFA and CH_2Cl_2). The initial nonsymmetrically esterified butanoate-based lipid **10e** is then prepared by formation of the carbamyl chloride with triphosgene followed by its reaction with 2-(dimethylamino)-ethanethiol-HCl in pyridine to provide **10e** in 69% yield. The remaining nonsymmetrically esterified, butanoate-based lipids of Table 5 (**10f−10i**) are prepared in a similar fashion. The synthesis of the first symmetrically esterified, bis-butanoate lipid **10j** proceeds in a similar fashion with the reaction of **7b** with 6-undecanol (EDC-HCl, *i*-Pr_2NEt, and DMAP), giving **8f** (48%). BOC removal (TFA and CH_2Cl_2) provides **9e** (67%), and the reaction of **9e** first with triphosgene and then with 2-(dimethylamino)-ethanethiol-HCl in pyridine yields **10j** (71%). The remaining bis-butanoate-based symmetrically esterified lipids (**10k**, **10m**, **10n**, **10o**, **10p**, and **10q**) are synthesized in a similar fashion. The syntheses of the nonsymmetrically esterified, bis-acetate-based lipids (**10r−10v**), and the symmetrically esterified bi-acetate-derived lipids (**10w−10z** and **10aa**) parallels the bis-butanoate methods discussed above, and these lipids were derived from commercially available 2-[*tert*-butoxycarbonyl-(carboxymethyl)amino]acetic acid.

Within the bis-butanoic acid manifold of lipids, our initial study would again (*vide supra*) examine the impact of the size of a branched chain alcohol utilized for esterification in a disymmetrically esterified molecule where (*Z*)-2-nonenol was introduced as the linear component (Table 6). The five disymmetric lipids of Table 6, **10e−10i**, have had 7-tridecanol, 8-pentadecnol, 8-pentadecanol, 9-heptadecanol, and 9-heptadecanol added as their branched components, respectively. The result is that **10e−10i** span a range of lipophilicities from a level far below that seen in Table 4 (**10e** cLog*D* = 9.89 through **10h** and **10i** cLog*D* = 11.93). The impact of lower lipophilicity is first seen upon formulation where **10e** (cLog*D* = 9.89) affords a larger nanoparticle size (93 nM) when compared to

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Table 5. Synthesis of Butanoate- and Acetate-Based Lipids

compound	*m*	R_1OH	R_2OH	$(CH_2)_o$	R_3	yield (%)
5b	2					73
6b	2					95
7b	2					89
8b	2	(*Z*)-2-nonenol	H			53
8c	2	(*Z*)-2-nonenol	7-tridecanol			71
9b	2	(*Z*)-2-nonenol	7-tridecanol			78
10e	2	(*Z*)-2-nonenol	7-tridecanol	1	CH_3	69
8d	2	(*Z*)-2-nonenol	8-pentadecanol			68
9c	2	(*Z*)-2-nonenol	8-pentadecanol			79
10f	2	(*Z*)-2-nonenol	8-pentadecanol	1	CH_3	74
10g	2	(*Z*)-2-nonenol	8-pentadecanol	2	CH_2CH_3	71
8e	2	(*Z*)-2-nonenol	9-heptadecanol			67
9d	2	(*Z*)-2-nonenol	9-heptadecanol			79
10h	2	(*Z*)-2-nonenol	9-heptadecanol	1	CH_3	72
10i	2	(*Z*)-2-nonenol	9-heptadecanol	2	CH_2CH_3	67
8f	2	6-undecanol	6-undecanol			48
9e	2	6-undecanol	6-undecanol			67
10j	2	6-undecanol	6-undecanol	1	CH_3	71
10k	2	6-undecanol	6-undecanol	2	CH_3	70
10m	2	6-undecanol	6-undecanol	2	CH_2CH_3	69
8g	2	7-tridecanol	7-tridecanol			68
9f	2	7-tridecanol	7-tridecanol			47
10n	2	7-tridecanol	7-tridecanol	2	CH_3	66
8h	2	8-pentadecanol	8-pentadecanol			60
9g	2	8-pentadecanol	8-pentadecanol			76
10o	2	8-pentadecanol	8-pentadecanol	1	CH_3	75
10p	2	8-pentadecanol	8-pentadecanol	2	CH_3	77
10q	2	8-pentadecanol	8-pentadecanol	2	CH_2CH_3	73
8i	0	(*Z*)-2-nonenol	H			29
8j	0	(*Z*)-2-nonenol	6-undecanol			57
9h	0	(*Z*)-2-nonenol	6-undecanol			82
10r	0	(*Z*)-2-nonenol	6-undecanol	1	CH_3	58
8k	0	(*Z*)-2-nonenol	8-pentadecanol			55
9i	0	(*Z*)-2-nonenol	8-pentadecanol			74
10s	0	(*Z*)-2-nonenol	8-pentadecanol	2	CH_3	63
8m	0	(*Z*)-2-nonenol	9-heptadecanol			56
9j	0	(*Z*)-2-nonenol	9-heptadecanol			84
10t	0	(*Z*)-2-nonenol	9-heptadecanol	1	CH_3	55
10u	0	(*Z*)-2-nonenol	9-heptadecanol	2	CH_3	61
10v	0	(*Z*)-2-nonenol	9-heptadecanol	2	CH_2CH_3	59
8n	0	6-undecanol	6-undecanol			54
9k	0	6-undecanol	6-undecanol			75
10w	0	6-undecanol	6-undecanol	2	CH_3	66
10x	0	6-undecanol	6-undecanol	2	CH_2CH_3	57
8o	0	8-pentadecanol	8-pentadecanol			58
9m	0	8-pentadecanol	8-pentadecanol			66
10y	0	8-pentadecanol	8-pentadecanol	2	CH_3	68
10z	0	8-pentadecanol	8-pentadecanol	2	CH_2CH_3	60
8p	0	9-heptadecanol	9-heptadecanol			60
9n	0	9-heptadecanol	9-heptadecanol			76
10aa	0	9-heptadecanol	9-heptadecanol	1	CH_3	64

those associated with **10f**–**10i** (70–78 nM). Lipids **10e**, **10f**, and **10h** have the 2-C spacing between S and N as well as *N*-methyl substitution. As was the case with the lipids of Table 4, this combination, with c-pK_a = 8.68, led to measured pK_a values in the TNS assay of **10e** pK_a = 6.05, **10f** pK_a = 6.04, and **10h** pK_a = 5.96, with Δ pK_a values for these three lipids of 2.63, 2.64, and 2.72, respectively. The introduction of the 3-C spacing between S and N along with *N*-ethyl substituents in the guise of lipids **10g** and **10i** results in a similar increase in calculated pK_a to 10.20, as seen in Table 4, and measured pK_a values of 6.66 for **10g** and 6.58 for **10i**. The environmental impact of this added lipophilicity at the basic terminus of the lipid in the shorter butanoate-derived lipids mirrors that reported in Table 4, resulting in Δ pK_a values of 3.54 and 3.62 for **10g** and **10i**, respectively. In this small series of nonsymmetrically esterified lipids, an increase in the size of

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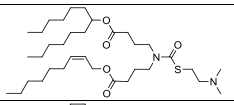
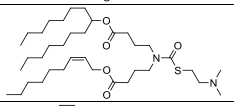
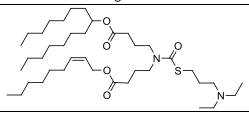
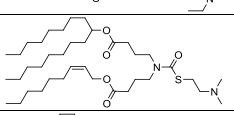
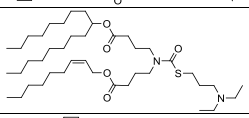
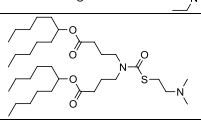
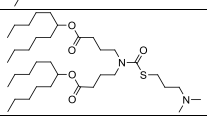
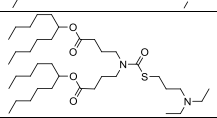
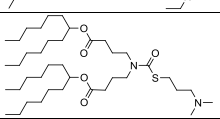
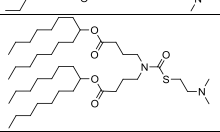
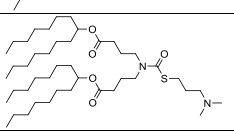
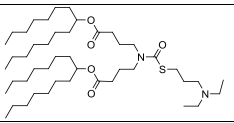
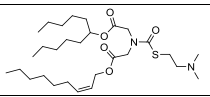
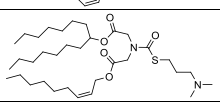
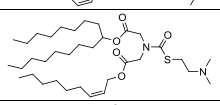
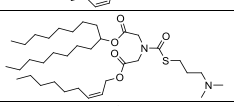
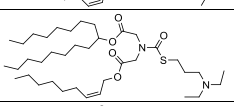
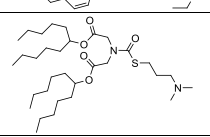
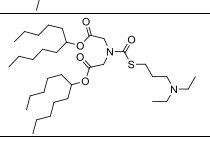
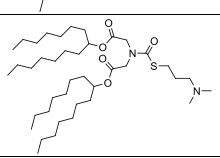
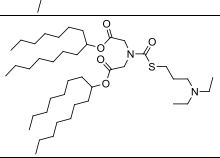
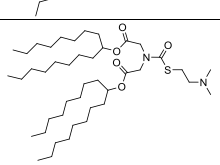
Table 6. Further Study of the Impact of Structure on Physicochemical Properties and *In Vivo* Activity in the FVII Knockdown Assay

#	Structure	cLogD[20]	c-pKa[20] (pKa)	Δ pKa	Particle Size (nm)	FVII Knockdown (%) 0.03mg/kg
10e		9.89	8.68 (6.05)	2.63	93	13
10f		10.91	8.68 (6.04)	2.64	70	82
10g		10.92	10.20 (6.66)	3.54	77	96
10h		11.93	8.68 (5.96)	2.72	78	80
10i		11.93	10.20 (6.58)	3.62	76	75
10j		9.94	8.68 (5.71)	2.97	89	5
10k		9.63	9.34 (6.80)	2.54	94	51
10m		9.95	10.20 (6.56)	3.64	86	61
10n		11.67	9.34 (6.56)	2.78	72	98
10o		14.01	8.68 (5.61)	3.07	64	70
10p		13.70	9.34 (6.38)	2.96	72	99

#	Structure	cLogD[20]	c-pKa[20] (pKa)	Δ pKa	Particle Size (nm)	FVII Knockdown (%) 0.03mg/kg
10q		14.03	10.20 (6.31)	3.89	82	97
10r		8.29	8.56 (5.39)	3.17	108	0
10s		9.98	9.30 (6.44)	2.86	72	62
10t		11.34	8.56 (5.13)	3.43	72	0
10u		11.00	9.30 (6.38)	2.92	73	97
10v		11.30	10.16 (6.20)	3.96	66	82
10w		8.96	9.30 (6.17)	3.13	92	0
10x		9.26	10.16 (6.21)	3.95	98	0
10y		13.04	9.30 (5.68)	3.92	62	31
10z		13.34	10.16 (5.62)	4.54	70	31
10aa		15.42	8.56 (4.65)	3.91	92	NT

the single branched component did not result in a significant impact on the measured pK_a. All lipids in the **10e−10i** subseries are sufficiently basic so as to expect a sizeable *in vivo* effect in the FVII knockdown assay. Lipids **10f−10i** afford FVII knockdown values of 82, 96, 80, and 75%, respectively; however, the less lipophilic lipid **10e** (cLog*D* = 9.89, pK_a = 6.05) performs poorly in the FVII knockdown assay (13%), suggesting the need for cLog*D* >10.00 for good *in vivo* activity in addition to the pK_a requirement (ca. 6.00 or greater).

The symmetrically bis-branched alcohol-based butanoate lipids **10j** through **10q** utilize 6-undecanol (**10j**, **10k**, and **10m**), 7-tridecanol (**10n**), and 8-pentadecanol (**10o**, **10p**, and **10q**) in the bis-esterification step. The impact of the placement of 2-branched chain ester in the butanoate lipid framework can be seen by a comparison of the 2-C spacing between S and N, with *N*-methyl mono-branch exhibiting lipids **10e** (pK_a = 6.05, Δ pK_a = 2.63), **10f** (pK_a = 6.04, Δ pK_a = 2.64), and **10h** (pK_a = 5.96, Δ pK_a = 2.72) with the related bis-branched lipids **10j** (pK_a = 5.71, Δ pK_a = 2.97) and **10o** (pK_a = 5.61, Δ pK_a = 3.07). While there was little impact on the measured pK_a and Δ pK_a with increasing branched moiety size in the **10e**/**10f**/**10h** series, moving to a bis-branched lipid paradigm results in a lower measured pK_a for **10j** by ca. 0.3 pK_a units and 0.4 pK_a units for **10o**. Within a series of bis-branched lipids, one can alter the measured pK_a by increasing the 2-C S-to-N spacing to three carbons and, as has been seen previously, lipophilicity can be increased and some of the increased basicity can be attenuated by altering from *N*-methyl to *N*-ethyl in the 3-C

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spacing moiety. Consider the series **10j** (6-undecanol, cLog*D* = 9.94, pK_a = 5.71), **10k** (6-undecanol, cLog*D* = 9.63, pK_a = 6.80), **10m** (6-undecanol, cLog*D* = 9.95, pK_a = 6.56) and the series **10o** (8-pentadecanol, cLog*D* = 14.01, pK_a = 5.61), **10p** (8-pentadecanol, cLog*D* = 13.70, pK_a = 6.38), and **10q** (8-pentadecanol, cLog*D* = 14.03, pK_a = 5.31). Lipophilicity, as measured by cLog*D*, can be altered toward desired ranges and pK_a can be altered from the <6.00 values of **10j** and **10o** to >6.00. These trends afford us latitude in design to achieve desired levels of lipophilicity and basicity for the applications of interest. The lipophilicity impact on formulatability is again seen in **10k**, where a cLog*D* value of 9.63 is associated with larger nanoparticle size of 94 nM. The lipids **10j**, **10m**, **10n**, **10o**, **10p**, and **10q** with cLog*D* values ranging from 9.94 to 14.03 formulate well and afford nanoparticles of 64−89 nM in size. The combination of lipophilicity and measured pK_a has an obvious effect on FVII knockdown in this short series of lipids. Lipid **10j** (cLog*D* = 9.94, pK_a = 5.71) has a poor FVII knockdown of 5%, while lipid **10k** (cLog*D* = 9.63, pK_a = 6.80), which has a lower c-pK_a but higher pK_a, is associated with an improved FVII knockdown of 51%. Moving toward greater lipophilicity and pK_a > 6.00 (**10n** cLog*D* = 11.67, pK_a = 6.56; **10p** cLog*D* = 13.70, pK_a = 6.38; **10q** cLog*D* = 14.03, pK_a = 6.31) leads to improved *in vivo* performance with FVII knockdown values of 98, 99, and 97% for these three lipids.

The bis-acetate-based lipids **10r−10aa** (Table 6) present an interesting arena to examine the impact of lipophilicity/basicity/branched alcohol size upon physicochemical calculated and measured parameters and *in vivo* performance. The mono-branched ester containing lipid **10r** (6-undecanol) has a low cLog*D* (8.29) and a low pK_a in formulation (5.39) likely due to the environmental impact of the branch in such close proximity to the basic-N (Δ pK_a = 3.17). The combination of low lipophilicity and low pK_a is also associated with formulatability issues, which are seen in an increased nanoparticle size (108 nM). The 9-heptadecanol containing mono-branched lipid **10t** is in better lipophilicity space (cLog*D* = 11.34); however, the larger branched chain ester results in further erosion of measured pK_a (5.13, Δ pK_a = 3.43) but a more normal nanoparticle size (72 nM) due to the increase in lipophilicity compared to **10r**. Neither of these lipids performs well *in vivo* (0% FVII knockdown) as **10r** is insufficiently lipophilic as well as too weakly basic, while **10t** exhibits low basicity in formulation. As shown above in Table 6 and also in Table 4, basicity in formulation can be altered by increasing the S-to-N spacing to three carbons, leading to **10s** (8-heptadecanol, cLog*D* = 9.98, pK_a = 6.44, Δ pK_a = 2.86), **10u** (9-heptadecanol, cLog*D* = 11.00, pK_a = 6.38, Δ pK_a = 2.92), and **10v** (cLog*D* = 11.30, pK_a = 6.20, Δ pK_a = 3.96). Lipids **10s**, **10u**, and **10v** formulate well (nanoparticle size, 66−72 nM) are sufficiently basic, and their use *in vivo* results in moderate (**10s**, 62%) to potent (**10u**, 97%; **10v**, 82%) FVII knockdown. The lower lipophilicity of **10s** is likely the cause of the modest *in vivo* activity.

The introduction of the second branched chain ester onto the bis-acetate core was expected to place us in poor calculated and measured physicochemical space given the data derived from **10r−10v**, and these expectations were met as shown toward the end of Table 6. Lipids **10w−10z** were constructed with the 3-C S-to-N spacing to increase c-pK_a, alternating between the *N*-methyl and *N*-ethyl motifs. Lipids **10w** and **10x** have bis-6-undecanoate esters on the bis-acetate core; they are less lipophilic than might be desired (**10w** cLog*D* = 8.96, **10x** cLog*D* = 9.26) but are sufficiently basic (**10w** pK_a = 6.17, Δ pK_a = 3.13; **10x** pK_a = 6.21, Δ pK_a = 3.95). The low lipophilicity associated with **10w** and **10x** leads to a 0% FVII knockdown despite good measured basicity. As increasing the size of the branched chain moiety has been shown to be associated with an environmental impact and lessened measured basicity (**15e** and **10o**), achieving appropriate levels of lipophilicity in this series by increasing the size of the branched moieties should push us toward decreasing basicity. This is exactly what is observed in lipids **10y**, **10z**, and **10aa**. The introduction of two 8-heptadecanol fragments into **10v** (3-C S-to-N spacer, *N*-methyl) leads to a cLog*D* = 13.04 but poor measured basicity (pK_a = 5.68, Δ pK_a = 3.92). Lipid **10z** bears identical branched chain fragments and S-to-N spacing but *N*-ethyl substitution, resulting in a cLog*D* = 13.34 and an even lower measured basicity (pK_a = 5.62, Δ pK_a = 4.54). As expected, lipids **10y** and **10z** are poorly active in the FVII knockdown at 31% each. Increasing the size of the branched alcohol moieties to 9-heptadecanol further increases the lipophilicity of lipid **10aa** to cLog*D* = 15.42 and the association of these fragments with a 2-C S-to-N linker (*N*-methyl) provides a very low measured pK_a = 4.65 (Δ pK_a = 3.91). Given the space **10aa** occupies, it was not examined *in vivo*.

To more clearly show the connection between two variables, pK_a and lipophilicity, upon *in vivo* activity, the lipids of the current study are presented in Table 7, sorted from higher to lower measured pK_a with associated cLog*D* and FVII

Table 7. Interrelationship between cLog*D* and Measured pK_a—Impact on *In Vivo* FVII Knockdown

lipid	cLog*D*	pK_a	FVII knockdown (%) (0.03 mg/kg)
10k	9.63	6.80	51
10k	9.63	6.80	51
10c	11.01	6.77	70
10g	10.92	6.66	96
10d	11.34	6.60	57
10i	11.93	6.58	75
10n	11.67	6.56	98
10m	9.95	6.56	61
10s	9.98	6.44	62
10p	13.70	6.38	99
10u	11.00	6.38	97
10q	14.03	6.31	97
10x	9.26	6.21	0
10v	11.30	6.20	82
10w	8.96	6.17	0
10e	9.89	6.05	13
10f	10.91	6.04	82
10a	11.32	6.03	63
10h	11.93	5.96	80
15d	15.02	5.89	71
15a	11.37	5.81	71
15b	12.38	5.81	68
15c	13.95	5.79	70
10j	9.94	5.71	5
10y	13.04	5.68	31
10z	13.34	5.62	31
10o	14.01	5.61	70
15e	16.08	5.59	40
10r	8.29	5.39	0
10t	11.34	5.13	0

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knockdown data presented. As mentioned previously, the pK_a range of 6.20−6.50 had been reported in the literature to be optimal for good FVII response.[5] These data clearly show that the *in vivo* response is a multivariable equation with strong ties to lipophilicity. At what should be the range of optimum pK_a impact, pK_a ca. 6.20−6.50 where maximal *in vivo* activity would be expected based on the assumptions mentioned above, two lipids **10w** and **10x** were found to be associated (Table 7) with FVII knockdown of 0%, and two lipids **10s** and **10m** exhibit FVII knockdown of 61−62%. These data track nicely with variable 2. The calculated cLog*D* of lipids **10w**, **10x**, **10s**, and **10m** is <10.00, while the remaining lipids of the optimal pK_a ca. 6.20−6.50 range afford greater FVII knockdown (75−99%) with greater lipophilicity (cLog*D* = 11.00−14.03).

We have reported[22] the utility of lipid **10a** in mRNA delivery *via* lipid nanoparticles to treat a factor IX (FIX)-deficient mouse model of hemophilia B. Delivery of human FIX (hFIX) mRNA encapsulated in lipid **10a**-containing LNPs results in the rapid appearance of FIX protein (within 4−6 h) that remains stable for up to 4−6 days and is therapeutically effective. Additionally, hFIX protein production with the **10a** LNP formulation (2 mg/kg) was noted to be ca. 2× of that found with the MC3 LNP formulation (2 mg/kg), 2500 ng/mL *vs* 1250 ng/mL. These data provide an initial correlation between siRNA (**10a** FVII knockdown, 63% at 0.03 mg/kg) and mRNA delivery for the lipids of the current study. The application of additional selected lipids of this study to the formulation and delivery of mRNA is underway and will be reported in due course.

Some lipids of the current study have been examined for their biodegradability in a mouse plasma assay, while a tolerability assay has been utilized to gain a preliminary reading on treatment toleration by intravenous injection of increasing doses of FVII LNPs. Mouse plasma stability was measured with time points at 0, 15, 30, 45, 60, and 120 min with a selected lipid used as an internal standard. Mouse plasma stability is listed as either $T_{1/2}$ or % remaining at the 2 h time point. In the tolerability assessment, nontargeting siRNA-containing LNPs are introduced *via* a single bolus injection. In life, clinical observations are made at 2, 6, 24, and 72 h post dose. Body weight and activity levels were measured/considered in life. After necropsy, the liver and spleen were examined for morphology changes. A lipid exemplar was selected from the bis-linear esters of Table 4 (**10a**), one from the mono-branched esters of Table 6 (**10f**) and a bis-branched ester congener from Table 6 (**10p**) to provide a preliminary look at biodegradability and response in the tolerability assay. Table 8 presents the cLog*D*, measured pK_a, FVII knockdown data, and mouse plasma stability measurement for lipids **10a**, **10f**, and **10p**. Bis-linear ester **10a** is rapidly cleaved by mouse plasma esterases, with $T_{1/2}$ = 6.9 min, not a surprising observation given the unhindered nature of the ester carbonyl moieties. As branched chain esters are added, one in the case of **10f** and two in **10p**, plasma esterase activity cleaves these more hindered esters more slowly with 73% of **10f** and 100% of **10p** remaining at the 2 h time point. More in-depth evaluations of the biodegradability of the lipids of this study are underway and the results will be presented in due course. All three lipids were tolerated at a dose of 3.0 mg/kg. The 3.0 mg/kg dose was selected in the tolerability study window to examine how a 100× dose increase (from a 0.03 mg/kg) was tolerated. No abnormal observations were noted for the tested lipids at either dose level.

Table 8. Biodegradability in the Mouse Plasma Stability Assessment and Lipid Levels in *In Vivo* Studies

lipid	cLog*D*	pK_a	FVII knockdown (%) (0.03 mg/kg)	mouse plasma stability $T_{1/2}$ or % remaining at 2 h
10a	11.32	6.03	63	6.9 min
10f	10.91	6.04	82	73%
10p	13.70	6.38	99	100%

CONCLUSIONS

The study of lipids with bis-octanoate chain lengths (**10a−10d** and **15a−15e**) provided an initial surprise when the modification of basic nitrogen substituents from methyl to ethyl at the same S-to-N chain length produced a decrease in measured basicity in formulation despite a ca. 1 pK_a unit increase in predicted basicity (**10a** to **10b**; **10c** to **10d**). An increase in the S-to-N chain length from two carbons to three carbons at the same nitrogen substitution (methyl or ethyl; **10a** to **10c** and **10b** to **10d**) does result in an expected increase in measured basicity. These variations in measured basicity are readily seen in the Δ pK_a parameter (the difference between predicted and measured basicities), and they suggest that there is an environmental impact on measured basicity by increasing lipophilicity at the basic terminus. In this bis-octanoate lipid series (**10a−10d** and **15a−15e**), another environmental impact on the measured pK_a in formulation is observed when the large branched chain alcohol, 9-heptadecanol, is introduced in the synthesis of **15e** (c-pK_a = 8.68, pK_a = 5.59, Δ pK_a = 3.09). As the bis-acid chains are shortened to butyrate (**10e−10q**) an identical *N*-methyl to *N*-ethyl impact on measured pK_a is seen, while as might be expected, the effect of the size of a single branched chain alcohol in ester formation is modest (**10e**/**10h**). Within this bis-butyrate series, a large change in measured *vs* calculated basicity is more readily apparent when 2-branched chain alcohols are introduced (**10o** and **10q**). In the bis-acetate derived lipids (**10r−10aa**), the environmental impact of a single branched chain alcohol is observed in **10t** and is more profoundly seen in the bis-branched chain lipids **10y−10aa**. Throughout the lipids of Tables 4 and 6, good *in vivo* activity in the FVII knockdown assay is observed when cLog*D* is 10.0 or greater and the measured pK_a is ca. 5.80 or greater. A higher measured pK_a at a similar cLog*D* generally leads to a greater FVII knockdown. Low cLog*D* values such as those reported for **10w** and **10x** lead to no FVII knockdown despite good measured basicity (6.17 and 6.21, respectively); these trends are shown for the entire series of lipids in Table 7.

These data suggest the potential to tune calculated and measured physicochemical parameters to achieve desired levels of lipophilicity and measured basicity by a combination of bis-acid chain length/number and size of branched chain ester moieties/S-to-N carbon chain length/N-substitution. The ability to bring targeted lipids presented in Table 6 into desirable physicochemical space and achieve good *in vivo* activity (FVII knockdown, >90%) is noteworthy and could not have been anticipated based on the literature data of Table 1. As we move forward with further changes in lipid design, this multivariable approach should allow us to realize a target lipophilicity and measured basicity with iterative structural alteration(s). Further studies of the impact of structure on calculated and measured physicochemical properties are underway and will be reported in due course.

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EXPERIMENTAL SECTION

Biology. *siRNA Formulation into Lipid Nanoparticles.* The LNPs were prepared, in a manner similar to that reported in reference **27**, by mixing appropriate volumes of lipids in ethanol with an aqueous phase containing siRNA duplexes using a Nanoassemblr microfluidic device, followed by downstream processing. For the encapsulation of siRNA, ~0.2 mg/mL siRNA was dissolved in 5 mM citrate buffer (pH 3.5). Lipids at the desired molar ratio were dissolved in ethanol. The molar percentage ratio for the constituent lipids is 58% ionizable amino lipids, 7% DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids), 33.5% cholesterol (Avanti Polar Lipids), and 1.5% DMG-PEG (1,2-dimyristoyl-*sn*-glycerol, methoxypolyethylene glycol; PEG chain molecular weight: 2000) (NOF America Corporation). At a flow ratio of 1:3 ethanol/aqueous phases, the solutions were combined in the microfluidic device using Nanoassemblr (Precision NanoSystems). The total combined flow rate was 12 mL/min per microfluidics chip and the total lipid-to-RNA weight ratio was ~30:1. The mixed material was then diluted three times with Tris buffer (pH 7.4) containing 50 mM sodium chloride and 9% sucrose after leaving the micromixer outlet, reducing the ethanol content to 6.25%. The diluted LNP formulation was concentrated and diafiltered by tangential flow filtration using hollow fiber membranes (mPES Kros membranes, Spectrum Laboratories) and Tris buffer (pH 7.4) containing 50 mM sodium chloride and 9% sucrose. A total of 10 diavolumes were exchanged, effectively removing the ethanol. The particle size and polydispersity index (PDI) were characterized using a Zen3600 (Malvern Instruments, with Zetasizer 7.1 software, Malvern, U.K.). A volume of 50 μL of formulations were diluted into 950 μL of Tris buffer at pH = 7.4 and equilibrated to 25 °C prior to analysis in a 1 mL cuvette using the following settings for measurement: material refractive index of 1.5, dispersant viscosity of 1.1 cP, and refractive index of 1.3. Each sample was analyzed for up to 30 runs. Encapsulation efficiency was calculated by determining the unencapsulated siRNA content by measuring the fluorescence intensity (Fi) upon the addition of RiboGreen (Molecular Probes) to the LNP and comparing this value to the total fluorescence intensity (Ft) of the RNA content that is obtained upon lysis of the LNPs by 1% Triton X-100, where % encapsulation = (Ft − Fi)/Ft × 100). All of the lipids of these studies were associated with encapsulation efficiencies of >90%.

Measurement of pK_a in Formulated Lipid Nanoparticles Using the TNS Assay. The apparent pK_a of ionizable lipid in the lipid nanoparticle was determined using 6-(*p*-toluidino)-2-naphthalenesulfonic acid sodium salt (TNS reagent, Sigma-Aldrich, St. Louis, MO). Lipid nanoparticles were diluted in PBS to a concentration of 1 mM total lipid. TNS was prepared as a 1 mg/mL stock solution in DMSO and then further diluted using distilled water to a working solution of 60 μg/mL (179 mM). Lipid nanoparticle samples were diluted to 90 μM lipid in 165 μL of buffered solutions containing 10 mM HEPES, 10 mM MES, 10 mM ammonium acetate, 130 mM NaCl, and final TNS working solution of 1.33 μg/mL (4 μM) where the pH ranged from 3.8 to 12. Following pipette mixing and incubation at room temperature in the dark for 15 min, fluorescence intensity was measured at room temperature in a BioTek Cytation3 imaging reader using excitation and emission wavelengths of 321 and 445 nm, respectively. The fluorescence signal was plotted as a function of the pH and analyzed using a nonlinear (Boltzmann) regression analysis with the apparent pK_a determined as the pH giving rise to half-maximal fluorescence intensity. Amino lipid apparent pK_a values were calculated using the Henderson−Hasselbalch equation.

In Vivo Activity—Factor VII Knockdown.[15,21] Seven to eight week-old, female Balb/C mice were purchased from Charles River Laboratories (Hollister, CA). Mice were held in a pathogen-free environment and all procedures involving animals were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC). Lipid nanoparticles containing factor VII siRNA[15] were administered intravenously at a dosing volume of 10 mL/kg. After 48 h, mice were anesthetized *via* isoflurane and blood was collected retro-orbitally into microtainer tubes coated with 0.109 M sodium citrate buffer (BD Biosciences, San Diego, CA) and processed to plasma. Plasma was tested immediately or stored at −80 °C for later analysis for factor VII levels. Measurement of FVII protein in plasma was determined using the colorimetric Biophen VII assay kit (Aniara Diagnostica, USA). Absorbance was measured at 405 nm and a calibration curve was generated using the serially diluted control plasma to determine levels of factor VII in plasma from treated animals, relative to the saline-treated control animals.

Mouse Plasma Stability. Lipid stock solution was prepared by dissolution of the lipid in isopropanol at the concentration of 5 mg/mL. A requisite volume of the lipid-isopropanol solution was then diluted to 100 μM concentration at a total volume of 1.0 mL with in 50:50 (v/v) ethanol/water. Ten microliters of this 100 μM solution was spiked into 1.0 mL of mouse plasma (BioIVT, cat. no. MSE00PLNHUNN, CD-1 mouse, anticoagulant: sodium heparin, not filtered) that was prewarmed to 37 °C and stirred at 50 rpm with a magnetic stir bar. The starting concentration of lipids in plasma was thus 1 μM. At time points 0, 15, 30, 45, 60, and 120 min, 0.1 mL of the plasma was withdrawn from the reaction mixture and the protein was precipitated by adding 0.9 mL of ice-cold 4:1 (v/v) acetonitrile/methanol with 1 μg/mL of a selected internal standard lipid added. After filtration through a 0.45 μm 96-well filtering plate, the filtrates were analyzed by LC−MS (Thermo Fisher's Vanquish UHPLC−LTQ XL linear ion trap mass spectrometer; Waters XBridge BEH Shield RP18 2.5 μm (2.1 × 100 mm) column with its matching guard column). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in 1:1 (v/v) acetonitrile/methanol. The flow rate was 0.5 min/min. Elution gradient was as follows: time, 0−1 min: 10% B; 1−6 min: 10−95% B; 6−8.5 min: 95% B; 8.5−9 min: 95−10% B; 9−10 min: 10% B. Mass spectrometry was in positive scanning mode from 600−1100 m/z. The peak of the molecular ion of the lipids was integrated in extracted ion chromatography (XIC) using Xcalibur software (Thermo Fisher). The relative peak area compared to T = 0, after normalization by the peak area of the internal standard, was used as the percentage of the lipid remaining at each time point. $T_{1/2}$ values were calculated using the first-order decay model.

Mouse Tolerability. Six to eight week-old, CD-1 female mice (Charles River Labs) were used for tolerability studies. All procedures used in animal studies were approved by the Institutional Animal Care and Use Committee. Lipid nanoparticles containing nontargeting siRNA (proprietary sequence based on a commercially available proprietary siRNA from Thermo Fisher Scientific) were administered to CD-1 females *via* intravenous injection at 1, 3, 10, and 30 mg/kg siRNA/kg dose. The general health condition of each animal was monitored for 72 h. Changes in life of >10% weight loss, and after necropsy (72 h), observations of gross liver and morphology changes (*e.g.*, liver color change, liver fibrosis, etc.) and/or spleen enlargement, would result in designating the run at that dose as not tolerated.

Chemistry. *General Methods.* Starting materials and other reagents were purchased from commercial suppliers and were used without further purification, unless otherwise indicated. All reactions were performed under a positive pressure of nitrogen or argon or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, unless otherwise indicated. The reactions were assayed by high-performance liquid chromatography (HPLC) and terminated as judged by the consumption of starting material. The ^{1}H-NMR spectra were recorded on Varian or Bruker instruments operating at the field strength indicated. the ^{1}H-NMR spectra are obtained as DMSO-d_6 or $CDCl_3$ solutions as indicated (reported in ppm) using TMS as the reference. Other NMR solvents were used as needed. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets. Coupling constants, when given, are reported in hertz. The NMR spectra of individual test compounds can be found in the Supporting Information. The mass spectra were obtained using liquid chromatography−mass spectrometry (LC−MS) on a Shimadzu instrument using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). The mass spectra were also

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measured by direct injection on a PerkinElmer PE-SCIEX API-150 instrument or Agilent-TRAP XCT instrument using electrospray (ESI) ionization. All test compounds showed >95% purity as determined by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: Agilent 1290 Infinity; Halo RP-Amide, 3.00 mm × 100 mm, 2.7 μm, 30 °C; 10% → 90% CH_3CN, 0.05% perchloric acid/H_2O to 90% → 10% CH_3CN, 0.05% perchloric acid/H_2O, 30 min run, flow rate of 1.0 mL/min, UV detection (λ = 205 nm) or 10% → 90% CH_3CN, 0.05% perchloric acid/H_2O to 90% → 10% CH_3CN, 0.05% perchloric acid/H_2O, 30 min run, flow rate of 1.0 mL/min, ELSD detection. HPLC analyses of test compounds can be found in the Supporting Information.

*8,8′-[(Phenylmethyl)imino]bis-octanoate 1,1′-Dimethyl Ester (**5a**).* To a solution of benzyl amine (50 g, 0.46 mol) in acetonitrile (750 mL) was added powdered potassium carbonate (148.3 g, 1.07 mol), followed by the addition of a solution of methyl-8-bromooctanoate (250.9 g ,1.058 mol, 2.3 equiv) in acetonitrile (250 mL) over a period of 10 min. The mixture was then placed at 80 °C and was stirred, under nitrogen, for 16 h. The mixture was cooled down to room temperature and water (1.0 L) was added. Stirring was continued for 10 min, then stirring was stopped, and the layers were allowed to separate. The organic phase was separated and concentrated *in vacuo* (bath temperature, 40 °C) and the residue was dissolved in ethyl acetate (1.0 L). The organic phase was washed with water (1.0 L) and brine (1.0 L) and dried over anhydrous sodium sulfate. Filtration and concentration *in vacuo* afforded crude **5a** as a pale yellow oil (201.3 g), which was utilized in the next reaction without further purification. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.20−7.36 (5), 3.67 (s, 6), 3.52 (s, 2), 2.25−2.42 (8), 1.20−1.70 (20).

*8,8′-[[(1,1-Dimethylethoxy)carbonyl]imino]bis-octanoate 1,1′-Dimethyl Ester (**6a**).* A solution of crude **5a** (201 g) in ethanol (1.7 L) in a Parr reactor was sparged with nitrogen for 10 min. Then, di-*t*-butyl-dicarbonate (115.7 g, 0.53 mol) and 10% Pd/C (15 g) were added and the vessel atmosphere was replaced with hydrogen (70 psi) and the mixture was stirred for 15 h at room temperature. The pressure was released, the mixture was again sparged with nitrogen for 20 min and then filtered through a pad of Celite, the filter cake was rinsed with ethanol (2 × 200 mL), and the combined filtrates were concentrated *in vacuo* to furnish crude **6a** as a pale yellow oil. Crude **6a** was purified by chromatography on a column of silica gel (1000 g, 100−200 mesh), packed with petroleum ether, and eluted with a gradient of petroleum ether/heptane (100:0 to 90:10) using the flash technique. Fractions containing **6a** were combined and concentrated *in vacuo* to afford **6a** as a clear, colorless oil (159 g, 0.37 mol, 81% from benzyl amine). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 3.63 (s, 6), 3.11 (m, 4), 2.28 (m, 4), 1.40 (s, 9), 1.23−1.74 (20).

*8,8′-[[(1,1-Dimethylethoxy)carbonyl]imino]bis-octanoic Acid (**7a**).* To a solution of **6a** (150 g, 0.349 mol) in THF (90 mL) and methanol (200 mL), cooled in an ice-water bath under nitrogen, was added a solution of NaOH (48.8 g, 1.2 mol) in water (300 mL) over 20 min. The mixture was allowed to warm to room temperature and stirred for 14 h. The solution was diluted with water (1.0 L) and washed with MTBE (0.75 L). The aqueous phase was separated and acidified to pH 4−5 with 1.5 N aq. HCl and then extracted with CH_2Cl_2 (2 × 1.0 L). The combined organic layers were washed with brine (2 × 1.0 L) and dried (Na_2SO_4). Filtration and concentration *in vacuo* afforded 148 g of crude **7a** as a pale yellow viscous liquid. This material was utilized in the subsequent reaction without further purification. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 3.06 (m, 4), 2.17 (t, *J* = 7.2 Hz, 4), 1.47 (s, 9), 1.10−1.60 (20).

*8,8′-[[(1,1-Dimethylethoxy)carbonyl]imino]bis-octanoic Acid, 1,1′-Di-(2Z)-2-nonen-1-yl Ester (**8a**).* To a solution of crude **7a** (145 g, 0.357 mol) in CH_2Cl_2 (1.5 L), stirring in an ice-water bath under nitrogen, were added DMAP (22 g, 0.18 mol) and (*Z*)-2-nonenol (101.56 g, 0.714 mol). Then, EDC-HCl (209 g, 1.09 mol) was added in portions over 2 h. The resulting solution was then allowed to warm to room temperature and stir for 16 h. The reaction was quenched by the addition of 10% aq. citric acid (250 mL) and the resulting mixture was washed with 10% aq. citric acid (1.5 L) and brine (1.5 L). The organic phase was separated, dried (Na_2SO_4), filtered, and concentrated *in vacuo* to provide crude **8a** as a yellow oil. The crude product was purified by chromatography (750 g, 100−200 mesh), packed in petroleum ether, and eluted with a gradient of petroleum ether/ethyl acetate from 100:0 to 90:10. Fractions containing **8a** were combined and concentrated *in vacuo* to give **8a** (167 g, 0.257 mol, 72%) as a clear, colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.63 (m, 2), 5.53 (m, 2), 4.61 (d, *J* = 6.4 Hz, 4), 3.12 (t, *J* = 7.2 Hz, 4), 2.28 (t, *J* = 7.6 Hz, 4), 2.09 (m, 4), 1.44 (s, 9), 1.15−1.70 (36), 0.85 (t, *J* = 7.2 Hz, 6); MS (ESI, Agilent-TRAP XCT): 594.9 (M^+ − BOC).

*8,8′-Iminobis-octanoic Acid, 1,1′-Di-(2Z)-2-nonen-1-yl Ester (**9a**).* To **8a** (20 g, 30.8 mmol) in CH_2Cl_2 (80 mL), cooled in an ice-water bath under nitrogen, was added trifluoroacetic acid (44.7 g, 0.39 mol, 30 mL) over 10 min. The mixture was then allowed to warm to room temperature and stir for 5 h. The mixture was concentrated *in vacuo*, diluted with ethyl acetate (300 mL), and washed with saturated aq. Na_2CO_3 (300 mL). The organic phase was separated, washed with brine (300 mL), and dried (Na_2SO_4). Concentration *in vacuo* afforded crude **9a** (15 g, 89%) as a pale yellow, viscous oil. This material was utilized in subsequent reactions without further purification. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 95:5 to 5:95 A/B at 2.00 min, hold 0.7 min): RT 2.335 min, *m*/*e* = 550.5 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.64 (m, 2), 5.54 (m, 2), 4.63 (d, *J* = 6.4 Hz, 4), 2.61 (m, 4), 2.31 (m, 4), 2.10 (m, 4), 1.64 (m, 4), 1.53 (m, 4), 1.20−1.45 (28), 0.90 (m, 6).

*8,8′-[[[[2-(Dimethylamino)ethyl]thio]carbonyl]imino]bis-octanoic Acid, 1,1′-Di-(2Z)-2-nonen-1-yl Ester (**10a**).* To a solution of **9a** (3.00 g, 5.40 mmol) in CH_2Cl_2 (60 mL), cooled in an ice water bath under nitrogen, was added triphosgene (1.62 g, 5.40 mmol) over 5 min followed by the addition of pyridine (2.16 g, 27.3 mmol, 5 equiv) over a period of 5 min. The mixture was allowed to warm to room temperature and stir for 4 h and then concentrated *in vacuo* (bath temperature, 23 °C). The residue was dissolved in pyridine (20 mL) and cooled in an ice-water bath under nitrogen, and then 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (3.87 g, 27 mmol) was added in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (200 mL). The solution was washed with 10% aq. citric acid (2 × 200 mL), saturated aq. $NaHCO_3$ (3 × 200 mL), and brine (200 mL) and dried (Na_2SO_4). Filtration and concentration *in vacuo* provided 3.1 g of crude **10a** as an orange oil. The orange oil was dissolved in heptane (60 mL) and to the resulting solution were added methanol (45 mL) and water (15 mL). The resulting two-phase mixture was stirred for 30 min and then allowed to stand to 10 min to separate. The aq. MeOH lower phase was removed and this procedure was repeated two additional times. The heptane layer was then treated with activated charcoal (DARCO, 100 mesh, 1.0 g) and the mixture was allowed to stir for 16 h. The charcoal was removed by filtration through a pad of Celite, the filter cake was rinsed with heptane (50 mL), and the solvent was removed *in vacuo* to give **10a** (2.9 g, 4.25 mmol, 79%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.039 min, *m*/*e* = 681.6 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.63 (m, 2), 5.56 (m, 2), 4.64 (d, *J* = 8.0 Hz, 4), 3.30 (brm, 2), 3.07 (t, *J* = 6.4 Hz, 2), 2.61 (brm, 2), 2.35 (s, 6), 2.28−2.34 (4), 2.12 (m, 4), 1.64 (m, 4), 1.20−1.40 (28), 0.91 (m, 6).

*8,8′-[[[[2-(Diethylamino)ethyl]thio]carbonyl]imino]bis-octanoic Acid, 1,1′-Di-(2Z)-2-nonen-1-yl Ester (**10b**).* On an identical scale as that reported for the synthesis of **10a**, utilization of 2-(diethylamino)-ethanethiol-HCl (Sigma-Aldrich, D86604) in place of the dimethyl variant led to the isolation of **10b** (2.72 g, 3.83 mmol, 71%) as a clear, pale yellow viscous liquid. ESI-MS (+ mode): 709.5 ($M + H^+$), 731.6 ($M + Na^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.64 (m, 2), 5.53 (m, 2), 4.62 (d, *J* = 6.4 Hz, 4), 3.30 (brm, 4), 2.98 (m, 2), 2.64 (m, 2), 2.57 (m, 4), 2.29 (m, 4), 2.09 (m, 4), 1.57 (brm ,4), 1.20−1.40 (28), 1.04 (m, 6), 0.88 (m, 6).

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*8,8′-[[[[3-(Dimethylamino)propyl]thio]carbonyl]imino]bis-octanoic Acid, 1,1′-Di-(2Z)-2-nonen-1-yl Ester (**10c**).* On an identical scale as that reported for the synthesis of **10a**, utilization of 3-(dimethylamino)-propanethiol-HCl[16] in place of the dimethyl-variant led to the isolation of **10c** (2.78 g, 3.99 mmol, 74%) as a clear, yellow viscous liquid. ESI-MS (+ mode): 696.1 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.63 (m, 2), 5.52 (m, 2), 4.61 (d, J = 6.4 Hz, 4), 3.27 (brm, 4), 2.91 (t, J = 6.0 Hz, 2), 2.27−2.37 (6), 2.22 (s, 6), 2.10 (m, 4), 1.78 (m, 2), 1.61 (brm, 4), 1.22−1.40 (30), 0.87 (m, 6).

*8,8′-[[[[3-(Diethylamino)propyl]thio]carbonyl]imino]bis-octanoic Acid, 1,1′-Di-(2Z)-2-nonen-1-yl Ester (**10d**).* On an identical scale as that reported for the synthesis of **10a**, utilization of 3-(diethylamino)-propanethiol-HCl[17] in place of the dimethyl variant led to the isolation of **10d** (3.05 g, 4.21 mmol, 78%) as a clear, yellow viscous liquid. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 40:60 to 5:95 A/B at 6.00 min, hold 2.00 min): RT 1.039 min, m/e = 723.6 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.65 (m, 2), 5.53 (m, 2), 4.61 (d, J = 6.4 Hz, 4), 3.27 (brs, 4), 2.90 (t, J = 6.0 Hz, 2), 2.44−2.51 (m, 6), 2.29 (m, 4), 2.06 (m, 4), 1.75 (m, 2), 1.45−1.70 (8), 1.20−1.40 (28), 1.00 (m, 6), 0.86 (m, 6).

*8-((tert-Butoxycarbonyl)amino)octanoate Nonan-5-yl Ester (**12a**).* To a solution of **11** (16.1 g, 62 mmol) in CH_2Cl_2 (200 mL), cooled in an ice-water bath under nitrogen, were added in order EDC-HCl (17.8 g, 93 mmol, 1.5 equiv), DMAP (0.76 g, 6 mmol), and Et_3N (17.2 mL, 12.48 g, 0.123 mol, 2.0 equiv). After stirring for 5 min, 5-nonanol (TCI America, #N0337) (9.0 g, 62 mmol) in CH_2Cl_2 (50 mL) was added over a period of 15 min. The mixture was allowed to warm to room temperature and stir for 16 h. The reaction was diluted with CH_2Cl_2 (200 mL), washed with water (250 mL), saturated aq. $NaHCO_3$ (400 mL), and brine (2 × 500 mL), and dried (Na_2SO_4). Concentration *in vacuo* afforded crude **12a**, which was utilized in the next step without further purification.

*8-Amino-octanoate Nonan-5-yl Ester (**13a**).* To a solution of crude **12a** in CH_2Cl_2 (250 mL), cooled in an ice-water bath under nitrogen, was added TFA (45.9 mL) over a period of 10 min. The mixture was allowed to warm to room temperature and then stir for 3 h. The solvent and TFA were then removed *in vacuo* and the residue was dissolved in EtOAc (250 mL). The resulting solution was washed with saturated aq. $NaHCO_3$ (250 mL) and brine (250 mL) and dried (Na_2SO_4). Filtration and concentration *in vacuo* gave crude **13a** as a viscous, yellow liquid. Crude **13a** was purified by chromatography on a column of silica gel (300 g, 60−120 mesh), packed, and eluted with CH_2Cl_2/MeOH/Et_3N (95:4:1) using the flash technique. Fractions containing **13a** were combined and concentrated *in vacuo* to afford **13a** (8.5 g, 49%) as a pale yellow viscous liquid.

*8-((8-(Nonan-5-yloxy)-8-oxooctyl)amino)octanoic Acid (Z)-Non-2-en-1-yl Ester (**14a**).* To **13a** (8.5 g, 29 mmol) in CH_3CN (60 mL) were added 8-bromo-octanoic acid (Z)-2-nonenylester[19] (10.3 g, 29 mmol) and K_2CO_3 (8.2 g, 59 mmol). The mixture was warmed in a 90 °C oil bath, under nitrogen, for 7 h. The mixture was cooled down to room temperature, the solids were removed by filtration through a pad of Celite, the filter cake was rinsed with CH_3CN (2 × 50 mL), and the combined filtrates were concentrated *in vacuo* to give crude **14a** as a dark brown, viscous oil. The crude product was purified by chromatography on a column of silica gel (250 g, 100−200 mesh), packed, and eluted with EtOAc/hexanes (70:30). Fractions containing **14a** were combined and concentrated *in vacuo*, providing **14a** (4.6 g, 28%) as a clear, pale yellow, viscous oil.

*8-((((2-(Dimethylamino)ethyl)thio)carbonyl)(8-(nonan-5-yloxy)-8-oxooctyl)amino)octanoic Acid (Z)-Non-2-en-1-yl Ester (**15a**).* To a solution of **13a** (1.50 g, 2.19 mmol) in CH_2Cl_2 (25 mL), cooled in an ice water bath under nitrogen, was added triphosgene (0.649 g, 2.19 mmol) over 5 min followed by the addition of pyridine (0.864 g, 10.9 mmol, 5 equiv) over a period of 5 min. The mixture was allowed to warm to room temperature and stir for 4 h and then concentrated *in vacuo* (bath temperature, 23 °C). The residue was dissolved in pyridine (10 mL) and cooled in an ice-water bath under nitrogen, and then 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (1.55 g, 10.9 mmol) was added in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (200 mL). The solution was washed with 10% aq. citric acid (2 × 200 mL), saturated aq. $NaHCO_3$ (3 × 200 mL), and brine (200 mL) and dried (Na_2SO_4). Filtration and concentration *in vacuo* provided crude **15a** as an orange oil. The orange oil was dissolved in heptane (50 mL) and to the resulting solution were added methanol (37.5 mL) and water (12.5 mL). The resulting two-phase mixture was stirred for 30 min and then was allowed to stand to 10 min to separate. The aq. MeOH lower phase was removed and this procedure was repeated two additional times. The heptane layer was then treated with activated charcoal (DARCO, 100 mesh, 1.0 g) and the mixture was allowed to stir for 16 h. The charcoal was removed by filtration through a pad of Celite, the filter cake was rinsed with heptane (50 mL), and the solvent was removed *in vacuo* to give **15a** (1.03 g, 1.51 mmol, 69%) as a pale yellow, viscous oil. ESI-MS (+ mode): 684.4 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.65 (m, 1), 5.52 (m, 1), 4.87 (m, 1), 4.62 (d, J = 6.4 Hz, 2), 3.27 (brm, 4), 3.02 (t, J = 7.0 Hz, 2), 2.53 (t, J = 7.0 Hz, 2), 2.26−2.34 (4), 2.30 (s, 6), 1.46−1.66 (10), 1.22−1.40 (32), 0.89 (m, 9).

*8-((tert-Butoxycarbonyl)amino)octanoate Undecan-6-yl Ester (**12b**).* To a solution of **11** (15.0 g, 58 mmol) in CH_2Cl_2 (200 mL), cooled in an ice-water bath under nitrogen, were added in order EDC-HCl (16.6 g, 86 mmol, 1.5 equiv), DMAP (0.706 g, 5 mmol), and Et_3N (16 mL, 11.61 g, 0.115 mol, 2.0 equiv). After stirring for 5 min, 6-undecananol (CombiBlocks, #QF-4463) (10.0 g, 58 mmol) in CH_2Cl_2 (50 mL) was added over a period of 15 min. The mixture was allowed to warm to room temperature and stir for 16 h. The reaction diluted with CH_2Cl_2 (200 mL), washed with water (250 mL), saturated aq. $NaHCO_3$ (400 mL), and brine (2 × 500 mL), and dried (Na_2SO_4). Concentration *in vacuo* afforded crude **12b**, which was utilized in the next step without further purification.

*8-Amino-octanoate Undecan-5-yl Ester (**13b**).* To a solution of crude **12b** in CH_2Cl_2 (250 mL), cooled in an ice-water bath under nitrogen, was added TFA (46.5 mL, 0.604 mol) over a period of 10 min. The mixture was allowed to warm to room temperature and then stir for 3 h. The solvent and TFA were then removed *in vacuo* and the residue was dissolved in EtOAc (250 mL). The resulting solution was washed with saturated aq. $NaHCO_3$ (250 mL) and brine (250 mL) and dried (Na_2SO_4). Filtration and concentration *in vacuo* gave crude **13b** as a viscous, yellow liquid. Crude **13b** was purified by chromatography on a column of silica gel (300 g, 60−120 mesh), packed, and eluted with CH_2Cl_2/MeOH/Et_3N (95:4:1) using the flash technique. Fractions containing **13b** were combined and concentrated *in vacuo* to afford **13b** (10.0 g, 56%) as a pale yellow viscous liquid.

*8-((8-(Undecan-6-yloxy)-8-oxooctyl)amino)octanoic Acid (Z)-Non-2-en-1-yl Ester (**14b**).* To **13b** (10.0 g, 29 mmol) in CH_3CN (80 mL) were added 8-bromo-octanoic acid Z-2-nonenylester[19] (11.0 g, 31 mmol) and K_2CO_3 (8.8 g, 62 mmol). The mixture was warmed in a 90 °C oil bath, under nitrogen, for 7 h. The mixture was cooled down to room temperature, the solids were removed by filtration through a pad of Celite, the filter cake was rinsed with CH_3CN (2 × 60mL), and the combined filtrates were concentrated *in vacuo* to give crude **14b** as a dark brown, viscous oil. The crude product was purified by chromatography on a column of silica gel (275 g, 100−200 mesh), packed, and eluted with EtOAc/hexanes (70:30). Fractions containing **14b** were combined and concentrated *in vacuo*, providing **14b** (4.0 g, 22%) as a clear, pale yellow, viscous oil.

*8-((((2-(Dimethylamino)ethyl)thio)carbonyl)(8-(undecan-6-yloxy)-8-oxooctyl)amino)octanoic Acid (Z)-Non-2-en-1-yl Ester (**15b**).* To a solution of **13b** (1.50 g, 2.00 mmol) in CH_2Cl_2 (20 mL), cooled in an ice water bath under nitrogen, was added triphosgene (0.593 g, 2.00 mmol) over 5 min followed by the addition of pyridine (0.790 g, 10.0 mmol, 5 equiv) over a period of 5 min. The mixture was allowed to warm to room temperature and stir for 4 h and then concentrated *in vacuo* (bath temperature, 23 °C). The residue was dissolved in pyridine (10 mL) and cooled in an ice-water bath under nitrogen, and then 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (1.42 g, 10.0 mmol) was added in one

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portion. The solution was allowed to warm to room temperature and stir for 20 h. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (200 mL). The solution was washed with 10% aq. citric acid (2 × 200 mL), saturated aq. $NaHCO_3$ (3 × 200 mL), and brine (200 mL) and dried (Na_2SO_4). Filtration and concentration *in vacuo* provided crude **15b** as an orange oil. The orange oil was dissolved in heptane (50 mL) and to the resulting solution were added methanol (37.5 mL) and water (12.5 mL). The resulting two-phase mixture was stirred for 30 min and then allowed to stand to 10 min to separate. The aq. MeOH lower phase was removed and this procedure was repeated two additional times. The heptane layer was then treated with activated charcoal (DARCO, 100 mesh, 1.0 g) and the mixture was allowed to stir for 16 h. The charcoal was removed by filtration through a pad of Celite, the filter cake was rinsed with heptane (50 mL) and the solvent was removed *in vacuo* to give **15b** (0.996 g, 1.40 mmol, 70%) as a pale yellow, viscous oil. ESI-MS (+ mode): 711.5 ($M + H^+$, base); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.63 (m, 1), 5.51 (m, 1), 4.85 (m, 1), 4.61 (d, J = 7.0 Hz, 2), 3.27 (brm, 4), 3.02 (t, J = 7.0 Hz, 2), 2.53 (t, J = 7.0 Hz, 2), 2.26−2.34 (4), 2.30 (s, 6), 2.09 (m, 2), 1.61 (brm, 4), 1.46−1.55 (6), 1.20−1.42 (34), 0.88 (m, 9).

*4,4′-[(Phenylmethyl)Imino]Bis-Butanoate-1, 1′-Dimethyl Ester (**5b**).* To a solution of methyl 4-bromobutanoate (100.0 g, 0.552 mol) in anhydrous CH_3CN (600 mL) was added benzyl amine (29.5 g, 0.276 mol) followed by K_2CO_3 (152.6 g, 1.104 mol). The mixture was warmed to 80 °C and stirred under nitrogen for 16 h. The mixture was then cooled down to room temperature and diluted with ice water (1.5 L). The resulting solution was extracted with EtOAc (3 × 350 mL), and the combined organic layers were dried (Na_2SO_4). Filtration and concentration *in vacuo* provided crude **5b** as a viscous, yellow liquid. The crude product was purified by chromatography on a column of silica gel (1.0 kg, 100−200 mesh) using a gradient of EtOAc/hexanes (25:75 to 35:65). Fractions containing **5b** were pooled and concentrated *in vacuo* to give **5** (61.93 g, 0.201 mol, 73%) as a colorless, viscous oil. ESI-MS (+ mode): 308.2 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 7.32 (m, 5), 3.65 (s, 6), 3.58 (s, 2), 2.80 (m, 4), 2.47 (m, 4).

*4,4′-[[(1,1-Dimethylethoxy)carbonyl]imino]bis-butanoate 1,1′-Dimethyl Ester (**6b**).* A solution of **5b** (61.00 g, 0.198 mol) in ethanol (600 mL), in a Paar vessel, was sparged with nitrogen for 15 min. Then, di-*t*-butyl-dicarbonate (47.6 g, 0.218 mol) and 10% Pd/C (6 g) were added and the vessels atmosphere was replaced with hydrogen (70 psi) and the mixture was stirred for 15 h at room temperature. The pressure was released, the mixture was again sparged with nitrogen for 20 min and then filtered through a pad of Celite, the filter cake was rinsed with ethanol (2 × 100 mL), and the combined filtrates were concentrated *in vacuo* to furnish crude **6b** as a pale yellow oil. Crude **6b** was purified by chromatography on a column of silica gel (250 g, 100−200 mesh), packed with petroleum ether, and eluted with a gradient of petroleum ether/heptane (100:0 to 90:10) using the flash technique. Fractions containing **6b** were combined and concentrated *in vacuo* to afford **6b** as a clear, colorless oil (59.7 g, 0.188 mol, 95%). ESI-MS (+ mode): 318.2 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 3.62, (s, 6), 3.29 (m, 4), 2.37 (m, 4), 1.85 (m, 4), 1.49 (s, 9).

*4,4′-[[(1,1-Dimethylethoxy)carbonyl]imino]bis-butanoic Acid (**7b**).* To a solution of **6b** (59.0 g, 0.185 mol) in THF (275 mL) was added 6 N aq. NaOH (125 mL). The mixture was warmed in a 60 °C oil bath and allowed to stir under nitrogen for 4 h. The solution was cooled down to room temperature and the THF was removed *in vacuo* to afford a viscous, pale yellow oil, which was diluted with water (300 mL) and extracted with EtOAc (3 × 250 mL). The aqueous phase was carefully acidified with 2 N aq. HCl to ca. pH = 5, then the mixture was extracted with EtOAc (2 × 300 mL), and the combined organic phases were dried (Na_2SO_4). The mixture was filtered, the filter cake was rinsed with EtOAc (100 mL), and the combined filtrates were concentrated *in vacuo* to give **7b** (47.64 g, 0.165 mol, 89%) as a white solid. ESI-MS (+ mode): 290.2 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 3.05 (m, 4), 2.18 (m, 4), 1.67 (m, 4), 1.27 (s, 9).

*4-((tert-Butoxycarbonyl)(4-(Z)-non-2-en-1-yloxy)-4-oxobutyl)-amino)butanoic Acid (**8b**).* To a suspension of **7b** (21.70 g, 75.00 mmol) in dry dichloromethane (250 mL) were added in order EDC-HCl (7.67 g, 40.00 mmol), *Z*-2-nonen-1-ol (5.84 g, 40.00 mmol), diisopropylethyl amine (5.17 g, 40.0 mmol), and DMAP (0.490 g, 4.00 mmol). The mixture was allowed to stir at room temperature, under nitrogen, overnight, then was cast into dichloromethane (250 mL) and water (500 mL). The organic layer was separated, washed with brine (500 mL), and dried (Na_2SO_4). Filtration and concentration *in vacuo* afforded crude **8b** as a viscous semisolid, which was purified by chromatography on a column of silica gel (750 g) using a gradient of dichloromethane/EtOAc (100:0 to 60:40). Fractions containing **8b** were pooled and concentrated *in vacuo* to give **8b** (16.44 g, 39.75 mmol, 53%) as a clear, viscous liquid. MS (PerkinElmer): 412.0 ($M - H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.63 (m, 1), 5.50 (m, 1), 4.61 (d, J = 7.0 Hz, 2), 3.14−3.25 (brm, 4), 2.33 (m, 2), 2.29 (m, 2), 2.08 (m, 2), 1.78−1.86 (4), 1.43 (s, 9), 1.33 (m, 2), 1.23−1.30 (6), 0.86 (t, J = 7.2 Hz, 3).

*4-((tert-Butoxycarbonyl)(4-oxo-4-(tridecan-7-yloxy)butyl)-amino)butanoic Acid (Z)-Non-2-en-1-yl Ester (**8c**).* To a solution of **8b** (3.10 g, 7.50 mmol) in anhydrous dichloromethane (50 mL) were added in order EDC-HCl (2.15 g, 11.25 mmol), 7-tridecanol (1.50 g, 7.5 mmol), *i*-Pr_2NEt (0.97 g, 7.5 mmol, 1.31 mL), and DMAP (92 mg, 0.75 mmol). The mixture was allowed to stir for 4 h at room temperature under nitrogen and then cast into dichloromethane (50 mL) and water (100 mL). The organic phase was separated, washed with brine (100 mL), and dried (Na_2SO_4). Filtration and concentration *in vacuo* afforded crude **8c**, which was purified by chromatography on a column of silica gel (150 g, 230−400 mesh) eluting with a gradient of hexanes/EtOAc from 95:5 to 50:50. Fractions containing **8c** were pooled and concentrated *in vacuo* to give **8c** (3.17 g, 5.36 mmol, 71%) as a clear, pale yellow, viscous oil. MS (PerkinElmer): 596.4 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.63 (m, 1), 5.51 (m, 1), 4.86 (m, 1), 4.63 (d, J = 7.0 Hz, 2), 3.26−3.25 (4), 2.24−2.33 (4), 2.09 (m, 2), 1.79−1.88 (4), 1.46−1.55 (4), 1.45 (s, 9), 1.36 (m, 2), 1.22−1.33 (22), 0.85−0.89 (9).

*4-((4-Oxo-4-(tridecan-7-yloxy)butyl)amino)butanoic Acid (Z)-Non-2-en-1-yl Ester (**9b**).* To a solution of **8c** (3.10 g, 5.24 mmol) in dry dichloromethane (50 mL) was added trifluoroacetic acid (12 mL). The mixture was stirred at room temperature, under nitrogen, for 3 h. Concentration *in vacuo* resulted in crude **9b**-TFA salt as a viscous, yellow oil, which was dissolved in dichloromethane (150 mL), washed with saturated aq. $NaHCO_3$ (3 × 150 mL), and dried (Na_2SO_4). Filtration and concentration *in vacuo* gave crude **9b**, which was purified by chromatography on a column of silica gel (100 g, 230−400 mesh) using a gradient of hexanes/EtOAc (60:40 to 10:90). Fractions containing **9b** were pooled and concentrated *in vacuo*, affording **9b** (2.03 g, 4.09 mmol, 78%) as a clear, colorless, viscous liquid. MS (PerkinElmer): 495.9 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.63 (m, 1), 5.48 (m, 1), 4.84 (m, 1), 4.62 (d, J = 7.0 Hz, 2), 3.60 (brs, 1), 3.01−3.09 (4), 2.40−2.48 (4), 2.08 (m, 2), 1.98−2.04 (4), 1.46−1.54 (4), 1.35 (m, 2), 1.20−1.31 (22), 0.84−0.88 (9).

*4-((((2-(Dimethylamino)ethyl)thio)carbonyl)(4-oxo-4-(tridecan-7-yloxy)butyl)amino)butanoate (Z)-Non-2-en-1-yl Ester (**10e**).* To a solution of **9b** (2.00 g, 4.00 mmol) in CH_2Cl_2 (50 mL), cooled in an ice water bath under nitrogen, was added triphosgene (1.19 g, 4.00 mmol) over 5 min followed by the addition of pyridine (1.58 g, 20.0 mmol, 5 equiv) over a period of 5 min. The mixture was allowed to warm to room temperature and stir for 4 h and then concentrated *in vacuo* (bath temperature, 23 °C). The residue was dissolved in pyridine (15 mL) and cooled in an ice-water bath under nitrogen, and then 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20 mmol) was added in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (175 mL). The solution was washed with 10% aq. citric acid (2 × 175 mL), saturated aq. $NaHCO_3$ (3 × 175 mL), and brine (175 mL) and dried (Na_2SO_4). Filtration and concentration *in vacuo* provided crude **10e** as an orange oil. The orange oil was dissolved in heptane (60 mL) and to the resulting solution was added methanol (45 mL) and

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water (15 mL). The resulting two-phase mixture was stirred for 30 min and then allowed to stand to 10 min to separate. The aq. MeOH lower phase was removed and this procedure was repeated two additional times. The heptane layer was then treated with activated charcoal (DARCO, 100 mesh, 0.75 g) and the mixture was allowed to stir for 16 h. The charcoal was removed by filtration through a pad of Celite, the filter cake was rinsed with heptane (50 mL), and the solvent was removed *in vacuo* to give **10e** (1.73 g, 2.76 mmol, 69%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.045 min, m/e = 627.5 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.63 (m, 1), 5.53 (m, 1), 4.87 (m, 1), 4.63 (d, J = 7.0 Hz, 2), 3.38 (brm, 4), 3.02 (t, J = 7.0 Hz, 2), 2.52 (t, J = 7.0 Hz, 2), 2.28−2.37 (4), 2.27 (s, 6), 2.09 (m, 2), 1.90 (brm, 4), 1.51 (brm, 4), 1.36 (m, 2), 1.22−1.34 (22), 0.88 (m, 9).

*4-((tert-Butoxycarbonyl)(4-oxo-4-(pentadecan-8-yloxy)butyl)-amino)butanoic Acid (Z)-Non-2-en-1-yl Ester (**8d**).* As described for the preparation of **8c**, **8b** (6.20 g, 15.00 mmol) in dry dichloromethane (100 mL) was treated with EDC-HCl (4.30 g, 22.50 mmol), 8-pentadecanol (3.43 g, 15.00 mmol), *i*-Pr_2NEt (1.94 g, 15.00 mmol, 2.62 mL), and DMAP (0.184 g, 1.50 mmol), leading to **8d** (6.36 g, 10.20 mmol, 68%) as a clear, pale yellow, viscous oil after work-up and purification by column chromatography. MS (PerkinElmer): 646.4 ($M + Na^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.64 (m, 1), 5.52 (m, 1), 4.86 (m, 1), 4.62 (d, J = 7.0 Hz, 2), 3.15−3.24 (4), 2.25−2.34 (4), 2.10 (m, 2), 1.80−1.86 (4), 1.47−1.54 (4), 1.45 (s, 9), 1.36 (m, 2), 1.22−1.32 (26), 0.86−0.89 (9).

*4-((4-Oxo-4-(pentadecan-8-yloxy)butyl)amino)butanoic Acid (Z)-Non-2-en-1-yl Ester (**9c**).* As described for the preparation of **9b**, a solution of **8d** (6.23 g, 10.00 mmol) in dry dichloromethane (100 mL) was treated with trifluoroacetic acid (24 mL) to give **9c** (4.14 g, 7.90 mmol, 79%) as a clear, colorless viscous liquid after work-up and purification by chromatography on a column of silica gel. MS (PerkinElmer): 524.1 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.64 (m, 1), 5.49 (m, 1), 4.84 (m, 1), 4.62 (d, J = 7.0 Hz, 2), 3.36 (brs, 1), 3.00−3.06 (brm, 4), 2.40−2.48 (4), 2.09 (m, 2), 1.97−2.04 (4), 1.46−1.53 (4), 1.37 (m, 2), 1.21−1.32 (28), 0.85−0.89 (9).

*4-(((((2-(Dimethylamino)ethyl)thio)carbonyl)(4-oxo-4-(pentadecan-8-yloxy)butyl)amino)butanoate (Z)-Non-2-en-1-yl Ester (**10f**).* As described for the synthesis of **10e**, **9b** (2.10 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10f** (1.93 g, 2.96 mmol, 74%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.052 min, m/e = 655.5 ($M + H^+$); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.56 (m, 1), 5.44 (m, 1), 4.77 (m, 1), 4.54 (d, J = 6.8 Hz, 2), 3.27 (brm, 4), 2.89 (t, J = 7.2 Hz, 2), 2.38 (t, J = 7.2 Hz, 2), 2.19 (m, 4), 2.14 (s, 6), 2.02 (m, 2), 1.77 (brm, 4), 1.45 (m, 4), 1.10−1.30 (28), 0.80 (m, 9).

*4-(((((2-(Diethylamino)propyl)thio)carbonyl)(4-oxo-4-(pentadecan-8-yloxy)butyl)amino)butanoate (Z)-Non-2-en-1-yl Ester (**10g**).* As described for the synthesis of **10e**, **9c** (2.10 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10g** (1.98 g, 2.84 mmol, 71%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.067 min, m/e = 697.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 5.60 (m, 1), 5.52 (m, 1), 4.86 (m, 1), 4.63 (d, J = 6.6 Hz, 2), 3.37 (brm, 4), 2.90 (t, J = 7.2 Hz, 2), 2.42−2.55 (6), 2.33 (m, 4), 2.10 (m, 2), 3.86 (brm, 4), 1.73 (m, 2), 1.50 (m, 4), 1.15−1.40 (28), 0.98 (t, J = 7.0 Hz, 6), 0.86 (m, 9).

*4-((tert-Butoxycarbonyl)(4-oxo-4-(heptadecan-9-yloxy)butyl)-amino)butanoic Acid (Z)-Non-2-en-1-yl Ester (**8e**).* As described for the preparation of **8c**, **8b** (6.20 g, 15.00 mmol) in dry dichloromethane (100 mL) was treated with EDC-HCl (4.30 g, 22.50 mmol), 9-heptadecanol (3.85 g, 15.00 mmol), *i*-Pr_2NEt (1.94 g, 15.00 mmol, 2.62 mL), and DMAP (0.184 g, 1.50 mmol), leading to **8e** (6.55 g, 10.05 mmol, 67%) as a clear, pale yellow, viscous oil after work-up and purification by column chromatography. MS (PerkinElmer): 674.5 ($M + Na^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.63 (m, 1), 5.51 (m, 1), 4.86 (m, 1), 4.62 (d, J = 7.0 Hz, 2), 3.16−3.24 (4), 2.25−2.34 (4), 2.09 (m, 2), 1.80−1.87 (4), 1.46−1.54 (4), 1.45 (s, 9), 1.36 (m, 2), 1.21−1.32 (30), 0.85−0.90 (9).

*4-((4-Oxo-4-(heptadecan-9-yloxy)butyl)amino)butanoic Acid (Z)-Non-2-en-1-yl Ester (**9d**).* As described for the preparation of **9b**, a solution of **8e** (6.52 g, 10.00 mmol) in dry dichloromethane (100 mL) was treated with trifluoroacetic acid (24 mL) to give **9d** (4.36 g, 7.90 mmol, 79%) as a clear, colorless viscous liquid after work-up and purification by chromatography on a column of silica gel. MS (PerkinElmer): 552.2 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.65 (m, 1), 5.51 (m, 1), 4.84 (m, 1), 4.63 (d, J = 7.0 Hz, 2), 3.02−3.10 (brm, 4), 2.93 (brs, 1), 2.40−2.48 (4), 2.08 (m, 2), 1.97−2.05 (4), 1.46−1.54 (4), 1.35 (m, 2), 1.22−1.33 (30), 0.85−0.89 (9).

*4-(((((2-(Dimethylamino)ethyl)thio)carbonyl)(4-oxo-4-(heptadecan-9-yloxy)butyl)amino)butanoate (Z)-Non-2-en-1-yl Ester (**10h**).* As described for the synthesis of **10e**, **9d** (2.00 g, 3.62 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.08 g, 3.62 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10h** (1.78 g, 2.61 mmol, 72%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.064 min, m/e = 683.5 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.64 (m, 1), 5.52 (m, 1), 4.87 (m, 1), 4.63 (d, J = 6.9 Hz, 2), 3.38 (brm, 4), 3.02 (t, J = 7.2 Hz, 2), 2.52 (t, J = 7.2 Hz, 2), 2.32 (m, 4), 2.27 (s, 6), 2.10 (m, 2), 1.90 (brm, 4), 1.51 (m, 4), 1.36 (m, 2), 1.20−1.33 (30), 0.87 (m, 9).

*4-(((((2-(Diethylamino)propyl)thio)carbonyl)(4-oxo-4-(heptadecan-9-yloxy)butyl)amino)butanoate (Z)-Non-2-en-1-yl Ester (**10i**).* As described for the synthesis of **10e**, **9d** (2.00 g, 3.62 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.08 g, 3.62 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10i** (1.75 g, 2.42 mmol, 67%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.065 min, m/e = 725.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 5.62 (m, 1), 5.53 (m, 1), 4.86 (m, 1), 4.62 (d, J = 6.6 Hz, 2), 3.69 (brm, 4), 2.90 (t, J = 7.2 Hz, 2), 2.54 (m, 6), 2.31 (m, 4), 2.09 (m, 2), 1.78 (m, 2), 1.50 (m, 4), 1.20−1.40 (36), 1.04 (t, J = 7.2 Hz, 6), 0.88 (m, 9).

*4,4′-((tert-Butoxycarbonyl)azanediyl)dibutyric Acid Bis-6-undecanoyl Ester (**8f**).* To a suspension of **7b** (14.46 g, 50 mmol) in anhydrous dichloromethane (500 mL) were added in order EDC-HCl (23.96 g, 125.0 mmol), 6-undecanol (17.24 g, 100.0 mmol), *i*-Pr_2NEt (12.92 g, 100.0 mmol, 8.71 mL), and DMAP (1.22 g, 10.00 mmol). The resulting mixture was stirred for 18 h at room temperature, under nitrogen, and then cast into dichloromethane (300 mL) and water (1.0 L). The organic phase was separated, washed with brine (1.0 mL), and dried (Na_2SO_4). Filtration and concentration *in vacuo*

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afforded the crude mixture as a pale yellow, viscous semisolid, which was purified by chromatography on a column of silica gel (1000 g, 230−400 mesh) using a gradient of hexanes/EtOAc (90:10 to 50:50), and fractions containing **8f** were pooled and concentrated *in vacuo* to provide **8f** (14.36 g, 24.00 mmol, 48%) as a clear, colorless, viscous liquid. MS (PerkinElmer): 620.5 ($M + Na^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.86 (m, 2), 3.16−3.24 (brm, 4), 2.27 (m, 4), 1.83 (m, 4), 1.46−1.54 (4), 1.43 (s, 9), 1.35−1.41 (4), 1.22−1.34 (24), 0.85−0.90 (12).

*4,4′-Azanediyldibutyric Acid Bis-undecan-6-yl Ester (**9e**).* As described for the preparation of **9b**, a solution of **8f** (13.76 g, 23.00 mmol) in dry dichloromethane (250 mL) was treated with trifluoroacetic acid (50 mL) to give **9e** (7.67 g, 15.41 mmol, 67%) as a clear, colorless viscous liquid after work-up and purification by chromatography on a column of silica gel. MS (PerkinElmer): 498.0 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.85 (m, 2), 3.04−3.12 (brm, 4), 2.44 (m, 4), 2.02 (m, 4), 1.45−1.54 (5), 1.21−1.32 (28), 0.88 (t, J = 7.2 Hz, 12).

*4,4′-[[[[3-(Dimethylamino)ethyl]thio]carbonyl]imino]bis-butanoic Acid Bis-6-undecanoyl Ester (**10j**).* As described for the synthesis of **10e**, **9e** (1.99 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10j** (1.78 g, 2.84 mmol, 71%) as a pale yellow, viscous oil. MS (PerkinElmer): 629.4 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.86 (m, 2), 3.33−3.41 (brm, 4), 3.01 (t, J = 6.8 Hz, 2), 2.51 (t, J = 6.8 Hz, 2), 2.27−2.33 (brm, 4), 2.26 (s, 6), 1.82−1.94 (4), 1.47−1.54 (8), 1.21−1.34 (24), 0.87 (t, J = 7.2 Hz, 12).

*4,4′-[[[[3-(Dimethylamino)propyl]thio]carbonyl]imino]bis-butanoic Acid Bis-6-undecanoyl Ester (**10k**).* As described for the synthesis of **10e**, **9e** (1.99 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10k** (1.80 g, 2.80 mmol, 70%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.048 min, m/e = 643.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.87 (m, 2), 3.37 (brm, 4), 2.91 (t, J = 7.2 Hz, 2), 2.26−2.40 (6), 2.22 (s, 6), 1.80 (m, 4), 1.43−1.56 (8), 1.20−1.37 (26), 0.88 (m, 12).

*4,4′-[[[[3-(Diethylamino)propyl]thio]carbonyl]imino]bis-butanoic Acid Bis-6-undecanoyl Ester (**10m**).* As described for the synthesis of **10e**, **9e** (1.99 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10m** (1.85 g, 2.76 mmol, 69%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.061 min, m/e = 671.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.87 (m, 2), 3.37 (brm, 4), 2.90 (t, J = 7.2 Hz, 2), 2.51 (q, J = 7.2 Hz, 4), 2.31 (m, 4), 1.89 (brm, 4), 1.75 (m, 4), 1.45−1.58 (8), 1.20−1.46 (24), 1.01 (t, J = 7.2 Hz, 6), 0.88 (m, 12).

*4,4′-((tert-Butoxycarbonyl)azanediyl)dibutyric Acid Bis-7-tridecanoyl Ester (**8g**).* A described for the preparation of **8f**, a suspension of **7b** (5.78 g, 20 mmol) in anhydrous dichloromethane (200 mL) was treated with EDC-HCl (9.58 g, 50.0 mmol), 7-tridecanol (8.00 g, 40.0 mmol), *i*-Pr_2NEt (5.16 g, 40.0 mmol, 6.96 mL), and DMAP (0.488 g, 2.00 mmol) to provide **8g** (8.90 g, 13.60 mmol, 68%), after work-up and chromatography on a column of silica gel, as a clear, pale yellow, viscous oil. MS (PerkinElmer): 676.4 ($M + Na^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.86 (m, 2), 3.16−3.25 (brm, 4), 2.27 (m, 4), 1.83 (m, 4), 1.47−1.54 (8), 1.45 (s, 9), 1.22−1.34 (32), 0.88 (t, J = 7.2 Hz, 12).

*4,4′-Azanediyldibutyric Acid Bis-tridecan-7-yl Ester (**9f**).* As described for the preparation of **9b**, a solution of **8g** (8.50 g, 13.0 mmol) in dry dichloromethane (100 mL) was treated with trifluoroacetic acid (20 mL) to give **9f** (3.38 g, 6.12 mmol, 47%) as a clear, colorless viscous liquid after work-up and purification by chromatography on a column of silica gel. MS (PerkinElmer): 554.3 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.84 (m, 2), 3.00−3.08 (brm, 4), 2.42 (m, 4), 2.01 (m, 4), 1.46−1.53 (8), 1.19−1.32 (33), 0.87 (t, J = 7.2 Hz, 12).

*4,4′-[[[[3-(Dimethylamino)propyl]thio]carbonyl]imino]bis-butanoic Acid Bis-7-tridecanoyl Ester (**10n**).* As described for the synthesis of **10e**, **9f** (2.21 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10n** (1.84 g, 2.64 mmol, 66%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.054 min, m/e = 699.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.86 (m, 2), 3.37 (brm, 4), 2.92 (t, J = 7.2 Hz, 2), 2.42 (m, 2), 2.24−2.44 (10), 1.80−1.96 (6), 1.46−1.57 (8), 1.18−1.37 (32), 0.87 (m, 12).

*4,4′-[[tert-Butoxycarbonyl]imino]bis-butanoic Acid Bis-8-pentadecanoyl Ester (**8h**).* To a solution of **7b** (13.0 g, 44.9 mmol) in dichloromethane (150 mL), cooled in an ice-water bath under nitrogen, was added EDC-HCl (24.1 g, 125 mmol). The mixture was allowed to stir for 15 min, and then Et_3N (12.6 g, 125 mmol), DMAP (2.7 g, 22.45 mmol), and 8-heptadecanol (20.3 g, 89.0 mmol) were added in order. The resulting mixture was allowed to warm to room temperature and stir for 12 h. The reaction was quenched with water (150 mL) and the organic phase was separated. The aq. phase was extracted with dichloromethane (3 × 100 mL) and the combined organic phases were washed with 10% aq. $NaHCO_3$ (250 mL) and brine (250 mL) and dried (Na_2SO_4). Concentration *in vacuo* provided crude **8h** as a light brown, viscous oil. The crude product was purified by chromatography on a column of silica gel (400 g, 100−200 mesh), packed with hexanes, and eluted with a gradient of hexanes/ethyl acetate from 100:0 to 75:25 using the flash technique. Fractions containing **8h** were cooled down and concentrated *in vacuo* to give **8h** (19.0 g, 26.9 mmol, 60%) as a clear, pale yellow, viscous oil. ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 4.87 (m, 2), 3.22 (brm, 4), 2.24 (m, 4), 1.85 (m, 4), 1.48−1.64 (8), 1.46 (s, 9), 1.13−1.32 (40), 0.88 (m, 12).

*4,4′-[Imino]bis-butanoic Acid Bis-8-pentadecanoyl Ester (**9g**).* To a solution of **8h** (18.9 g, 26.5 mmol) in dichloromethane (190 mL), cooled in an ice-water bath under nitrogen, was added CF_3CO_2H (16.39 mL, 214.2 mmol, 8 equiv) at such a rate so as to maintain the internal temperature of 0−5 °C. The mixture was then allowed to warm to room temperature and stir for 6 h. The solvent and excess CF_3CO_2H were removed *in vacuo* and the residue was taken up in dichloromethane (150 mL), washed with 10% aq. $NaHCO_3$ (3 × 150 mL) and brine (250 mL), and dried (Na_2SO_4). Filtration and concentration *in vacuo* provided crude **9g** as a viscous yellow oil, which was purified by chromatography on a column of silica gel (400 g, 100−200 mesh), packed with hexanes/EtOAc (75:25), and eluted with a gradient of hexanes/EtOAc (75:25 to 25:75) using the flash technique. Fractions containing **9g** were pooled and concentrated *in vacuo* to afford **9g** as a pale yellow, viscous oil (12,4 g, 20.2 mmol, 76%). LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min,

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hold 3.00 min): RT 1.756 min, m/e = 610.6 (M + H^+); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 4.86 (m, 2), 2.88 (m, 4), 2.40 (m, 4), 1.96 (m, 4), 1.46−1.55 (8), 1.20−1.34 (40), 0.86 (m, 12).

*4,4′-[[[[3-(Dimethylamino)ethyl]thio]carbonyl]imino]bis-butanoic Acid Bis-8-pentadecanoyl Ester (**10o**).* As described for the synthesis of **10e**, **9g** (2.44 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10o** (2.22 g, 3.00 mmol, 74%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.077 min, m/e = 741.4 (M + H^+); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 4.87 (m, 2), 3.39 (brm, 4), 3.04 (t, J = 6.8 Hz, 2), 2.56 (t, J = 6.8 Hz, 2), 2.20−2.35 (10), 1.86 (brm, 4), 1.42−1.55 (8), 1.16−1.32 (40), 0.88 (m, 12).

*4,4′-[[[[3-(Dimethylamino)propyl]thio]carbonyl]imino]bis-butanoic Acid Bis-8-pentadecanoyl Ester (**10p**).* As described for the synthesis of **10e**, **9g** (2.44 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10p** (2.32 g, 3.08 mmol, 77%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.058 min, m/e = 755.5 (M + H^+); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 4.89 (m, 2), 3.39 (brm, 4), 2.93 (t, J = 7.2 Hz, 2), 2.30−2.46 (6), 2.28 (s, 6), 1.75−1.93 (6), 1.45−1.60 (8), 1.20−1.35 (40), 0.92 (m, 12).

*4,4′-[[[[3-(Diethylamino)propyl]thio]carbonyl]imino]bis-butanoic Acid Bis-8-pentadecanoyl Ester (**10q**).* As described for the synthesis of **10e**, **9g** (2.44 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10q** (2.29 g, 2.92 mmol, 73%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.052 min, m/e = 783.6 (M + H^+); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 4.86 (m, 2), 3.36 (brm, 4), 2.90 (t, J = 7.2 Hz, 2), 2.47−2.55 (6), 2.31 (m, 4), 1.89 (brm, 4), 1.75 (m, 2), 1.45−1.56 (8), 1.20−1.40 (40), 1.01 (t, J = 7.2 Hz, 6), 0.87 (m, 12).

*(Z)-N-(tert-Butoxycarbonyl)-N-(2-(non-2-en-1-yloxy)-2-oxoethyl)glycine (**8i**).* To a solution of 2-[*tert*-butoxycarbonyl-(carboxymethyl)amino]acetic acid (CombiBlocks, #SS-1100) (40.0 g, 0.172 mol) in dry dichloromethane (500 mL) were added in order EDC-HCl (32.97 g, 0.172 mol), (*Z*)-non-2-en-1-ol (24.47 g, 0.172 mol), *i*-Pr_2NEt (22.23 g, 0.172 mol, 29.96 mL), and DMAP (2.10 g, 17.2 mmol). The mixture was allowed to stir for 5 h at room temperature under nitrogen, then diluted with dichloromethane (150 mL), washed with water (0.75 L) and brine (0.75 L), and dried (Na_2SO_4). Filtration and concentration *in vacuo* afforded crude **8i** as a viscous liquid, which was purified by chromatography on a column of silica gel (750 g, 230−400 mesh) using a gradient of dichloromethane/methanol from 100:0 to 95:5. Fractions containing **8i** were pooled and concentrated *in vacuo* to furnish **8i** (17.78 g, 49.9 mmol, 29%) as a clear, pale yellow, viscous liquid. MS (PerkinElmer): 356.1 (M − H); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 10.10 (brs, 1), 5.66 (m, 1), 5.50 (m, 1), 4.70 (m, 2), 4.11 (s, 2), 4.00 (s, 2), 2.16 (m, 2), 1.43 (s, 4.5), 1.42 (s, 4.5), 1.35 (m, 2), 1.20−1.32 (6), 0.86 (t, J = 7.2 Hz, 3).

*(Z)-Non-2-en-1-yl N-(tert-butoxycarbonyl)-N-(2-oxo-2-(undecan-6-yloxy)ethyl)glycinate (**8j**).* To a solution of **8i** (5.36 g, 15.00 mmol) in anhydrous dichloromethane (100 mL) were added in order EDC-HCl (2.18 g, 15.00 mmol), 6-undecanol (2.58 g, 15.00 mmol), *i*-Pr_2NEt (1.94 g, 15.00 mmol, 2.61 mL), and DMAP (0.183 g, 1.50 mmol). The mixture was allowed to stir for 5 h at room temperature, under nitrogen, and then diluted with dichloromethane (100 mL). The mixture was washed with water (200 mL) and brine (200 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo* to provide **8j** as a yellow, viscous liquid. Crude **8j** was purified by chromatography on a column of silica gel (250 g, 230−400 mesh) using a gradient of hexanes/ethyl acetate from 100:0 to 70:30. Fractions containing **8j** were pooled and concentrated *in vacuo* to yield **8j** (4.38 g, 8.55 mmol, 57%) as a clear, pale yellow viscous oil. MS (PerkinElmer): 511.4 (M − H); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.64 (m, 1), 5.50 (m, 1), 4.90 (m, 1), 4.68 (m, 2), 4.11 (s, 1), 4.09 (s, 1), 3.99 (s, 2), 2.09 (m, 2), 1.48−1.56 (6), 1.44 (s, 4.5), 1.43 (s, 4.5), 1.36 (m, 2), 1.22−1.32 (16), 0.84−0.90 (9).

*(Z)-Non-2-en-1-yl (2-Oxo-2-(undecan-6-yloxy)ethyl)glycinate (**9h**).* To a solution of **8j** (4.09 g, 8.00 mmol) in anhydrous dichloromethane (60 mL) was added trifluoroacetic acid (35 mL), and the resulting mixture was stirred at room temperature under nitrogen for 3 h. The solvent and excess CF_3CO_2H were removed *in vacuo* and the residue was taken up in dichloromethane (75 mL), washed with 10% aq. $NaHCO_3$ (3 × 75mL) and brine (125 mL), and dried (Na_2SO_4). Filtration and concentration *in vacuo* provided crude **9h** as a viscous yellow oil, which was purified by chromatography on a column of silica gel (75 g, 230−400 mesh) and eluted with a gradient of hexanes/EtOAc (75:25 to 20:80). Fractions containing **9h** were pooled and concentrated *in vacuo* to provide **9h** (2.70 g, 6.56 mmol, 82%) as a clear, pale yellow, viscous oil. MS (PerkinElmer): 412.4 (M + H^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.67 (m, 1), 5.50 (m, 1), 4.96 (m, 1), 4.75 (d, J = 7.0 Hz, 2), 3.98 (s, 2), 3.96 (s, 2), 2.08 (m, 2), 1.52−1.58 (4), 1.36 (m, 2), 1.20−1.32 (19), 0.85−0.90 (9).

*(Z)-Non-2-en-1-yl N-(((2-(Dimethylamino)ethyl)thio)carbonyl)-N-(2-oxo-2-(undecan-6-yloxy)ethyl)glycinate (**10r**).* As described for the synthesis of **10e**, **9h** (1.64 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10r** (1.25 g, 2.32 mmol, 58%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.041 min, m/e = 543.4 (M + H^+); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.66 (m, 1), 5.50 (m, 1), 4.92 (m, 1), 4.70 (t, J = 7.2 Hz, 2), 4.19−4.24 (4), 3.05 (m, 2), 2.53 (m, 2), 2.27 (s, 6), 2.08 (m, 2), 1.43 (m, 4), 1.20−1.40 (20), 0.88 (m, 9).

*(Z)-Non-2-en-1-yl N-(tert-Butoxycarbonyl)-N-(2-oxo-2-(pentadecan-8-yloxy)ethyl)glycinate (**8k**).* As described for the synthesis of **8j**, the reaction of **8i** (5.36 g, 15.00 mmol) in anhydrous dichloromethane (100 mL) with 8-pentadecanol (3.43 g, 15.00 mmol), EDC-HCl (2.18 g, 15.00 mmol), *i*-Pr_2NEt (1.94 g, 15.00 mmol, 2.61 mL), and DMAP (0.183 g, 1.50 mmol) led to **8k** (4.68 g, 8.25 mmol, 55%) as a clear, pale yellow, viscous oil after purification by chromatography on a column of silica gel. MS (PerkinElmer): 590.3 (M + Na^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.65 (m, 1), 5.51 (m, 1), 4.90 (m, 1), 4.69 (m, 2), 4.11 (s, 1), 4.08 (s, 1), 3.99 (s, 2), 2.09 (m, 2), 1.46−1.56 (4), 1.44 (s, 4.5), 1.43 (s, 4.5), 1.37 (m, 2), 1.22−1.32 (26), 0.86−0.90 (9).

*(Z)-Non-2-en-1-yl (2-Oxo-2-(pentadecan-8-yloxy)ethyl)glycinate (**9i**).* As described for the synthesis of **9h**, a solution of **8k** (4.54 g, 8.00 mmol) in dry dichloromethane (60 mL) was treated with trifluoroacetic acid (35 mL), leading to **9i** (2.77 g, 5.92 mmol, 74%) as a clear, yellow, viscous oil after work-up and purification by column

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chromatography. MS (PerkinElmer): 468.2 (M + H^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.69 (m, 1), 5.50 (m, 1), 4.96 (m, 1), 4.75 (d, J = 7.0 Hz, 2), 3.98 (s, 2), 3.95 (s, 2), 2.08 (m, 2), 1.52−1.58 (4), 1.36 (m, 2), 1.20−1.33 (27), 0.86−0.89 (9).

*(Z)-Non-2-en-1-yl N-(((2-(Dimethylamino)propyl)thio)carbonyl)-N-(2-oxo-2-(pentadecan-8-yloxy)ethyl)glycinate (**10s**).* As described for the synthesis of **10e**, **9i** (1.87 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10s** (1.54 g, 2.52 mmol, 63%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.049 min, m/e = 613.5 (M + H^+); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 5.65 (m, 1), 5.50 (m, 1), 4.92 (m, 1), 4.69 (m, 2), 4.13−4.30 (4), 2.95 (t, J = 7.2 Hz, 2), 2.32 (t, J = 7.2 Hz, 2), 2.20 (s, 6), 2.08 (m, 2), 1.78 (m, 2), 1.18−1.42 (32), 0.87 (m, 9).

*(Z)-Non-2-en-1-yl N-(tert-Butoxycarbonyl)-N-(2-oxo-2-(heptadecan-9-yloxy)ethyl)glycinate (**8m**).* As described for the synthesis of **8j**, the reaction of **8i** (5.36 g, 15.00 mmol) in anhydrous dichloromethane (100 mL) with 9-heptadecanol (3.85 g, 15.00 mmol), EDC-HCl (2.18 g, 15.00 mmol), *i*-Pr_2NEt (1.94 g, 15.00 mmol, 2.61 mL), and DMAP (0.183 g, 1.50 mmol) led to **8m** (5.00 g, 8.40 mmol, 56%) as a clear, pale yellow, viscous oil after purification by chromatography on a column of silica gel. MS (PerkinElmer): 596.4 (M + H^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.66 (m, 1), 5.50 (m, 1), 4.90 (m, 1), 4.69 (m, 2), 4.11 (s, 1), 4.08 (s, 1), 3.99 (s, 2), 2.09 (m, 2), 1.47−1.56 (4), 1.44 (s, 4.5), 1.43 (s, 4.5), 1.35 (m, 2), 1.21−1.33 (30), 0.85−0.89 (9).

*(Z)-Non-2-en-1-yl (2-Oxo-2-(heptadecan-9-yloxy)ethyl)glycinate (**9j**).* As described for the synthesis of **9h**, a solution of **8m** (4.77 g, 8.00 mmol) in dry dichloromethane (60 mL) was treated with trifluoroacetic acid (35 mL), leading to **9j** (3.33 g, 6.72 mmol, 84%) as a clear, yellow, viscous oil after work-up and purification by column chromatography. MS (PerkinElmer): 496.2 (M + H^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 5.69 (m, 1), 5.50 (m, 1), 4.97 (m, 1), 4.76 (d, J = 7.0 Hz, 2), 3.95 (s, 2), 3.93 (s, 2), 2.09 (m, 2), 1.51−1.58 (4), 1.35 (m, 2), 1.22−1.32 (31), 0.85−0.89 (9).

*(Z)-Non-2-en-1-yl N-(((2-(Dimethylamino)ethyl)thio)carbonyl)-N-(2-oxo-2-(heptadecan-9-yloxy)ethyl)glycinate (**10t**).* As described for the synthesis of **10e**, **9j** (1.98 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10t** (1.38 g, 2.20 mmol, 55%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.059 min, m/e = 627.5 (M + H^+); ^{1}H-NMR (400 mHz, $CDCl_3$): δ = 5.67 (m, 1), 5.51 (m, 1), 4.91 (m, 1), 4.70 (m, 2), 4.17−4.27 (4), 3.07 (m, 2), 2.52 (m, 2), 2.27 (s, 6), 2.08 (m, 2), 1.53 (brm, 4), 1.20−1.41 (32), 0.88 (m, 9).

*(Z)-Non-2-en-1-yl N-(((2-(Dimethylamino)propyl)thio)carbonyl)-N-(2-oxo-2-(heptadecan-9-yloxy)ethyl)glycinate (**10u**).* As described for the synthesis of **10e**, **9j** (1.98 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10u** (1.56 g, 2.44 mmol, 61%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.047 min, m/e = 641.5 (M + H^+); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 5.66 (m, 1), 5.50 (m, 1), 4.92 (m, 1), 4.70 (m, 2), 4.15−4.27 (4), 2.96 (t, J = 6.9 Hz, 2), 2.38 (t, J = 7.2 Hz, 2), 2.46 (s, 6), 2.08 (m, 2), 1.81 (m, 2), 1.52 (brm, 4), 1.18−1.42 (32), 0.88 (m, 9).

*(Z)-Non-2-en-1-yl N-(((2-(Diethylamino)propyl)thio)carbonyl)-N-(2-oxo-2-(heptadecan-9-yloxy)ethyl)glycinate (**10v**).* As described for the synthesis of **10e**, **9j** (1.98 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10v** (1.58 g, 2.36 mmol, 59%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.063 min, m/e = 669.5 (M + H^+); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 5.64 (m, 1), 5.50 (m, 1), 4.92 (m, 1), 4.15−4.38 (4), 2.94 (t, J = 7.2 Hz, 2), 2.43−2.53 (6), 2.09 (m, 2), 1.73 (m, 4), 1.52 (brm, 4), 1.16−1.42 (32), 1.00 (t, J = 7.2 Hz, 6), 0.87 (m, 9).

*2,2′-((tert-Butoxycarbonyl)azanediyl)diacetic Acid Bis-undecan-6-yl Ester (**8n**).* To a solution of 2-[*tert*-butoxycarbonyl-(carboxymethyl)amino]acetic acid (CombiBlocks, #SS-1100) (40.0 g, 0.172 mol) in dry dichloromethane (750 mL) were added in order EDC-HCl (65.94 g, 0.344 mol), 6-undecanol (59.27 g, 0.344 mol), *i*-Pr_2NEt (44.46 g, 0.344 mol, 59.92 mL), and DMAP (4.20 g, 34.4 mmol). The mixture was allowed to stir for 5 h at room temperature under nitrogen, then diluted with dichloromethane (250 mL), washed with water (1.0 L) and brine (1.0 L), and dried (Na_2SO_4). Filtration and concentration *in vacuo* afforded crude **8n** as a yellow, viscous liquid, which was purified by chromatography on a column of silica gel (750 g, 230−400 mesh) using a gradient of dichloromethane/methanol from 100:0 to 95:5. Fractions containing **8n** were pooled and concentrated *in vacuo* to furnish **8i** (50.32 g, 92.9 mmol, 54%) as a clear, pale yellow, viscous liquid. MS (PerkinElmer): 564.3 (M + Na^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.87−4.94 (2), 4.07 (s, 2), 3.97 (s, 2), 1.49−1.56 (8), 1.43 (s, 9), 1.22−1.34 (24), 0.84−0.90 (12).

*2,2′-(Azanediyl)diacetic Acid Bis-undecan-6-yl Ester (**9k**).* As described for the synthesis of **9h**, a solution of **8n** (4.33 g, 8.00 mmol) in dry dichloromethane (60 mL) was treated with trifluoroacetic acid (35 mL), leading to **9k** (3.33 g, 6.72 mmol, 84%) as a clear, yellow, viscous oil after work-up and purification by column chromatography. MS (PerkinElmer): 442.2 (M + H^+); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.98 (m, 2), 3.92 (s, 4), 1.52−1.60 (8), 1.20−1.35 (25), 0.88 (t, J = 7.2 Hz, 12).

*2,2′-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)-diacetic Acid Bis-undecan-6-yl Ester (**10w**).* As described for the synthesis of **10e**, **9k** (1.77 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10w** (1.55 g, 2.64 mmol, 66%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.053 min, m/e = 587.4 (M + H^+); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.92 (m, 2), 4.22 (s, 2), 4.16 (s, 2), 2.96 (t, J = 7.2 Hz, 2), 2.44 (m, 2), 2.30 (s, 6), 1.85 (m, 2), 1.47−1.62 (8), 1.17−1.40 (24), 0.87 (m, 12).

*2,2′-((((3-(Diethylamino)propyl)thio)carbonyl)azanediyl)diacetic Acid Bis-undecan-6-yl Ester (**10x**).* As described for the synthesis of **10e**, **9k** (1.77 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15

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mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10x** (1.40 g, 2.28 mmol, 57%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.049 min, m/e = 615.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.92 (m, 2), 4.44 (s, 2), 4.16 (s, 2), 2.94 (t, J = 7.2 Hz, 2), 2.50−2.62 (6), 1.80 (m, 2), 1−47-1.62 (8), 1.18−1.37 (24), 1.04 (t, J = 7.2 Hz, 6), 0.88 (m, 12).

*2,2′-((tert-Butoxycarbonyl)azanediyl)diacetic Acid Bis-pentadecan-8-yl Ester (**8o**).* As described for the synthesis of **8n**, a solution of 2-[*tert*-butoxycarbonyl(carboxymethyl)amino]acetic acid (Combi-Blocks, #SS-1100) (40.0 g, 0.172 mol) in dry dichloromethane (750 mL) was treated with EDC-HCl (65.94 g, 0.344 mol), 8-pentadecanol (78.57 g, 0.344 mol), *i*-Pr_2NEt (44.46 g, 0.344 mol, 59.92 mL), and DMAP (4.20 g, 34.4 mmol) to give, after work-up and purification by column chromatography, **8o** (65.24 g, 99.8 mmol, 58%) as a clear, pale yellow, viscous oil. MS (PerkinElmer): 652.6 (M − H); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.90 (m, 2), 4.06 (s, 2), 3.97 (s, 2), 1.47−1.44 (8), 1.43 (s, 9), 1.20−1.32 (40), 0.84−0.88 (12).

*2,2′-(Azanediyl)diacetic Acid Bis-pentadecan-8-yl Ester (**9m**).* As described for the synthesis of **9h**, a solution of **8o** (5.23 g, 8.00 mmol) in dry dichloromethane (60 mL) was treated with trifluoroacetic acid (35 mL), leading to **9m** (3.33 g, 6.72 mmol, 84%) as a clear, yellow, viscous oil after work-up and purification by column chromatography. MS (PerkinElmer): 554.4 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.97 (m, 2), 3.95 (s, 4), 1.50−1.60 (8), 1.20−1.32 (41), 0.88 (t, J = 7.2 Hz, 12).

*2,2′-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)diacetic Acid Bis-pentadecan-8-yl Ester (**10y**).* As described for the synthesis of **10e**, **9m** (2.21 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(dimethylamino)-propanethiol-HCl[16] (3.11 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10y** (1.90 g, 2.72 mmol, 68%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.053 min, m/e = 699.5 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.91 (m, 2), 4.21 (s, 2), 4.16 (s, 2), 2.96 (t, J = 7.2 Hz, 2), 2.44 (m, 2), 2.29 (s, 6), 1.84 (m, 2), 1.45−1.60 (8), 1.17−1.40 (40), 0.87 (m, 12).

*2,2′-((((3-(Diethylamino)propyl)thio)carbonyl)azanediyl)diacetic Acid Bis-pentadecan-8-yl Ester (**10z**).* As described for the synthesis of **10e**, **9m** (2.21 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 3-(diethylamino)-propanethiol-HCl[17] (3.67 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10z** (1.74 g, 2.40 mmol, 68%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.051 min, m/e = 727.6 ($M + H^+$); ^{1}H-NMR (300 mHz, $CDCl_3$): δ = 4.92 (m, 2), 4.21 (s, 2), 4.16 (s, 2), 2.95 (t, J = 7.2 Hz, 2), 2.50−2.62 (6), 1.79 (m, 2), 1.46−1.58 (8), 1.20−1.37 (40), 1.04 (t, J = 7.2 Hz, 6), 0.87 (m, 12).

*2,2′-((tert-Butoxycarbonyl)azanediyl)diacetic Acid Bis-heptadecan-9-yl Ester (**8p**).* As described for the synthesis of **8n**, a solution of 2-[*tert*-butoxycarbonyl(carboxymethyl)amino]acetic acid (Combi-Blocks, #SS-1100) (40.0 g, 0.172 mol) in dry dichloromethane (750 mL) was treated with EDC-HCl (65.94 g, 0.344 mol), 9-heptadecanol (88.22 g, 0.344 mol), *i*-Pr_2NEt (44.46 g, 0.344 mol, 59.92 mL), and DMAP (4.20 g, 34.4 mmol) to give, after work-up and purification by column chromatography, **8p** (73.29 g, 0.103 mmol, 60%) as a clear, pale yellow, viscous oil. MS (PerkinElmer): 732.4 ($M + Na^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.90 (m, 2), 4.07 (s, 2), 3.98 (s, 2), 1.47−1.55 (8), 1.44 (s, 9), 1.22−1.34 (48), 0.87 (t, J = 7.2 Hz, 12).

*2,2′-(Azanediyl)diacetic Acid Bis-heptadecan-9-yl Ester (**9n**).* As described for the synthesis of **9h**, a solution of **8p** (5.68 g, 8.00 mmol) in dry dichloromethane (60 mL) was treated with trifluoroacetic acid (35 mL), leading to **9n** (3.71 g, 6.08 mmol, 76%) as a clear, yellow, viscous oil after work-up and purification by column chromatography. MS (PerkinElmer): 610.4 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.98 (m, 2), 3.99 (s, 4), 1.51−1.59 (8), 1.20−1.32 (49), 0.88 (t, J = 7.2 Hz, 12).

*2,2′-((((3-(Dimethylamino)ethyl)thio)carbonyl)azanediyl)diacetic Acid Bis-heptadecan-9-yl Ester (**10aa**).* As described for the synthesis of **10e**, **9n** (2.44 g, 4.00 mmol) in CH_2Cl_2 (50 mL) was treated with triphosgene (1.19 g, 4.00 mmol) and pyridine (1.58 g, 20.0 mmol), the mixture was concentrated *in vacuo* and diluted with pyridine (15 mL), and to the resulting solution (ice-water bath under nitrogen) was added 2-(dimethylamino)-ethanethiol-HCl (Sigma-Aldrich, D141003) (2.83 g, 20.0 mmol) in one portion. The solution was allowed to warm to room temperature and stir for 20 h. The reaction was worked up and purified as described for the preparation of **10e** to afford **10aa** (1.90 g, 2.56 mmol, 64%) as a pale yellow, viscous oil. LC−MS (Shimadzu 2020; ELSD A: water/0.05% TFA: B: CH_3CN/0.05% TFA 80:20 to 0:100 A/B at 2.00 min, hold 3.00 min): RT 1.071 min, m/e = 741.6 ($M + H^+$); ^{1}H-NMR (500 mHz, $CDCl_3$): δ = 4.91 (m, 2), 4.21 (s, 2), 4.17 (s, 2), 3.05 (t, J = 7.0 Hz, 2), 2.51 (t, J = 7.0 Hz, 2), 2.26 (s, 6), 1.46−1.55 (8), 1.19−1.33 (48), 0.87 (t, J = 7.2 Hz, 12).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01407.

Molecular formula strings (CSV)

HPLC traces and ^{1}H-NMR spectra for final lipid structures (PDF)

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Notes

The authors declare the following competing financial interest(s): KR and other Arcturus Therapeutics employees have stock options some of which may have been exercised.

■ ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; BOC-anhydride, di-*tert*-butyl dicarbonate; BOC, tert-butoxy-carbonyl; cLog*D*, calculated Log*D*, octanol/buffer (pH 7.4); CRISPR, clustered regularly interspaced short palindromic repeats; DLin-DMA, 1,2-dilinoleyoxy-3-dimethylaminopropane; DLin-MC3-DMA, (6*Z*,9*Z*,28*Z*,31*Z*)-heptatriacont-6,9,28,31-tetraene-19-yl 4-(dimethylamino)butanoate; DMAP, 4-dimethylamino-pyridine; DMG-PEG, (1,2-dimyristoyl-*sn*-glycerol, methoxypolyethylene glycol; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DMSO, dimethyl sulfoxide; ED_{50}, median effective dose; EDC-HCl, ethylcarbodimide hydrochloride; ELSD, evaporative light scattering detection; ESI, electrospray ionization; FIX, factor IX; FVII, factor VII; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; LC−MS, liquid chromatography−mass spectroscopy; LDL-R, low-density lipoprotein receptor; LNP, lipid nanoparticle; LUNAR, Lipid-enabled and Unlocked Nucleomonomer Agent-modified RNA; MES, 2-(*N*-morpholino)ethanesulfonic acid; miRNA, microRNA; mRNA, messenger RNA; MS, mass spectroscopy; MTBE, methyl *tert*-butyl ether; pK_a, acid dissociation constant; RNA, ribonucleic acid; siRNA, small interfering RNA; TFA, trifluoroacetic acid; TNS, 6-(*p*-toluidinyl)naphthalene-2-sulfonic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol

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