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Novel potent apoA-I peptide mimetics that stimulate cholesterol efflux and pre-β particle formation in vitro

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ABSTRACT

Reverse cholesterol transport (RCT) is believed to be the primary mechanism by which HDL and its major protein apoA-I protect against atherosclerosis. Starting from the inactive 22-amino acid peptide representing the consensus sequence of the class A amphipathic helical repeats of apoA-I, we designed novel peptides able to mobilize cholesterol from macrophages in vitro, and to stimulate the formation of 'nascent HDL' particles, with potency comparable to the entire apoA-I protein.

High-density lipoproteins (HDL) protect against atherosclerosis by multiple mechanisms[1] one of which is considered to be reverse cholesterol transport (RCT), a process that involves the efflux of cholesterol from arterial macrophages to HDL, ultimately resulting in elimination of excess cholesterol to the liver for excretion in the bile and feces.[2] RCT is a regulated process promoted by specific transporters in vivo,[3] such as the ATP-binding cassette A1 (ABCA1), that mediates cholesterol efflux from macrophages to the main protein component of HDL, the apolipoprotein A-I (apoA-I), to generate nascent HDL particles.[4] The anti-atherogenic effects of apoA-I have been documented in animal models[5] and in humans,[6] and efforts are being devoted to develop treatments based on direct infusion of apoA-I, or pharmacological agents that can upregulate apoA-I production.[7] Due to difficulties in manufacturing apoA-I at the scale required for therapeutic intervention, one alternative, promising approach is to develop apoA-I peptide mimetics.[8]

apoA-I is composed of multiple repeats of a unique secondary structural element defined as the class A amphipathic α-helix.[9] In this helix, negatively charged amino acid residues are clustered at the center of the polar face, while positively charged residues are at the interface between the hydrophilic and the hydrophobic face. Designed peptides with amphipathic helical character or peptides based on more than one repeat of apoA-I have been shown to promote cholesterol efflux from cells and to have antioxidant and anti-inflammatory effects.[10] One of these peptides, D-4F, was shown to have anti-atherogenic effect in animal models[11] and has advanced to human clinical trials.

Our approach to develop apoA-I peptide mimetics was to start from the 22-mer 'apoA-I consensus sequence' (apoA-I_{cons}, Fig. 1 and compound **1**, Table 1) based on the tandem repeat sequences of apoA-I.[12] When tested in an in vitro cholesterol efflux assay, apoA-I_{cons} (**1**) showed no ability to promote cholesterol efflux from macrophages (Table 1). Therefore we explored a series of substitutions aimed at (i) stabilizing the class A α-helical motif, (ii) modulating lipophilicity, and (iii) modulating the negative charge density. The peptides were tested in an in vitro cholesterol efflux assay and screened for cytotoxicity in macrophages (RAW cell) and red blood cells.

apoA-I_{cons} has a negative charged residue at position 13 (E^{13}, black arrow in Fig. 1) which occurs on the hydrophobic face of the helix. To test the hypothesis that increasing the hydrophobic character in the context of stabilizing the α-helical conformation might promote cholesterol efflux from macrophages, E^{13} was substituted with α-aminoisobutyric acid (Aib).

The analog with the substitution E^{13}→Aib (peptide **2**, Table 1) was unable to stimulate cholesterol efflux in vitro. However the

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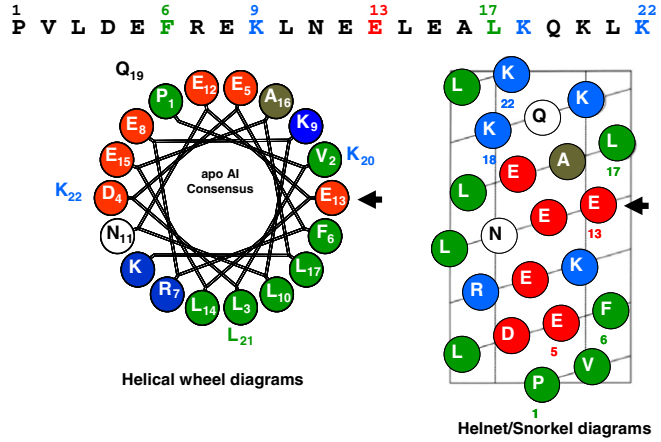

Figure 1. Helical wheel (left) and Helnet/Snorkel (right) diagrams of apoA-I_{cons}. The sequence of the consensus peptide is given above the helical wheel and helical net diagrams. Color code: red, acidic; blue, basic; green, hydrophobic; bold black, Asn and Gln. The negative charged E^{13} at position 13 (arrow) occurs on the hydrophobic face of the helix.

Table 1
In vitro cholesterol efflux from macrophages and cytotoxicity of compounds **1**–**8**

Compd	Sequence	CE[a] EC_{50} (μM)	Cytotoxicity[b,c] IC_{50}[b] (μM)
1	ApoA-I_{cons}	na	40
2	PVLDEFREKLNEAibLEALKQKLK	6%@100	>160
3	PVLDEFREKLNEAibLEAibLKQKLK	23	>160
4	PVLDEFREKLNEhFLLEALKQKLK	2.3	>160
5	PVLDEFRGlaKLNEAibLEALKQKLK	44	75
6	PVLDEFREKLNEAibLGlaALKQKLK	41	75
7	PVLDEFRGlaKLNEALEALKQKLK	142	40
8	PVLDEFREKLNEALGlaALKQKLK	234	75

[a] In vitro cholesterol efflux from RAW cells. Values are means of three experiments (na = not active).
[b] RBC lysis assay.
[c] No cytotoxicity up to 160 μM in macrophage RAW cells.

analog with the double substitutions Aib^{13}/Aib^{16} (E^{13}→Aib and A^{16}→Aib (**3**) showed some activity with an EC_{50} = 23μM (Table 1).

Surprisingly, the introduction of just one single bulky hydrophobic residue such as hexafluoroleucine (hF-Leu) in position 13 brought a large beneficial effect on potency (peptide **4**, Table 1), with EC_{50} = 2.3 μM.

To maintain the total negative charge density of the consensus sequence and compensate the loss of the carboxylate at position 13, the substitution E^{13}→Aib^{13} was combined with the introduction of γ-carboxyglutamic acid (Gla) either in place of E^8 or E^{16}, both on the hydrophilic face of the helix.

Both analogs Gla^8/Aib^{13} and Gla^{15}/Aib^{13}, peptides **5** and **6** (Table 1), showed a gain in potency compared to the single-substituted Aib^{13} analog (**2**), with EC_{50} = 44 and 41 μM, respectively. The effect on cholesterol efflux activity depended on both the Gla and Aib residues, since the corresponding control peptides with Gla/Ala at the same positions, peptides **7** and **8**, were less potent, with EC_{50} = 142 and 234 μM, respectively (Table 1).

However, all peptides containing Gla (**5–8**) were cytotoxic, indicating that their efflux activity could be due to disruption of the membrane integrity.

To further explore the effect of altering hydrophobicity, we introduced long-chain (C_{5-7}) hydrocarbon amino acids containing olefin-bearing tethers, in either *R*- or *S*-configuration (R_{5H} S_{5H}, R_{6H}, S_{7H}, Fig. 2) at specific positions of the consensus sequence. In addition to increasing the hydrophobic character of the peptide, these residues offer the possibility of stabilizing its helical conformation by formation of a cyclic, stapled structure between residues on the same face of the α-helix (positions $i - i + 4$ or $i - i + 7$).

This can be accomplished by ring-closing metathesis[13] (RCM, Fig. 2). For this purpose the fully elongated resin-bound peptide was exposed to a ruthenium catalyst that promotes cross-linking of the alkenyl chains through olefin metathesis,[14] thereby forming an all-hydrocarbon macrocyclic cross-link.

The hydrocarbon bridge was engineered on the hydrophobic face of the helix of the consensus sequence, and position 13 (E^{13}) was kept constant for replacement, based on previous results. We initially formed an eight-member ($i - i + 4$) ring, by linking amino acids at position 9–13 (K^9/E^{13}) (peptides **10** and **12**, Table

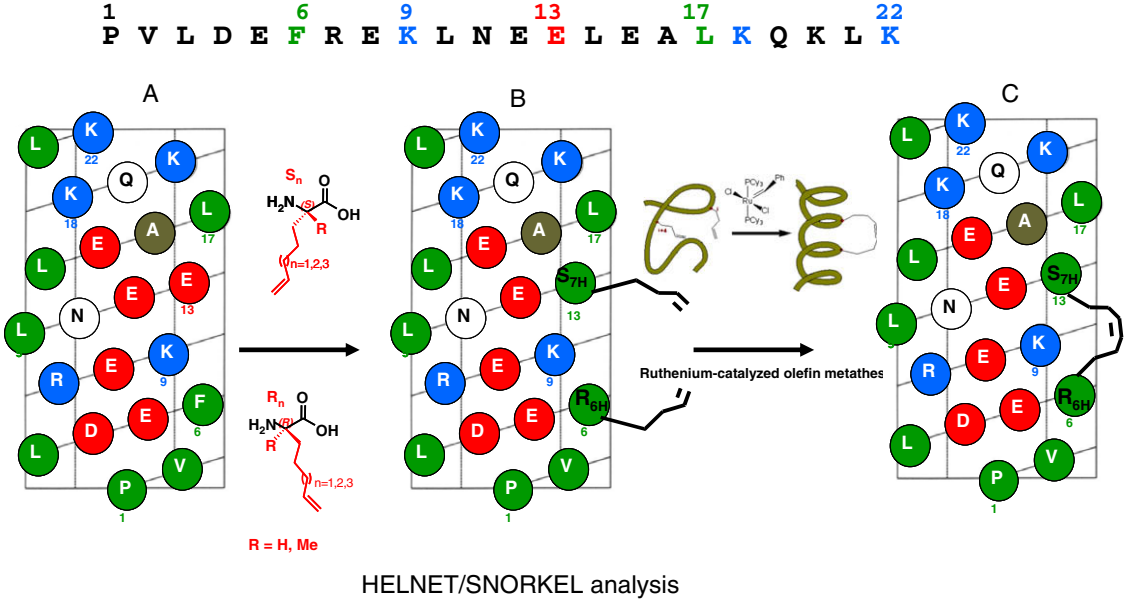

Figure 2. apoA-I_{cons} (A), with the olefinic amino acids R_{6H}^6 and S_{7H}^{13} before (B), and after (C) RCM to form the hydrocarbon bridge ('staple'). The sequence of apoA-I_{cons} is given above. Color code: red, acidic; blue, basic; green, hydrophobic; bold black, Asn and Gln.

2) or 13–17 (E^{13}/L^{17}) (peptide **14**, Table 2) through RCM of C_5-olefin amino acids of both *R*- and *S*-configuration (R_{5H}, S_{5H}; Fig. 2). Both the open and the stapled peptides were tested for their ability to promote cholesterol efflux in vitro (Table 2). The stapled peptide obtained with R_{5H} between K^9 and E^{13} (peptide **10**, Table 2) showed good cholesterol efflux capacity with an EC_{50} = 10 μM, while the corresponding open precursor (peptide **9**, Table 2) was also active, albeit about fivefold less potent, hinting at the importance of structural stabilization in addition to increased hydrophobicity.

We found that the D-configuration of the C_5-olefinic amino acid (R_{5H}) was preferred over the L-configuration (S_{5H}), for both the open and the stapled form. Furthermore, the position of the olefinic amino acids was critical for activity, since substitution of R_{5H} at positions E^{13}/L^{17} yielded inactive peptides, both in the open and the stapled form (Table 2).

We then explored a larger ring size ($i - i + 7$) with C_{6-7} olefin amino acids of both L- and D-configuration. We found that the cholesterol efflux capacity was increased, particularly with C_6-olefin (R_{6H}) at F^6 and C_7-olefin (S_{7H}) at E^{13} and vice versa (peptides **15–17**, Table 3).

Interestingly, the open sequences **15** and **17**, where the positions of R_{6H} and S_{7H} are exchanged, showed the same activity as the corresponding stapled peptide **16** (Table 3). We were unable to produce the stapled form of peptide **17** in acceptable yields. The potency shown by peptides **15–17** in the cholesterol efflux assay is the same as the peptide mimetic D-4F (**18**) and importantly, only eightfold lower than the full-length apoA-I protein (**19**) (Table 3). Further studies are ongoing to clarify the preferred configuration at each position.

Table 2
In vitro cholesterol efflux from macrophages and cytotoxicity of compounds **9–14**

Compd	Sequence[a]	CE[b] EC_{50} (μM)	Cytotoxicity[c,d] IC_{50}[c] (μM)
1	$ApoAI_{cons}$	na	40
9	$PVLDEFRER_{5H}LNER_{5H}LEALKQKLK$	46	>160
10	$PVLDEFRER_{5H}{}^{*}LNER_{5H}{}^{*}LEALKQKLK$	10	>160
11	$PVLDEFRES_{5H}LNES_{5H}LEALKQKLK$	7%@30	>160
12	$PVLDEFRESS_{5H}{}^{*}LNES_{5H}{}^{*}LEALKQKLK$	NA	>160
13	$PVLDEFREKLNER_{5H}LEAR_{5H}KQKLK$	3%@30	>160
14	$PVLDEFREKLNER_{5H}{}^{*}LEAR_{5H}{}^{*}KQKLK$	2%@30	>160

[a] The asterisk indicates the presence of a side-chain to side-chain ring (stapling) between the two residues.
[b] Cholesterol efflux from RAW cells. Values are means of three experiments (na = not active).
[c] RBC lysis assay.
[d] No cytotoxicity up to 160 μM in macrophage RAW cells.

Table 3
In vitro cholesterol efflux from macrophages and cytotoxicity of compounds **1**, **15–18**, and the apoA-I protein (**19**)

Compd	Sequence[a]	CE[b] EC_{50} (μM)	Cytotoxicity[c,d] IC_{50}[c] (μM)
1	$ApoA\text{-}I_{cons}$	na	na
15	$PVLDER_{6H}REKLNES_{7H}LEALKQKLK$	0.8 ± 0.25	>160
16	$PVLDER_{6H}{}^{*}REKLNES_{7H}{}^{*}LEALKQKLK$	2.0 ± 0.3	>160
17	$PVLDES_{7H}REKLNER_{6H}LEALKQKLK$	1.3 ± 0.20	>160
18	dwfkafydkvaekfkeaf	2.0 ± 0.25	75
19	h-ApoA-I	0.1 ± 0.01	>160

[a] The asterisk indicates the presence of a side-chain to side-chain ring (stapling) between the two residues; lower case indicates (D)-amino acids.
[b] Cholesterol efflux from RAW cells. Values are means of three experiments (na = not active).
[c] RBC lysis assay.
[d] No cytotoxicity up to 160 μM in macrophage RAW cells.

Circular dichroism spectroscopy studies revealed that the 22-mer apoA-I_{cons} peptide displays about 36% helicity in solution. Substitution of the long hydrocarbon chain amino acids R_{6H} and S_{7H} at positions 6 and 13 of the sequence resulted in a large increase in the helical content, which was essentially the same for the open and stapled forms (62% and 65%, respectively, Fig. 3). The lack of additional helical stabilization by stapling offers an explanation for the finding that **16** is not more potent than **15**. Overall optimal cholesterol efflux activity depends on a balance of factors including position, side-chain length, and stereochemistry of the olefinic amino acids.

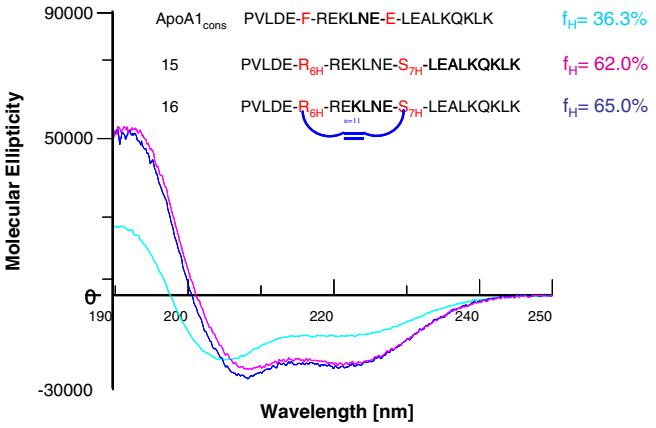

Figure 3. Circular dichroism analysis of peptides **15** and **16** in phosphate buffer, in comparison with apoA-I_{cons}. The percent helicity was calculated according to Chen et al.[16]

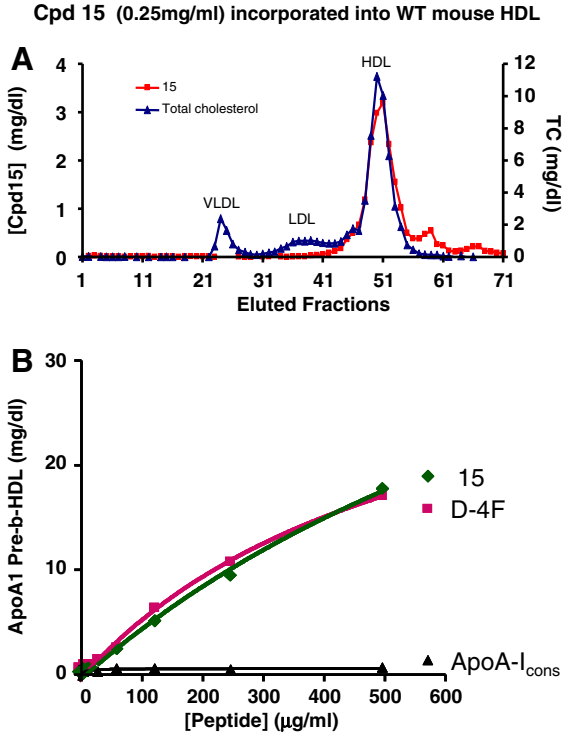

Figure 4. (A) Incubation of peptide **15** with mouse plasma at 37 °C for 2 h in vitro followed by size exclusion chromatography lipoprotein analysis; in red peptide distribution among the lipoprotein fractions (HDL, LDL, and VLDL). (B) Titration curves (ELISA with pre-β-Ab) for peptide **15**, consensus sequence and D-4F incubated with human plasma.

Having identified peptide **15** as the most potent analog, we further explored its mechanism of action. Pre-β-HDL particles ('nascent HDL') have been shown to play a key role in RCT[5], and D-4F in particular has been shown to cause an increase in pre-β-HDL.[5] When peptides **1** and **15** were incubated in plasma for 2 h at 37 °C, only peptide **15** was selectively incorporated into the HDL lipoprotein fraction (as monitored by mass-spectrometry of each fraction after size exclusion chromatography lipoprotein analysis, Fig. 4).

Moreover, similarly to D-4F, peptide **15** induced an increase in the level of pre-β-HDL particles as revealed by size exclusion chromatography analysis followed by ELISA with an apoA-I pre-β-specific antibody[15] (Fig. 4).

In conclusion we have shown here that by using as a starting point the consensus sequence of apoA-I, it is possible to design apoA-I peptide mimetics able to mobilize cholesterol from macrophages in an ABCA1-dependent pathway, and to promote the formation of 'nascent HDL' particles, with potency superior to other peptide mimetics in development and, importantly, comparable to the apoA-I protein, which is known to be efficacious in humans.

Key to our success is the use of α-alkenyl amino acids at suitable positions of the consensus sequence.

Although more studies are required to elucidate the mechanism of action of the apoA-I mimetic peptides described here, and to determine whether the observed in vitro activity correlates with an effect on atherosclerotic plaques in vivo, these results are encouraging, and represent a step towards the development of peptide-based therapies aimed at reducing cardiovascular risk in humans.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.128.

References and notes

1. Linsel-Nitschke, P.; Tall, A. R. *Nat. Rev. Drug Disc.* **2005**, *4*, 193.
2. Rader, D. J. *J. Clin. Invest.* **2006**, *116*, 3090.
3. Wang, X.; Collins, H. L.; Ranalletta, M.; Fuki, I. V.; Billheimer, J. T.; Rothblat, G. H.; Tall, A. R.; Rader, D. J. *J. Clin. Invest.* **2007**, *117*, 2216.
4. Wang, N.; Silver, D. L.; Costet, P.; Tall, A. R. *J. Biol. Chem.* **2000**, *275*, 33053.
5. (a) Rubin, E. M.; Krauss, R. M.; Spangler, E. A.; Verstuyft, J. G.; Clift, S. M. *Nature* **1991**, *353*, 265; (b) Plump, A. S.; Scott, C. J.; Breslow, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9607.
6. (a) Nissen, S. E.; Tsunoda, T.; Tuzcu, E. M.; Schoenhagen, P.; Cooper, C. J.; Yasin, M.; Eaton, G. M.; Lauer, M. A.; Sheldon, W. S.; Grines, C. L.; Halpern, S.; Crowe, T.; Blankenship, J. C.; Kerensky, R. *JAMA* **2003**, *290*, 2292; (b) Tardif, J. C.; Heinonen, T.; Noble, S. *Curr. Atheroscler. Rep.* **2009**, *11*, 58.
7. (a) Plump, A. S.; Scott, C. J.; Breslow, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9607; (b) Berthou, L.; Staels, B.; Saldicco, I.; Berthelot, K.; J Casey, J.; Fruchart, J. C.; Denefle, P.; Branellec, D. *Arterioscler. Thromb. Vasc. Biol.* **1994**, *14*, 1657; (c) Pastore, L.; Belalcazar, L. M.; Oka, K.; Cela, R.; Lee, B.; Chan, L.; Beaudet, A. L. *Gene* **2004**, *327*, 153.
8. (a) Navab, M.; Anantharamaiah, G. M.; Reddy, S. T.; Van Lenten, B. J.; Datta, G.; Garber, D.; Fogelman, A. M. *Curr. Opin. Lipidol.* **2004**, *15*, 645; (b) Shah, P. K.; Chyu, K. Y. *Trends Cardiovasc. Med.* **2005**, *15*, 291.
9. (a) Segrest, J. P. *FEBS Lett.* **1974**, *38*, 247; (b) Segrest, J. P.; Jones, M. K.; De Loof, H.; Brouillette, C. G.; Venkatachalapathi, Y. V.; Anantharamaiah, G. M. *J. Lipid Res.* **1992**, *33*, 141; (c) Segrest, J. P.; Li, L.; Anantharamaiah, G. M.; Harvey, S. C.; Liadaki, K. N.; Zannis, V. *Curr. Opin. Lipidol.* **2000**, *11*, 105; (d) Shah, P. K.; Kaul, S.; Nilsson, J.; Cercek, B. *Circulation* **2001**, *104*, 2498.
10. (a) Datta, G.; Chaddha, M.; Hama, S.; Navab, M.; Fogelman, A. M.; Garber, D. W.; Mishra, V. K.; Epand, R. M.; Epand, R. F.; Lund-Katz, S.; Phillips, M. C.; Segrest, J. P.; Anantharamaiah, G. M. *J. Lipid Res.* **2001**, *42*, 1096; (b) Remaley, A. T.; Thomas, F.; Stonik, J. A.; Demosky, S. J.; Bark, S. E.; Neufeld, E. B.; Bocharov, A. V.; Vishnyakova, T. G.; Patterson, A. P.; Eggerman, T. L.; Santamarina-Fojo, S.; Brewer, H. B. *J. Lipid Res.* **2003**, *44*, 828; (c) Navab, M.; Anantharamaiah, G. M.; Srinivasa, T. R.; Hama, S.; Hough, G.; Grijalva, V. R.; Wagner, A. C.; Frank, J. S.; Datta, G.; Garber, D.; Fogelman, A. M. *Circulation* **2004**, 3215; (d) Natarajan, P.; Forte, T. M.; Chu, B.; Phillips, M. C.; Oram, J. F.; Bielicki, J. K. *J. Biol. Chem.* **2004**, *279*, 24044; (e) Sethi, A. A.; Stonik, J. A.; Thomas, F.; Demosky, S. J.; Amar, M.; Neufeld, E.; Brewer, H. B.; Davidson, W. S.; D'Souza, W.; Sviridov, D.; Remaley, A. T. *J. Biol. Chem.* **2008**, *283*, 32273.
11. (a) Navab, M.; Anantharamaiah, G. M.; Hama, S.; Garber, D. W.; Chaddha, M.; Hough, G.; Lallone, R.; Fogelman, A. M. *Circulation* **2002**, *105*, 290; (b) Li, X.; Chyu, K. Y.; Faria Neto, J. R.; Yano, J.; Nathwani, N.; Ferreira, C.; Dimayuga, P. C.; Cercek, B.; Kaul, S.; Shah, P. K. *Circulation* **2004**, *110*, 1701.
12. Anantharamaiah, G. M.; Venkatachalapathi, Y. V.; Brouillette, C. G.; Segrest, J. P. *Arteriosclerosis* **1990**, *10*, 95.
13. (a) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **1998**, *37*, 3281; (b) Schafmeister, C. E.; Po, J.; Verdine, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 5891.
14. Grubbs, R. H.; Miller, S. J.; Fu, G. C. *Acc. Chem. Res.* **1995**, *28*, 446.
15. Troutt, J. S.; Alborn, W. E.; Mosior, M. K.; Dai, J.; Murphy, A. T.; Beyer, T. P.; Zhang, Y.; Cao, G.; Konrad, R. J. *J. Lipid Res.* **2008**, *49*, 581.
16. Chen, Y. H.; Yang, J. T.; Chau, K. H. *Biochemistry* **1974**, *13*, 3350–3359.