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ARTICLE

The Synthesis of the Very Long Chain Polyunsaturated Fatty Acid (VLC-PUFA) 32:6 n-3

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Abstract: This article describes the synthesis of VLC-PUFA 32:6 n-3, D_2-labeled 32:6 n-3, and the uptake of 32:6 n-3 into mouse retinal tissue.

Introduction

VLC-PUFAs are fatty acids that contain ≥24 carbon atoms and consist of an interesting combination of saturated and unsaturated hydrocarbon subunits.[1] They exist in membranes as phospholipids and are present in very low concentrations in the retina and testes.[2,3,4] Generally VLC-PUFAs do not come from dietary sources but are instead generated endogenously from the chain extension of long chain fatty acids, typically docosahexaenoic acid (DHA), arachidonic acid (AA), or eicosapentaenoic acid (EPA) by the enzyme ELOVL4 (elongation of very long chain fatty acids-4).[5] Our interest in these targets not only came from their intriguing structures but also from the substantial but still anecdotal evidence that suggests that they play a role in age-related macular degeneration (AMD) and Stargardt's disease.

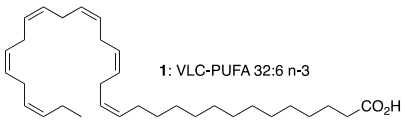

Figure 1. VLC-PUFA 32:6 n-3

As the name suggests, AMD is a degenerative eye disease that leads to blurred or loss of vision in the center of the visual field.[6] In light of our aging population, that AMD mostly afflicts the elderly makes this disease a significant problem. In general, there are two forms of the disease, wet and dry. While therapies have been effective in some patients suffering from choroidal neovascularization (CNV) a form of wet AMD,[7] these same therapies have not been effective for other forms of AMD.[8] Not only are there currently no therapies for dry AMD, but the etiology of the disease is not well understood.[9] Along these lines, it is interesting that the symptoms associated with Stargardt's disease, a hereditary disease caused by mutations on the ELOVL4 gene, are similar to those seen in the dry form of AMD.[10] These mutations have been shown to lead to the down-regulation of the ELOVL4 enzyme and, as a consequence, the presence of lower concentrations of VLC-PUFAs in the retina.[11] It is also worth noting that VLC-PUFA concentrations are lower in patients who suffer from AMD.[12] Thus, although the evidence is anecdotal at this point, arguments can be made for VLC-PUFAs playing a role in AMD. To date, the lack of a ready supply of VLC-PUFAs has kept researchers from gaining a better understanding of their role in both healthy eyes and patients suffering from AMD.[13] In an effort to overcome this, we set out to synthesize and study one member of the VLC-PUFA family, C32:6 n-3 (**1**) along with a deuterium labeled analog of **1**.[14]

When we began our efforts, we were aware of two independent syntheses of our initial target, VLC-PUFA C32:6 n-3.[15] One synthesis came from Raman and co-workers and involved the coupling of a cuprate derived from THP protected 10-bromo-1-decanol and an alkyl bromide derived from DHA. In a similar fashion to Raman, a synthesis from Maharvi and co-workers involved the coupling of a saturated C-10 sulfone with a polyunsaturated tosylate derived from DHA. Attractive to both approaches was that the generation of the requisite 32 carbon chain involved the coupling of two readily available precursors. As part of our overall plan involved tracking the uptake of synthetic VLC-PUFAs from a dietary source to the retina, a problem with both of these approaches had to do with the challenge of using them to synthesize labeled substrates. In spite of this, we did initially explore the Raman synthesis but were unable to repeat the reported coupling reaction. We did not pursue the Maharvi route both because of our desire to generate labeled material and because we envisioned that the

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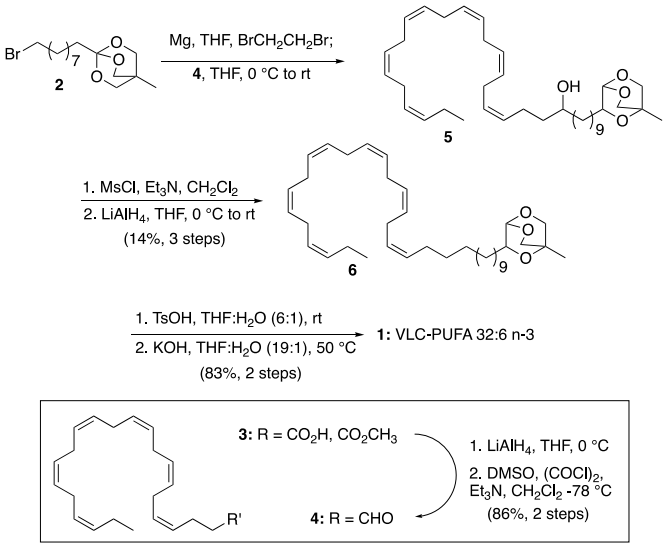
requisite reductive desulfonation would be limiting. Thus, we sought out a modified synthetic strategy to these targets. This work is described here.

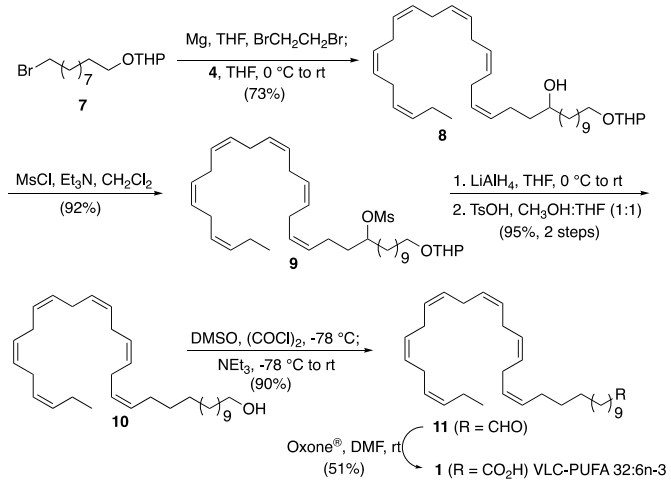
Methods and Discussion

Concerns that we had when planning our synthetic approach to the VLC-PUFA substrates were centered around their susceptibility to oxidation and/or olefin isomerization/migration. Because of this, we felt that it was necessary to generally avoid strongly oxidizing, reducing, and acidic conditions. The approach to **1** that we ultimately identified was related to those mentioned above with one small but significant difference. We set out to examine the coupling of a masked decanoic acid nucleophile with a docosahexaenoic acid (DHA) derived aldehyde. Not only might the coupling protocol employing the aldehyde be more successful and amenable to scale-up but the resulting 2° alcohol would serve as a precursor to an isotopic (D) label during the subsequent deoxygenation sequence. Mindful of these goals and in an attempt to avoid a late-stage oxidation we generated the coupling precursor **2** representing the saturated VLC-PUFA subunit from 10-bromodecanoic acid (Scheme 1).[16] DHA derived aldehyde **4** was synthesized from DHA or the corresponding ester by employing a reduction, oxidation sequence. The addition of the Grignard reagent derived from orthoester **2** to aldehyde **4** gave the desired 2° alcohol **5** but in low yield and as an inseparable mixture with the product of homo coupling of the Grignard reagent from **2**. Reduction of the mesylate that came from **5** allowed us to access orthoester protected VLC-PUFA **6** in a modest 14% overall yield for the three steps. In contrast to our problems with the coupling chemistry, the hydrolysis of the orthoester proved uneventful and provided VLC-PUFA 32:6 n-3 **1** in 83% yield over the final two steps. The analytical data (^{1}H NMR, ^{13}C NMR, HRMS, GC-MS) for **1** matched both the reported data and authentic samples of **1**. What diminished our enthusiasm for this approach was its relatively low efficiency (8 steps, 6% overall yield). We attributed the efficiency problem to both the dimerization problem and the somewhat surprising instability of the orthoester. In our hands it turned out to be particularly sensitive subsequent to its coupling with DHA. For example, the C-32 orthoester containing substrates generally underwent decomposition when exposed to any acidic conditions including SiO_2 during chromatography. These issues seemed to be even more problematic on scale up.



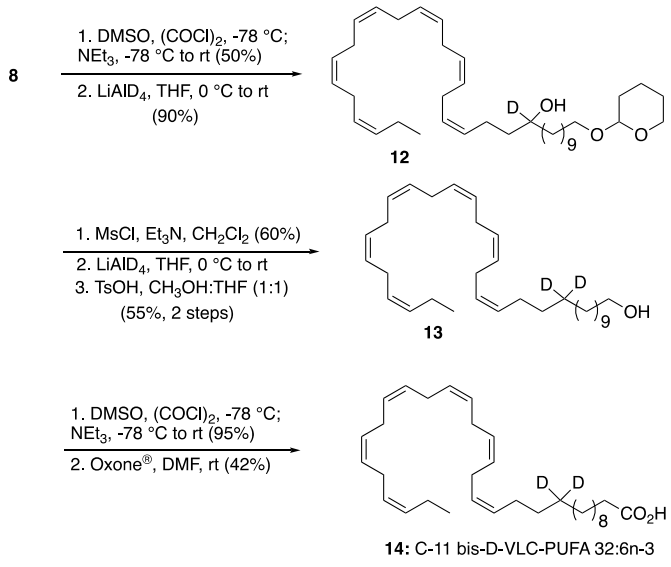
Scheme 1. 1st Generation Synthesis of VLC-PUFA 32:6 n-3

In light of the difficulties mentioned above, we sought out an alternate approach to 32:6 n-3 that avoided the orthoester and settled on THP protected bromodecanol **7**. The obvious downside to the use of **7** was that it would require a late-stage oxidation reaction. Regardless, we were pleased to find that the C-32 THP protected substrates were generally better behaved than the orthoesters had been in our hands. For example, the coupling of the Grignard reagent from **7** with DHA aldehyde **4** afforded **8** in 73% yield and was successfully carried out on a multi-gram scale. Mesylate formation gave **9**. Reductive removal of the mesylate gave 32:6 n-3 alcohol **10** after THP acetal hydrolysis. We were somewhat surprised when the oxidation of **10** proved to be challenging. The use of Raman's protocol and CrO_3 and H_5IO_6 resulted in unrecognizable mixtures of products. Reagents like TEMPO or $PhI(OAc)_2$ either resulted in the decomposition of **10** or the recovery of starting material, presumably due to solubility issues.[17] We eventually found that a two-step procedure that combined a Swern oxidation with a subsequent Oxone® oxidation gave VLC-PUFA 32:6n-3 in 46% yield from **10**. Overall, the synthesis of **1** required 6 steps from **7** and was achieved in 29% overall yield.[18,19] To date we have used this sequence to generate >10 g of **1** for uptake and visual acuity studies.[14]



Scheme 2. 2nd Generation Synthesis of VLC-PUFA 32:6 n-3

As was stated above, one of the advantages of the coupling with DHA derived aldehyde **4** was that the resulting alcohol could be converted into an isotopically labelled VLC-PUFA whose uptake could be tracked using mass spectrometry.[20] To this goal, we targeted C-11 bis-deuterated VLC-PUFA **14**. The treatment of the ketone that comes from the oxidation of alcohol **8** with $LiAlD_4$ resulted in mono-deuterated alcohol **12**. Mesylate formation and a second $LiAlD_4$ reduction allowed us to access C-11 bis-deuterated alcohol **13** after THP acetal hydrolysis. Oxidation per our previously established conditions gave bis-deuterated VLC-PUFA **14**. We are currently examining the properties of **14** including its uptake into the retina.



Scheme 3. Synthesis of C-11 bis-Deuterium VLC-PUFA 32:6 n-3

In view of the physiological benefits of VLC-PUFAs and the dysfunction associated with low retinal levels of VLC-PUFAs, we examined the bioavailability of synthetic 32:6 n-3 delivered to mice via gavage feeding.[21] The mice received a single dose of 11 mg of 32:6 n-3 and were sacrificed at 0, 6, and 12 h (n=2 mice/time interval). As shown in Figure 2, even though peripheral tissue like serum, Red Blood Cells (RBC), and the liver do not normally contain VLC-PUFAs, in our studies VLC-PUFAs became detectable in the serum 6 h after oral administration and reached their highest concentration after 12 h. We were also excited to find a significant increase in 32:6 n-3 in the retina 12 h after feeding. VLC-PUFAs remained undetectable in the liver, brain, and RBC membranes throughout the 12 h time frame. Additional studies in wild type and EVOVL4 knock-out mice are ongoing.

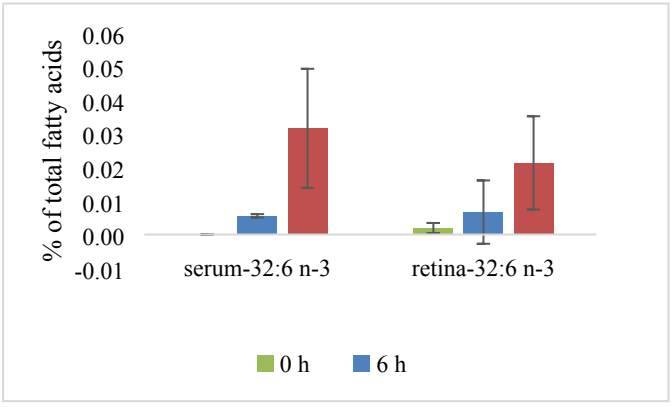


Figure 2. Bioavailability of VLC-PUFA 32:6 n-3. **(a)** Serum and retinal uptake of 32:6 n-3 after single-dose gavage feeding of 11 mg/mouse (n=2 mice/time point). N.D. = non-detectable.

Conclusions

In conclusion, described here has been a synthesis of VLC-PUFA 32:6n-3 and a deuterium labelled derivative of this compound from the coupling of saturated and polyunsaturated precursors. This approach has the advantage of being amenable to multi-gram scale-up and that it should be amenable to the synthesis of other members of the VLC-PUFA family. In addition to the synthesis, we have studied the bioavailability of VLC-PUFAs and have observed a rapid increase in retinal VLC-PUFAs in response to acute supplementation. This result clearly demonstrates that orally administered VLC-PUFAs are bioavailable to ocular tissues. In addition to optimizing the synthesis, studying the properties of VLC-PUFA 32:6 n-3, and examining labelled derivatives, we are currently examining the synthesis and properties of other members of the VLC-PUFA family. These efforts will be communicated in due course.

Author Contributions

A.G., P.S.B., and J.D.R. designed research; A.W., R.R., S.L., C.O., and A.G. performed research. A.W., R.R., S.L., C.O. and J.D.R.contributed new reagents/analytical tools; all authors analyzed data; and A.G., P.S.B., and J.D.R. wrote the manuscript.

Conflicts of interest

A.G., R.R., J.D.R., P.S.B., and the University of Utah have filed a provisional patent application on the chemical synthesis of VLC-PUFAs and their therapeutic effects.

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All animal handling procedures used in this study were approved by appropriate institutional animal care and use committees and were carried out according to NIH guidelines.

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[21]. Mice were housed in the vivarium at the Moran Eye Center and maintained in a standard 12 h light and 12 h dark cycle environment, with water and food ad libitum (Teklad Global Soy-Free Rodent Diet, Envigo). All animal handling procedures used in this study were approved by appropriate institutional animal care and use committees and were carried out according to NIH guidelines.