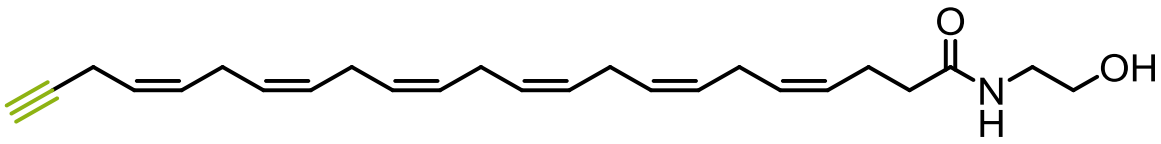
Synthesis of a chemical probe based on *N*-docosahexaenoylethanolamide

Exploring anti-inflammatory compounds derived from cyclooxygenase activity



MSc Thesis report
Septembre 2015 - April 2016

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Contents

List of abbreviations

ACN	acetonitrile
ARA	arachidonic acid
br.	broad (in NMR)
COX-1/COX-2	cyclooxygenase 1/cyclooxygenase 2
d	doublet (in NMR)
DCM	dichloromethane
DHA	docosahexaonic acid
DHEA	N-docosahexanoyl ethanolamide
DIC	diisopropylcarbodiimide
DMF	dimethylformamide
EDC	N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide HCl
EDTA	ethylenediaminetetraacetic acid
epi	epinephrine hydrogen tartrate
Et_2O	diethylether
EtOAc	ethylacetate
EtOH	ethanol
eq.	equivalents
g	gram
h	hour
HOBt	1-hydroxybenzotriazole hydrate
(U)HPLC	(ultra) high performance liquid chromatography
Hz	Hertz
M	molar
m	multiplet (in NMR)
MeOH	methanol
min	minutes
MQ	milli-Q (ultrapure water)
MS	mass spectrometry
NMR	nuclear magnetic resonance
NSAIDs	non-steroidal anti-inflammatory drugs
PE	petroleum-ether 40-60
PUFA’s	polyunsaturated fatty acids
q	quartet (in NMR)
quint	quintet (in NMR)
s	singlet (in NMR)
t	triplet (in NMR)
t-Bu	*tert*-butyl
rt	room temperature
TEA	triethylamine
TFA	trifluoroacetic acid
THP	2-tetrahydropyran
TLC	thin-layer chromatography
TMS	trimethylsilyl
tris	tris(hydroxymethyl)aminoethane

Abstract

Docosahexaenoic acid, a polyunsaturated fatty acid, is metabolized in humans to its *N*-acylethanolamide derivative, *N*-docosahexaenoyl ethanolamide (DHEA). DHEA is found to have anti-inflammatory properties by modulating the activity of cyclooxygenase 2 (COX-2, but the exact molecular mechanism is unknown. In order to investigate which DHEA metabolites are formed by COX-2, a chemical probe has been designed to selectively 'fish' for these metabolites. Since the methyl terminus of DHEA is not involved in any known metabolic pathway, the structure was altered here. The probe design places an alkyne here to enable a selective and efficient possibility for fishing, using click chemistry. Using a copper catalysed alkyne-azide cycloaddition (CuAAC) isolation of alkyne-DHEA metabolites in cell lysates should become possible.

The two building blocks required for the modular synthetic strategy towards alkyne-DHEA were successfully prepared and condensed. Thereby most of the reaction condition needed for the subsequent steps in the complete synthesis are now known, only the selective reduction of the alkynes is still a step that is currently now working. Several conditions were tested to selectively reduce the internal alkynes to Z-alkenes, but none of these proved successful at this point. There are still potential conditions for this reaction that might work, using nickeldiacetate or zinc as a reagent, but more research has to be performed.

Additionally, natural DHEA was used to investigate the metabolites in an earlier developed enzyme assay for COX-2 activity, using purified enzyme. After incubation with DHEA and arachidonic acid, the natural substrate for COX-2, the samples were analysed by UHPLC. Due to inconsistent results, probably due to low or no enzyme activity of the used batch of enyzme, no conclusions could be drawn.

If the selective reduction reaction will be successful in the future, alkyne-DHEA can be synthesized. More tests with the enzyme assay are necessary in order to look for metabolites of native DHEA derived by COX-2. Eventually the alkyne-DHEA probe can be used in cells to look for specific anti-inflammatory DHEA metabolites that are formed in the more relevant complex in vivo environment.

Introduction

Docosahexaenoic acid (DHA; 22:6(n-3) is a polyunsaturated fatty acid (PUFA), mostly present in fatty fish. Several PUFA's, like DHA, are incorporated in human cells and are part of several essential cellular pathways. It is even advised to incorporate a certain amount of DHA containing products in a healthy diet, because of the positive effects of DHA. For example, DHA lowers the risks for developing cardiovascular diseases, like thrombosis and coronary artery disease. [1] There are also several correlations found for an increased intake of DHA and a decreased risk of inflammation based diseases, like asthma and arthritis. [1] Based on a study where the influence of a derivative of DHA was studied, this derivative was found to have an influence on inflammation processes, but the exact molecular mechanism is not known. [2] This molecular mechanism is of interest for a better understanding of inflammation processes and how it is inhibited by DHEA metabolites and its unravelling is the overall goal of the study of which this thesis project was a part.

Inflammation is a complex biological process, which involves many different kinds of cells and cellular pathways. One of the important enzymes in inflammation is cyclooxygenase-2 (COX-2). The natural substrate of COX-2 is another PUFA, arachidonic acid (ARA). COX-2 metabolizes ARA in prostaglandin G_2 (PGG_2), a precursor in inflammation processes, which can be seen in Figure 1. [3]

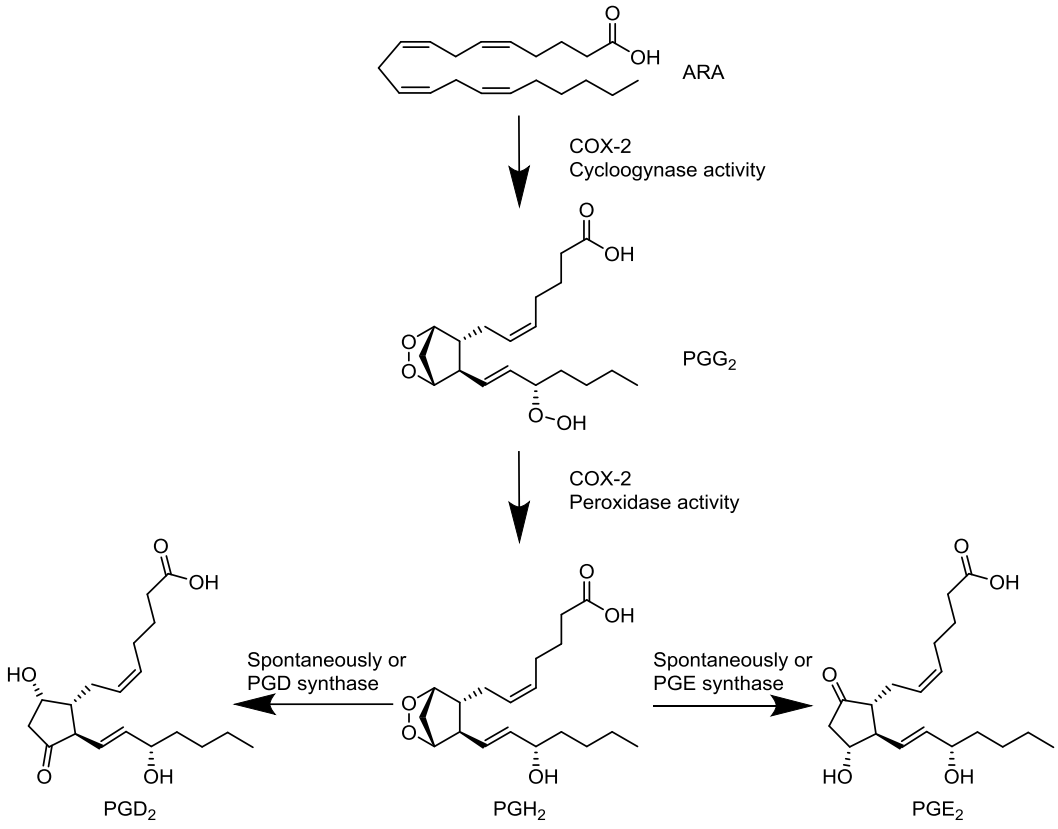


Figure 1: Natural metabolism of arachidonic acid by cyclooxygenase-2 in prostaglandin E_2 or D_2.

Besides of the spontaneously or enzymatically rearrangement of PGG_2 to PGD_2 or PGE_2, also other enzymes are involved in the production of other prostaglandins or tromboxanes from PGG_2. ARA is metabolized by COX-2 in PGH_2, but also COX-1 can metabolize ARA into the same prostaglandin, where the role of these two enzymes differs. COX-1 maintains tissue homeostasis and therefore is generally active in cells. This in contrast to COX-2, which expression can be induced in case of for example inflammation. [3] Inhibition of COX-2, so lowering the levels of PGG_2 or PGH_2, relieves symptoms of pain and inflammation. Some non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-2, but also have side effects due to inhibition of other enzymes, like COX-1. [4] In this case NSAIDs interfere with homeostatic processes. The use of some NSAIDs can also increase for example risks of cardiovascular problems. [5]

DHA is metabolized in humans into its *N*-acylethanolamide derivative, *N*-docosahexaenoylethanolamide (DHEA), which has been shown to modulate COX-2 activity. [2] DHEA could be of interest for developing alternatives for COX-2 inhibition. When more insight in the mechanism of modulation of COX-2 by DHEA oxidative metabolites is gained, possibly new insights in inflammatory mechanisms can be obtained. Inflammation plays a role in multiple diseases, which leads to a high demand for anti-inflammatory drugs. More insight into the influence of DHEA on the COX-2 activity could lead to new insights for novel compounds for the treatment of these diseases. These putative anti-inflammatory oxidative metabolites of DHEA would represent endogenously generated inhibitors and therefore might have an advantage over NSAIDs by causing less side effects.

To find an answer to the question what the anti-inflammatory properties of DHEA and its oxidative metabolites are, the influence of these compounds on the oxidative activity of COX-2 by DHEA needs to be investigated. This gives rise to another question: what are the oxidative metabolites produced by COX-2 from DHEA? The metabolites of DHEA can be analysed after incubation with purified COX-2, but especially for the more relevant experiment of incubation within intact COX-2 producing cells, the isolation of these specific metabolites is not possible. Therefore strategies are needed that make it possible to selectively isolate DHEA metabolites. A convenient way of only isolating the intended metabolites is by using click chemistry to install an affinity tag onto only the DHEA metabolites. A small alteration in the structure of DHEA already enables this selective and efficient click chemistry, essentially turning it into a chemical probe for DHEA oxidative metabolism. A click reaction makes it possible to selective ‘fish’ for only the metabolites of DHEA in a cell lysate. Therefore a DHEA based chemical probe will be used to take the first step in identifying the metabolites of DHEA produced by COX-2.

The methyl terminus of DHEA is not directly involved in any known metabolic processes, which should makes it the position of choice for modification of DHEA.

We have designed alkyne-DHEA as a chemical probe analogue of DHEA. By using a terminal alkyne, click chemistry can be performed on its COX-2 metabolites, using a copper catalysed alkyne-azide cycloaddition (CuAAC). It is not known whether COX-2 will recognize and metabolize alkyne-DHEA. However, a similar alkyne-terminated analogue of DHA has already been reported by Petasis *et al.* to be metabolized by lipoxygenase (LOX), another oxidative enzyme. [6] This previously synthesized analogue and our target chemical probe, alkyne-DHEA, can be seen in **Error! Reference source not found.**.

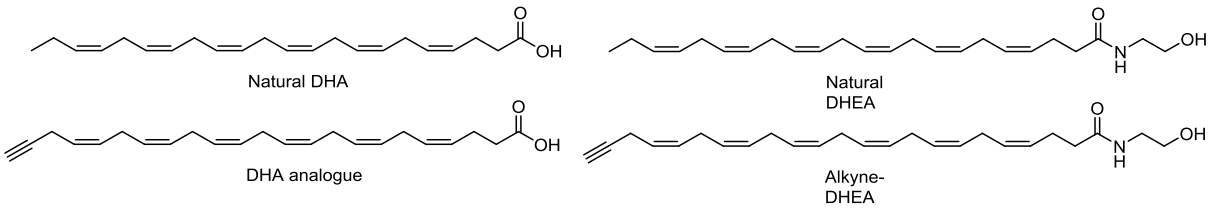


Figure 2 Natural DHA and DHEA in comparison with an already synthesized DHA chemical probe analogue and the target alkyne-DHEA chemical probe.

Experimental approach

Synthesis

For the DHEA-based chemical probe an alkyne terminated DHEA is the target. Retrosynthetic analysis reveals that only two building blocks are needed to synthesize alkyne-DHEA. The structure of alkyne-DHEA and its building blocks can be seen in Figure 3. Building block **2** is needed three times in combination with building block **1** to synthesize alkyne-DHEA.

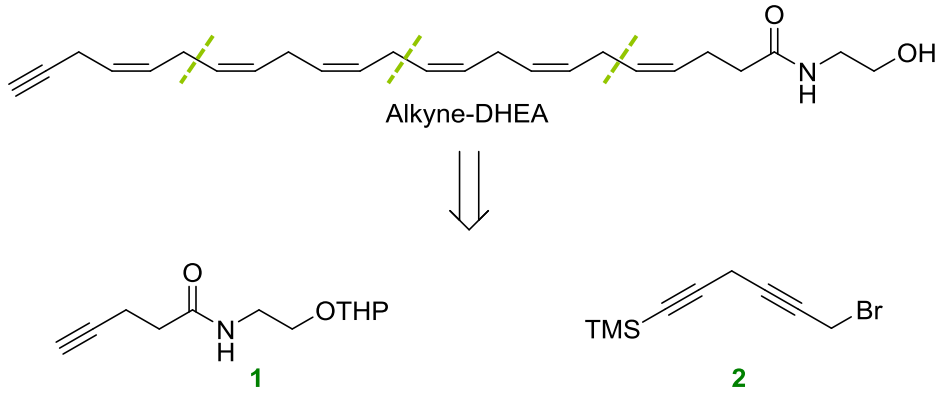


Figure 3: the alkyne-DHEA chemical probe and its two building blocks. Building block 2 is needed three times in combination with building block 1.

The chemical synthesis of PUFA's is quite a well-established field in organic chemistry. [7] Therefore the synthesis of alkyne-DHEA involves some well-known robust chemical reactions for its construction, but alkyne-DHEA itself has not been made before.

Using the two depicted building blocks, the total alkyne-terminated DHEA can be synthesized in modular fashion. These building blocks are not commercially available and will be synthesized according to the route depicted in Figure 4

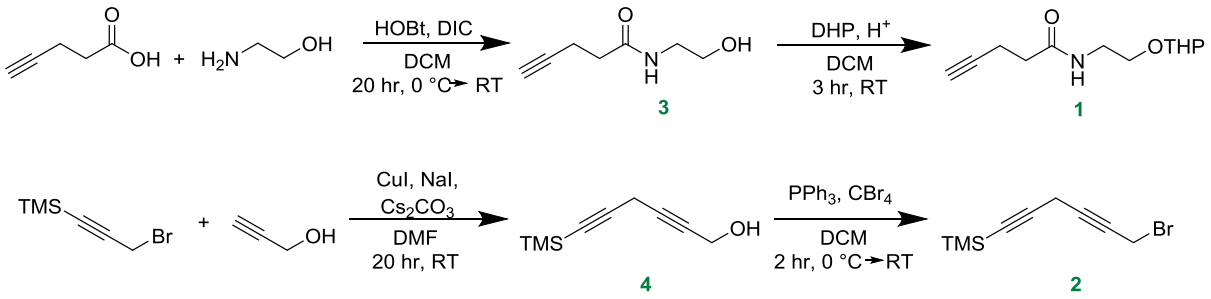


Figure 4: Synthesis of building block 1 and 2.

The starting materials for the synthesis of the two building blocks are commercially available. The synthesis of building block **1** starts with an amide bond formation. The synthesis of intermediate **3** has previously been reported by Shieh *et al.* [8] [9] and we adopted their procedure. The alcohol terminus of ethanolamine will be protected with tetrahydropyran (THP) to prevent side reactions of building block **2** later on in the synthesis. The coupling of trimethylsilyl propargylbromide and propargylalcohol is copper catalysed and cesiumcarbonate mediated, an optimized reaction for the formation of skipped diynes. [10] A carbonate metal base is commonly used in these kind of chemistry and Caruso *et al.* showed that cesiumcarbonate is more effective than potassium- and sodiumcarbonate. Therefore cesiumcarbonate will be used, although intermediate **4** has also previously been successfully made before using potassiumcarbonate. [11] Building block **2** is synthesized through an Appel reaction. Tetrabromomethane is used in combination with triphenylphosphine in order to replace the hydroxyl terminus of intermediate **4** with a halide. The terminal bromide is necessary for the coupling of two building blocks further on in the synthesis. [12, 13]

When these two building blocks are successfully prepared, the total alkyne-DHEA can be synthesized. The overall synthesis strategy is depicted in Figure 5.

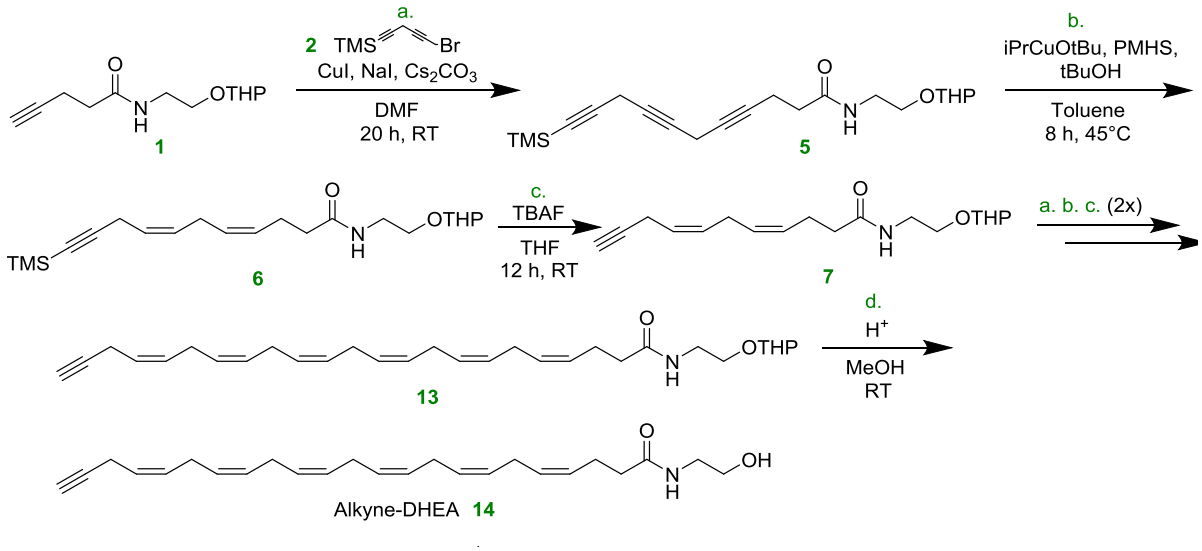


Figure 5: total synthesis of alkyne-DHEA, starting with building block 1 and 2.

The first step, coupling of the two building blocks, **1** and **2**, to form compound **5**, is also a copper catalysed reaction, mediated by cesiumcarbonate, and follows the procedure as mentioned above. The coupling of a bromide compound with an alkyne in presence of an amide is possible. [14, 15] The next step is a copper catalysed Z-selective semi-reduction of the formed skipped diyne. [7] Since alkynes are sensitive to oxidation, each addition step will be followed by reduction of the alkynes, instead of reducing all alkynes at the end of the synthesis. However, DHEA should be alkyne-terminated. Therefore a trimethylsilyl group (TMS) will be used as protection of the terminal alkyne. There are several examples available where internal alkynes are reduced to alkenes in presence of a terminal TMS protected alkyne. [16, 17] Before another addition of building block **2**, the TMS protecting group will be removed. The addition, semi-reduction and removal of TMS is repeated another two times, after which the THP ether is hydrolysed, yielding alkyne-DHEA, **14**. If the reduction and removal of TMS are combined, alkyne-DHEA can be synthesized in six steps from only the two earlier mentioned building blocks.

All the mentioned chemical reaction are known and have been used before on similar compounds, but not on exactly these intermediates and not towards alkyne-DHEA itself. In order to evaluate and optimize all reaction conditions of this novel strategy, a shorter version, compound **7,** will therefore be synthesized as a test case, before alkyne-DHEA itself is fully synthesized.

COX-2 enzyme assay

The synthesized alkyne-DHEA will be used in an enzyme assay with COX-2. Using this assay, it can be checked whether alkyne-DHEA is metabolized. This assay is developed at the department of Human nutrition and based on an assay described by

Reininger et *al.* [18] This enzyme assay was used to determine the effect of addition of DHEA to COX-2 in presence of ARA. After incubation of different concentrations of DHEA with ARA and COX-2, an ELISA method is used to calculate the activity levels of COX-2. PGE_2 levels are measured by the degree of colouration of the mixture with respect to the incubation of only ARA with COX-2. In this complete assay significant effects of DHEA are observed.

In order to investigate the molecular mechanism of this effect, the enzyme assay itself will be used to incubate alkyne-DHEA with COX-2 in presence and absence of ARA. The enzyme will be removed and the metabolites will be separated by HPLC, flash chromatography or preparative TLC, depending on the amount of material. Subsequently these metabolites can be identified by HRMS. If a sufficient amount of metabolites is obtained, also NMR can be used to elucidate its structure. This procedure can be repeated by incubation DHEA in order to compare the formed metabolites. In this way the reliability of the results of the incubation with alkyne-DHEA can be checked.

The next step is incubating lipopolysaccharide (LPS) stimulated RAW2 cells with alkyne-DHEA to compare produced metabolites with the metabolites isolated from the enzyme assay. A schematic representation of this procedure can be found in Figure 6.

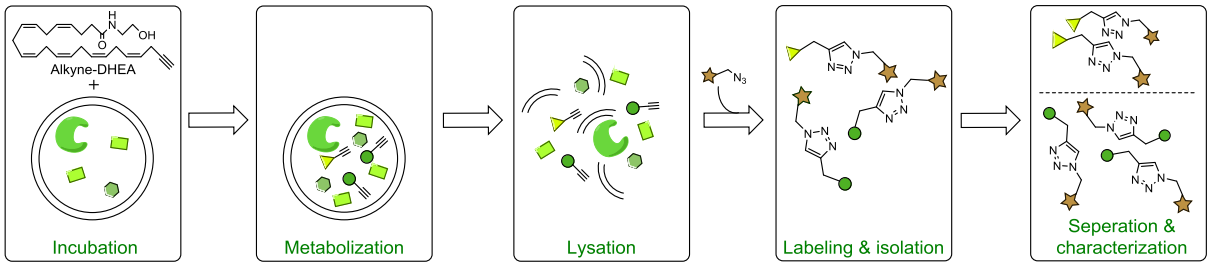


Figure 6: Schematic representation of the incubation of alkyne-DHEA in RAW2 cells and analysing of the produced metabolites

After incubation, COX-2 can oxidatively metabolise alkyne-DHEA. These cells will be lysed and the metabolites, represented by triangles and circles, can be isolated by click chemistry, using a copper catalysed alkyne-azide cycloaddition. Using a azido-biotin derivative, represented by stars in Figure 6, these metabolites can be easily isolated from the cell lysate by streptavidin, for example using streptavidin beads. The formed metabolite-biotin complexes can be separated by HPLC, flash chromatography or preparative TLC, depending on the amount of material. Finally, these metabolite-biotin complexes can be analysed by HRMS. If a sufficient amount of metabolite-biotin complexes is obtained, also NMR can be used to elucidate its structure.

Objective

First of all, the DHEA-alkyne chemical probe has to be synthesized. Secondly, when this has been successfully completed, the chemical probe will be used in an enzyme assay with COX-2 to check whether alkyne-DHEA is accepted and metabolized and to subsequently identify its metabolites. If possible, alkyne-DHEA will also be tested on cells and its metabolites will be isolated by click chemistry and identified.

Results & discussion

Synthesis of building block 1

For the synthesis of building block **1**, first intermediate **3** had to be prepared. The procedure described by Shieh *et al.* was used. [8] In a first attempt EDC was used instead of DIC. Because the described work up was most suitable for the use of DIC, this resulted in loss of material corresponding to 48% yield (0.17 g). In a second attempt, the DIC procedure was followed and resulted in a yield of 76% (0.56 g).

Intermediate **3** was used to synthesize building block **1** by a protection of the terminal hydroxyl moiety. Based on the procedure described by Zhang *et al.* a first attempt reached incomplete conversion. [19] After work up of the reaction mixture, by quenching with TEA and evaporation, and purification by flash chromatography 0.10 g (63%) of the product was obtained. A next attempt was performed 20 times more concentrated (1.43 M), the same concentration used by Zhang *et al.* The conversion improved, but still no full conversion was reached. Addition of an additional 0.5 eq. of DHP and stirring for another hour resulted in full conversion. After work up and purification of the reaction mixture the product was obtained in 84% yield (0.37 g). In a third attempt the higher concentration was maintained and instead of 1.1 equivalents, 1.5 equivalents of DHP were added at start the reaction. The reaction reached full conversion in three hours. After work up and purification of the reaction mixture the product was obtained in 78% yield (0.29 g).

An explanation for this good but not excellent yield is that in general product is lost during purification due to diluted fractions. A plausible explanation could be that the crude product partly precipitated on the column. Due to the large volume of fractions, not all the purified product was observed in TLC and collected.

Synthesis of building block 2

Building block **2** was synthesized through intermediate **4**. As described in the experimental approach, cesiumcarbonate was used instead of more common used sodium- or potassiumcarbonate. Caruso *et al.* showed that the use of cesiumcarbonate increases the obtained yield and their procedure also fits an unprotected hydroxyl moiety. [10] In this case a yield of 78% (0.24 g), 67% (1.16 g) and 87% (1.33 g) was obtained.

Building block **2** was synthesized through an Appel reaction. For each further use of building block **2** a fresh batch was made to prevent potential degradation. The used procedure was based on the earlier synthesis of alkyne containing molecules. In contrast to two described methods, after purification by flash chromatography triphenylphosphineoxide was still present. [13] [12] This yielded **2** in 66% (0.16 g). In a next attempt the crude concentrated reaction mixture was dissolved in a minimal amount of DCM followed by slow addition of Et_2O/heptane (1:1). The precipitated

triphenylphosphineoxide was separated from the dissolved product by filtration and the filtrate was concentrated and analysed by NMR. This procedure was repeated another time to give pure product **2** in 82% (0.55 g).

Synthesis of compound 5

The completed building blocks **1** and **2** were used to synthesize compound **5**. The same procedure for the synthesis of compound **4** was used, since this involved the same chemistry. In this case the yield significantly dropped to 15% (0.03 g) and in a second attempt 24% (0.22 g). The reaction was monitored by TLC and due to the disappearance of building block **2** over time, high conversion was expected. Building block **1** was still present, but due to an inaccuracy in the amount of solution of the two added building blocks, a slightly excess of one of the two building blocks was probably present. After purification the amount of recovered building block **1** was more than expected (0.15 g). A probable reason for the low yields is that building block **2** degrades during the reaction. To optimize this reaction, an excess of building block **2** should be used in future attempts.

Reduction procedures

In order to selectively reduce the internal alkynes of compound **5**, to obtain the diene **6**, several methods were evaluated. Most of these methods were tested on intermediate **4.**

First a copper complex, iPrCuO*t*Bu, was used as catalyst. [20] This catalyst was freshly prepared before each reaction and the reaction was performed with the use of a Schlenk system. Whittaker *et al.* described the use of this catalyst and used 6-dodecyne as a model molecule. 6-Dodecyne was successfully reduced, however, 40 mol%, of the catalyst was used due to an error in the described procedure. Another attempt with intermediate **4** and 6-dodecyne with 4 mol% catalyst showed no conversion for 6-dodecyne and multiple spots during an attempt with intermediate **4** by TLC. These different spots were analysed by MS and NMR, but no conclusive mass and no alkene signals were found. The procedure described by Whitakker *et al.* uses a glove box for amounts less than 1 gram. Only gram-scale reactions are performed outside a glove box. Since these reactions are performed on 0.10 gram scale, water could be the problem. Only a large amount of catalyst is capable of reducing 6-dodecyne.

We next evaluated another type of reduction, using nickeldiacetate. [11] [16] Also in this case 6-dodecyne was first evaluated as a model molecule. In 90 minutes complete conversion was reached and NMR showed 6Z-dodecene was formed. When intermediate **4** was used, two spots were observed by TLC. These products

were analysed by ^{1}H NMR and the highest Rf spot of these showed reduction of the TMS protected alkyne. The lower one could not be completely analysed due to low amount, but clearly no alkene signals were visible by NMR. A second attempt, with a lowered reaction temperature of 0 °C, showed the same pattern but after purification by column chromatography, only the lowest spot was visible. Again, no alkene signals were visible by NMR.

A third catalyst we evaluated was Lindlar's catalyst, a heterogeneous catalyst that consists of palladium deposited on calcium carbonate and is poisoned with lead. [17] The used procedure reached full conversion in 2.5 hour for the reduction of 6-dodecyne to 6Z-dodecene in *tert*-butanol. When intermediate **4** was exposed to these conditions in methanol, no conversion was obtained after performing the reaction overnight. The solvent was changed for 6-dodecyne to *tert*-butanol due to insolubility in methanol.

Due to limited time available in the project, compound **5** was used directly. The procedure was repeated for compound **5**, still giving no conversion. Another common used solvent for this catalyst is ethylacetate. [21] The same procedure was repeated in ethylacetate but no conversion was visible in 2.5 hours. At first it seemed a product was formed, but after purification and analysis, this proved to be quinoline. A last attempt was attempted without quinoline, which normally prevents overreduction. Also in this case no conversion of **5** was observed by TLC.

The fourth and final reduction method we evaluated was zinc powder. [22] This reagent is used in selective alkyne reductions, for example reducing one alkyne and leaving another one intact. Also a method to leave a TMS-protected alkyne intact is described. This method was used to reduce compound **5**. After 2.5 hour no starting material was left. After purification by column chromatography multiple different products are visible. After analysis by NMR and TLC, one isolated spot turned out to consist of multiple products. These were separated another time. The amount of products was about 1 mg or less each, which makes accurate analysis by NMR impossible. There was also a moment during the reaction when all solvent had evaporated, which could have led to degradation. Another attempt led to less spots at first sight. After purification by column chromatography still multiple products were visible. One of them showed significant alkene signals in the NMR spectrum, but due to an insufficient amount, no further analysis could be performed. And due to limited time in this project, no other conditions are explored. More suggestion will be discussed in the **Error! Reference source not found.**.

Enzyme assay

The cell-free enzyme assay was used to look for the products formed with DHEA, without using alkyne-DHEA. In this assay four samples were made: one containing arachidonic acid (0.25 µM), two containing DHEA (2 µM and 40 µM) and one containing both (0.25 µM ARA and 2 µM DHEA). Those concentrations were chosen by considering results of an earlier experiment. In this experiment the concentration of DHEA that decreases the levels of PGE_2 was determined. The stated concentrations in these results seemed to be the initial added concentration instead of the final concentration. Because of this possible misinterpretation, two different concentrations of DHEA are used: the reported concentration of inhibition as added concentration and as final concentration.

The experiment was performed twice. The second time contained twice the amount of material. Therefore the last step was slightly changed. In the first experiment the enzyme was precipitated with 1.8 mL acetonitrile and the supernatant was separated from the pallet. The second time the pallet was suspended another time in 1.8 mL acetonitrile, because the limited volume of the used reaction vessel. After evaporation of the solvent under a stream of nitrogen, the samples were analysed by UHPLC.

Most of chromatograms showed the same pattern, regardless of the sample composition. Considering the addition of several co-factors, these compounds can be separated and visualized in this case. Only in the second experiment another pattern is observed for the sample containing DHEA and ARA. Also the starting materials, ARA and DHEA, were injected at the expected concentration of the assay. The same pattern was observed. To confirm that the used UV detection method is suitable for this starting material, the absorption spectrum of ARA was measured. In the **Error! Reference source not found.** it can be seen that ARA shows absorption in the used detection wavelength range of 190 to 360 nm.

It should be noted that after storing the samples for a couple of days at room temperature for measurement, they showed a remarkable difference in colour. All the samples of the first experiment turned dark orange in contrast to the almost colourless samples of the second experiment. In the first experiment no formic acid was available, so the difference in colour could be due to pH differences.

Future prospects

In order to synthesize alkyne-DHEA, the reduction of alkynes to Z-alkenes is the only step in the synthesis that is currently still not working. Several conditions were evaluated, where a zinc mediated reduction is the most promising at the moment. If more starting material is made, more information of the formed products can be obtained. In that case it can be said if the desired product is formed. Optimizing this reaction could lead to more efficient product formation, for example by using

completely dry circumstances and using sonification to obtain better mixing in the mixture.

Other used reagents, nickeldiacetate and Lindlar's catalyst, are more commonly used in selective reduction of alkynes to cis-alkenes, also in the case of fatty acids synthesis. Maybe reaction optimization can lead to successful reduction. These reagents are only used in mild conditions. Maybe changing the pressure gives more result. In case of nickeldiacetate the TMS-protected alkyne was also reduced. The temperature was lowered, but no clear result was found. Maybe on a larger scale this effect can be studied in more detail.

The enzyme assay gave no conclusive results. To know for sure whether the enzyme assay is still working, the enzyme activity should be determined. This can be done by using the enzyme assay in combination with the ELISA for PGE_2 formation. If enzyme is still active, the analysis of the incubations can be improved. Maybe using larger amounts or different concentrations can give more information. This assay can be used to see if the alkyne-DHEA is accepted as the natural DHEA.

Eventually, if the alkyne-DHEA probe is synthesized, the probe can be used in the enzyme assay and LPS stimulated RAW2 cells as described in the experimental approach.

Conclusion

Building blocks **1** and **2** and compound **5** were successfully synthesized. Therefore an important part of the synthesis towards the target alkyne-DHEA chemical probe has been developed, but the critical step of reducing alkynes to Z-alkenes is more challenging. If the future experiments identify conditions for this reduction, the complete synthesis can be performed. Since many prior studies into the synthese of skipped 1,3-diynes are known, including the selective reduction of alkynes, especially in fatty acid total synthesis, there are good prospects to find suitable reaction conditions for the reduction of **5**.

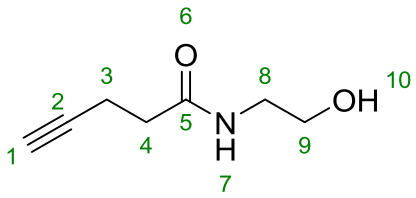
The enzyme assay gave no conclusive results in a first attempt. There are possibilities to improve the conditions for these incubations in order to check for metabolites and eventually if alkyne-DHEA is accepted and metabolized. If the probe is completely synthesized, the alkyne-DHEA probe can be used in an enzyme assay and eventually in cells to search for metabolites.

Materials and methods

General procedures

All chemicals were obtained from commercial sources and used without further purification. All reactions were stirred under an argon atmosphere at room temperature unless stated otherwise. For flash column chromatography silica gel (40-63 µm mesh) was used with the stated solvents and automated flash chromatography was performed on a Biotage Isolera system with prepacked silica columns. All the products were stored at -20°C under argon directly after purification to prevent potential degradation Thin layer chromatography was carried out on commercially available pre-coated aluminium sheets (Merck $60F_{254}$) and visualised using a $KMnO_4$ or cerium molybdate solution. NMR spectra were recorded on a Bruker 400 MHz spectrometer. NMR peak assignments were made on te basis of COSY, HSQC and HMBC experiments. The ^{1}H and ^{13}C chemical shifts were reported relative to the solvent residual ($CDCl_3$ δ 7.26) in ppm. UHPLC chromatograms were recorded on a agilent 1290 infinty UHPLC.

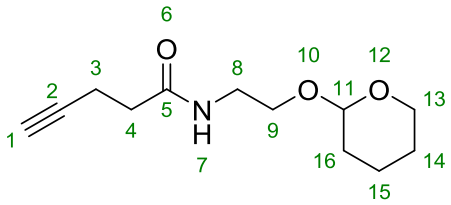
Synthesis of *N*-(2-hydroxyethyl)pent-4-ynamide (3)



4-Pentynoic acid (0.47 g, 4.79 mmol, 1 eq.) and 1-hydroxybenzotriazole hydrate (0.69 g, 5.11 mmol, 1 eq.) were dissolved in 50 mL dry DCM. After cooling of the reaction mixture to 0°C, diisopropylcarbodiimide (0.73 mL, 4.73 mmol, 1 eq.) was added and the resulting suspension was allowed to warm to room temperature. After 15 minutes of warming ethanolamine (0.40 mL, 6.63 mmol, 1.4 eq.) was added and the reaction mixture was stirred overnight. The suspension was filtered and the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (DCM/MeOH in 95:5 to 90:10 in presence of TEA) yielding a light yellow oil in 76% (0.51 g, 3.59 mmol). Rf = 0.50 in DCM/MeOH (9:1 in presence of TEA)

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (br s, 1H, H-7), 3.89 (br s, 1H, H-10), 3.64 (t, *J*=5.1 Hz, 2H, H-9), 3.35 (q, *J*=5.2 Hz, 2H, H-8) 2.44 (m, 2H, H-3), 2.38 (m, 2H, H-4), 1.96 (t, *J*=2.3 Hz, 1H, H-1).

Synthesis of *N*-(2-(tetrahydro-*2H*-pyran-2-yl)ethyl)pent-4-ynamide (1)

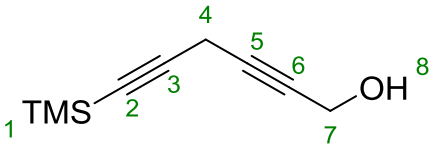


To compound **3** (0.28 g, 1.98 mmol, 1 eq.) *p*-toluenesulfonic acid (0.02 g, 0.10 mmol, 0.05 eq.) and 1.4 mL dry DCM were added. Subsequently 3,4-dihydro-*2H*-pyran (0.27 mL, 2.98 mmol, 1.5 eq.) was added slowly. After 3 hours the reaction mixture was quenched with a few drops of TEA and concentrated. The crude product was purified by column chromatography (PE/EtOAc 1:1) yielding a colourless oil in 78% (0,29 g, 1.31 mmol). Rf = 0.58 in EtOAc

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (br s, 1H, H-7), 4.40 (t, J=3.9, 1H, H-11), 3.73-3.67 (m, 1H, H-9), 3.60 (m, 1H, H-9), 3.43-3.26 (m, 4H, H-8-16), 2.36 (t, J=7.1, 2H, H-3), 2.26 (t, J=7.1, 2H, H-4), 1.86 (t, J=2.5, 1H, H-1), 1.68-1.62 (m, 1H, H-15), 1.59-1.52 (m, 1H, H-13), 1.37 (m, 4H, H-13-14-15),

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 99.3, 82.9, 69.1, 66.5, 62.7, 39.4, 35.1, 30.5, 25.2, 19.7, 14.7.

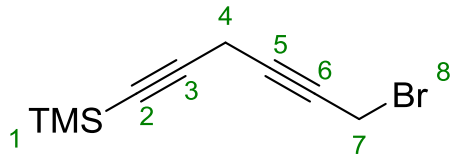
Synthesis of (6-trimethylsilyl)hexa-2,5-diyn-1-ol (4)



Cs_2CO_3 (3.01 g, 9.24 mmol, 1 eq.), NaI (1.39 g, 9.37 mmol 1 eq.) and CuI (1.79 g, 9.25 mmol, 1 eq.) were finely ground and dried overnight in a vacuum oven (50 °C) and afterwards suspended in dry DMF (20 mL). Subsequently, porpargyl alcohol (0.54 mL, 9.18 mmol, 1 eq.) was added and the suspension was stirred for 20 minutes. 3-bromo-1-(trimethylsilyl)-1-propyne (1.50 mL, 9.18 mmol, 1 eq.) was added dropwise and the suspension was stirred overnight. The reaction mixture was quenched with a sat. NH_4Cl solution (200 mL) and the mixture was extracted 3 times with 100 mL Et_2O. The combined organic layers were dried with $MgSO_4$ and concentrated. The crude product was purified by column chromatography (PE/EtOAc 95:5 to 85:15) yielding a brown oil in 87% (1.33 g, 7.99 mmol). Rf = 0.34 in PE/EtOAc (17:3)

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.26 (s, 2H, H-7), 3.25 (s, 2H, H-4), 0.15 (s, 9H, H-1).

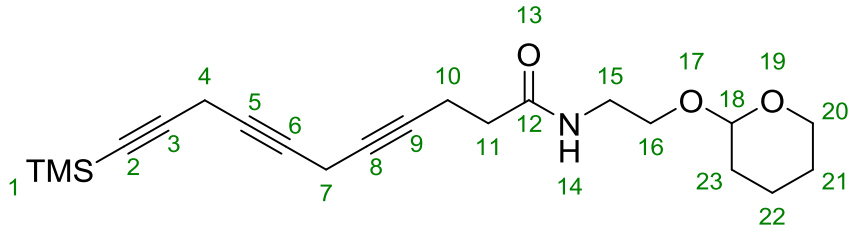
Synthesis of (6-bromohexa-1,4-diyn-1-yl)trimethylsilane (2)



After co-evaporation of compound **4** (0.49 g, 2.95 mmol, 1 eq.) CBr_4 (1.28 g, 3.85 mmol, 1.3 eq.) was added. The reaction mixture was dissolved in 8 mL dry DCM and cooled to 0 °C. PPh_3 (1.16 g, 4.43 mmol, 1.5 eq.) was dissolved in dry DCM and added slowly to the reaction mixture. Afterwards the reaction mixture was allowed to warm to rt. After 140 minutes the reaction mixture was concentrated. The residue was dissolved in a minimal amount of DCM (about 1 mL) and Et_2O/heptane (1:1) was added dropwise. The suspension was filtered and washed with Et_2O. After evaporation of the filtrate, the crude product was dissolved another time in little DCM and the addition of Et_2O/heptane was repeated. After evaporation the product **2** was obtained in a brown oil in 82% (0.55 g, 2.43 mmol). Rf = 0.44 in PE or 0.94 in PE/EtOAc (9:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90 (s, 2H, H-7), 3.25 (s, 2H, H-4), 0.15 (s, 9H, H-1).

Synthesis of *N*-(2-((tetrahydro-*2H*-pyran-2-yl)oxy)ethyl)-11-(trimethylsilyl)undeca-4,7,10-triynamide (5)



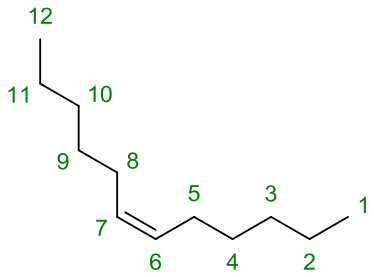
Cs_2CO_3 (0.82 g, 2.52 mmol, 1 eq.), NaI (0.49 g, 2.60 mmol, 1 eq.) and CuI (0.39 g, 2.64 mmol, 1 eq.) were finely ground and dried overnight in a vacuum oven (50 °C) and afterwards suspended in dry DMF (20 mL). Subsequently, building block **1** (0.55 g, 2.43 mmol, 1 eq.) was added as solution in DMF and the suspension was stirred for 20 minutes. Building block **2** (0.56 g, 2.43 mmol, 1 eq.) was added dropwise as solution in DMF and the suspension was stirred overnight. The reaction mixture was quenched with a sat. NH_4Cl solution (200 mL) and the mixture was extracted 3 times with 100 mL Et_2O. The combined organic layers were dried over $MgSO_4$ and concentrated. The crude product **5** was purified by column chromatography (PE/EtOAc 95:5 to 85:15) yielding a brown oil in 24% (0.22 g, 0.58 mmol). Rf = 0.34 in EtOAc/PE (3:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 (s, 1H, H-14), 4.55 (br s, 1H, H-18), 3.88 (m, 1H, H-23), 3.77 (quint, *J*=5.2, 1H, H-16), 3.62 (quint, *J*=5.2, 1H, H-16), 3.52 (m, 2H, H-23), 3.48 (m, 2H, H-15), 3.20 (m, 2H, H-4), 3.13 (m, 2H, H-7), 2.50 (t, *J*=7.1, 2H, H-10), 2.38 (t, *J*=7.1, 2H, H-11), 1.83 (m, 1H, H-23), 1.74 (m, 1H, H-20), 1.55 (m, 4H, H-20-21-22), 0.15 (s, 9H, H-1).

^{13}C NMR (100 MHz, $CDCl_3$) δ 168.9, 97.8, 81.1, 67.2, 65.0, 61.2, 37.6, 33.4, 28.8, 23.3, 18.0, 12.9.

Reduction

Method using a copper complex

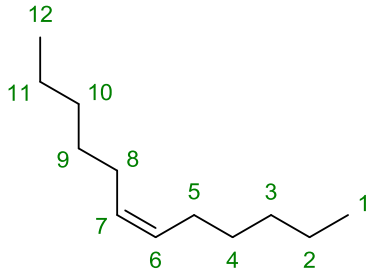


IPrCuCl (0.14 g, 0.28 mmol, 0.4 eq.) and anhydrous NaOt-Bu (0.03 g, 0.28 mmol, 0.4 eq.) were dissolved in 5 mL dry THF in a flame-dried round bottom flask. This catalyst solution was allowed to stir vigorously for 20 minutes, while 6-dodecyne (0.15 mL, 0.70 mmol, 1 eq.*), polymethylhydrooloxane (2.0 mL, 50 eq.) and *tert*-butanol (1.67 mL, 17 mmol, 25 eq.) were dissolved in 20 mL dry toluene in another flame-dried round bottom flask. The catalyst solution was transferred to the this latter mixture. The resulting reaction mixture was heated at 40 °C for 5 hour, after which the mixture was diluted with PE and filtered through a short silica pad. The product was eluted with ethylacetate/PE (1:1) and the filtrate was concentrated. Analysis by NMR confirmed formation of 6Z-dodecene, so no further purification was performed. Rf = 1 in PE

* The procedure as reported by Whittaker et *al.* contains an error: 0.148 mL of 6-dodecyne was mentioned where probably 1.480 mL was used. A tenfold of the starting material should thus be used when following their procedure.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, *J*=6.9, 6H, H-1 H-12), 1.27-1.38 (m, 12H, H-2 H-3 H-4 H-9 H-10 H-11), 2.03 (q, *J*=6.4, 4H, H-5 H-8), 5.36 (t, *J*=4.6, 2H, H-6 H-7)

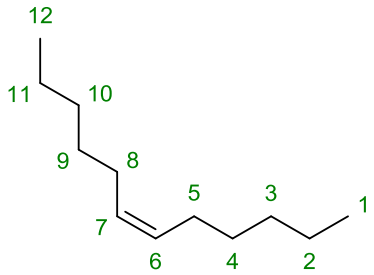
Method for using Lindlar's catalyst



To the Lindlar catalyst (0.10 g, 50 %wt), 3 mL *tert*-butanol was added under a argon atmosphere. Argon was replaced for H_2. After saturation of the catalyst, quinoline (0.14 mL, 1.2 mmol, 1 eq) was added followed by 6-dodecyne (0.25 mL, 1.2 mmol, 1 eq). After 2.5 hours the reaction mixture was filtered through celite and the filtrate was concentrated. Analysis by NMR confirmed formation of 6Z-dodecene, so no further purification is performed. Rf = 1 in PE

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (t, *J*=6.9, 6H, H-1 H-12), 1.28-1.37 (m, 12H, H-2 H-3 H-4 H-9 H-10 H-11), 2.03 (q, *J*=6.4, 4H), 5.37 (t, *J*=5.0, 2H, H-6 H-7)

Method using nickeldiacetate



$Ni(OAc)_2{\cdot}4\ H_2O$ (0.60 g, 2.41 mmol, 2 eq.) was dissolved in 5 mL EtOH (96%) and placed under a balloon of H_2. $NaBH_4$ (0.09 g, 2.41 mmol, 2 eq.) was added and was followed after 20 minutes by ethylenediamine (0.64 mL, 9.62 mmol, 8 eq.). After saturation of the reaction mixture 6-dodecyne (0.25 mL, 1.20 mmol, 1 eq.) was added. The reaction mixture was left stirring for another 90 minutes under a H_2 atmosphere. Subsequently the reaction mixture was filtered through a pad of celite. The residue was washed with EtOH and the filtrate was concentrated. Analysis by NMR confirmed formation of *6Z*-dodecene, so no further purification is performed. Rf = 1 in PE

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (t, *J*=6.9, 6H, H-1 H-12), 1.28-1.37 (m, 12H, H-2 H-3 H-4 H-9 H-10 H-11), 2.04 (q, *J*=6.4, 4H), 5.36 (t, *J*=5.7, 2H, H-6 H-7)

Method using zinc

1,2-Dibromoethane (10 µL, mmol, 0.8 eq.) was added to a mixture of zinc powder (0.09 g, 3 g/5 mmol) in 1 mL absolute ethanol in a three-necked round-bottom flask. The mixture was heated to reflux and kept there for another 10 minutes in a N_2 atmosphere. The reaction mixture was cooled to about 50 °C and compound **5** (0.06 g, 0.15 mmol, 1 eq.) was added. The reaction mixture was heated again to reflux and after 1.75 hour the reaction mixture was cooled to rt. The reaction mixture was quenched with saturated NH_4Cl (aq) (10 mL) and extracted seven times with Et_2O (5 mL). The combined organic layers were dried and concentrated. The mixture of products was purified by column chromatography (EtOAc/PE 1:1 to 13:7). The first obtained fractions are purified again (EtOAc/PE 1:9 to 2:8) yielding no conclusive results by NMR.

Enzyme assay

For the incubation mixture earlier made solutions of hemtin in DMSO (5 mM) and epi in MQ (1.8 mM) are used. These solutions were stored in -80 °C. An aqueous solution of Na_2EDTA (50 mM) and tris-HCl (1M, pH 8.0) were freshly prepared. For each series of incubations a new incubation mixture is made following Table 1.

Table 1: composition of the incubation mixture for the enzyme assay. Solution diluted with MQ.

Solution of	Concentration
Na_2EDTA	50 µM
Tris-HCl	100 mM
Hematin	5 µM
Epi	18 µM

The incubation mixture is used in each sample and the samples are prepared following table 2. The enzyme mixture is stored on ice.

Table 1: Composition of samples for the second incubation. All amounts are showed in µL. The first incubation consisted of half of the stated amounts.

Entry	Incubation mixture	Enzyme mixture	EtOH 40 µM	DHEA 800 µM	DHEA 80 µM	DHEA 80 µM	ARA 10 µM	ARA 5 µM
1	360	2	20	x	x	x	x	10
2	360	2	20	20	x	x	x	x
3	360	2	20	x	20	x	x	x
4	360	2	20	x	x	10	10	x

After addition of the incubation mixture, enzyme mixture and ethanol, the samples were incubated for 5 minutes at room temperature. In the meantime the dilutions of ARA and DHEA are made. After incubation, DHEA and/or ARA are added and the samples are incubated by 37 °C for 20 minutes. The reaction was stopped by adding 20 µL of 10% v/v formic acid in MQ. Subsequently the enzyme was precipitated by adding 1.8 mL acetonitrile and the supernatant is separated from the pallet. The pellet was suspended again in 1.8 mL acetonitrile and separated from the supernatant. The total supernatant was dried under a stream of nitrogen in warm water and stored in -20 °C

UHPLC analysis

The samples were analysed by UHPLC (agilent 1290 infinty series). Seperation was achieved by injecting 10 µL of the mixture on a Zorbax eclipse Xplus C18 column (2.1 mm × 100 mm, 1.8 µm) at room temperature and with a flow rate of 0.4 ml min-1. The eluens consisted of 0.05% trifluoroacetic acid in water (eluens A) and in 0.05% trifluoroacetic acid in acetonitrile (eluens B). The used method for separation of the samples was as follows: elution for 3 min at 5% B followed by a gradient towards 100% B in 22 minutes This was followed by a steep gradient back to 5% B in 1 minute and the system was then re-equilibrated by employing 9 minutes of 5% B. A photodiode array detector (DAD G4212A) was used (190 to 360 nm) to analyse the eluent.

Acknowledgments

First of all I would like to thank Tom for his daily supervision. I really learned a lot from all our conversations and I also learned a lot by making my own decisions. Furthermore I would like to thank Bas, Tjerk and Jorin for all the help in the lab. I learned some nice tricks and received good advice. I also would like to thank Jocelijn and Michiel for the help at the department of human nutrition, especially for all the answers to my questions. I also would like to thank Maurice for his help and reading this report. Finally I would like to thank everyone in the office, Jona, Wouter, Ruijo, Pepijn and Marian, for the nice talks and pleasant time. I had a great time!

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Supplementary information

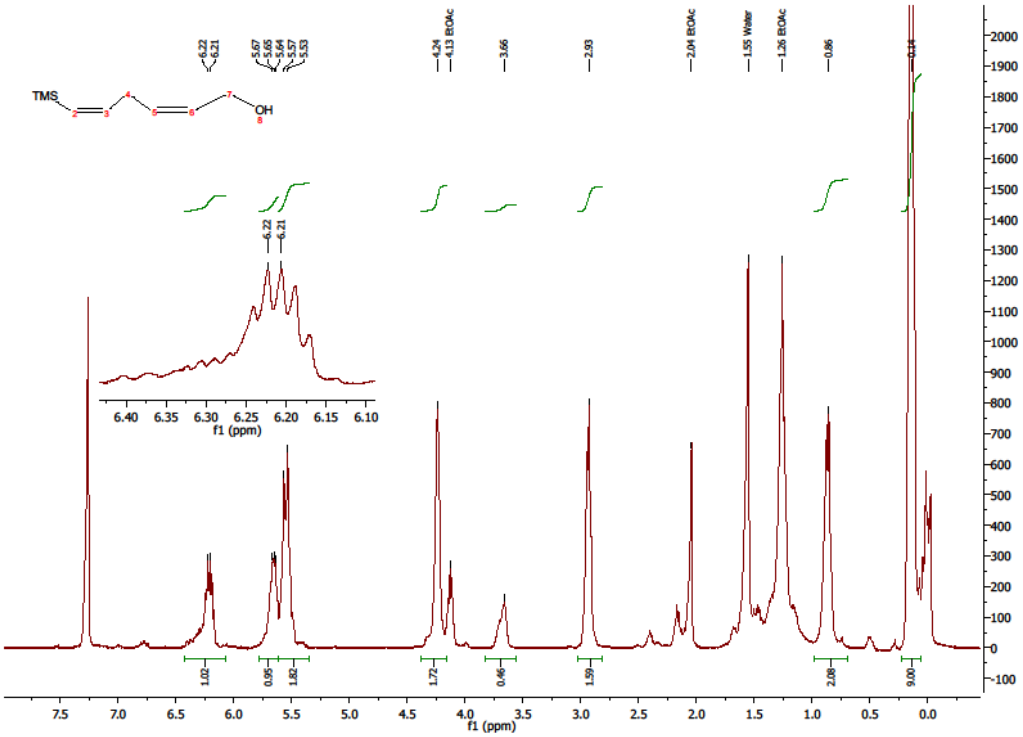

Figure 7: Spectrum of reduction of compound 4 using nickeldiacetate. Characteristic alkene-signals around 5.6 and 6.2 ppm.

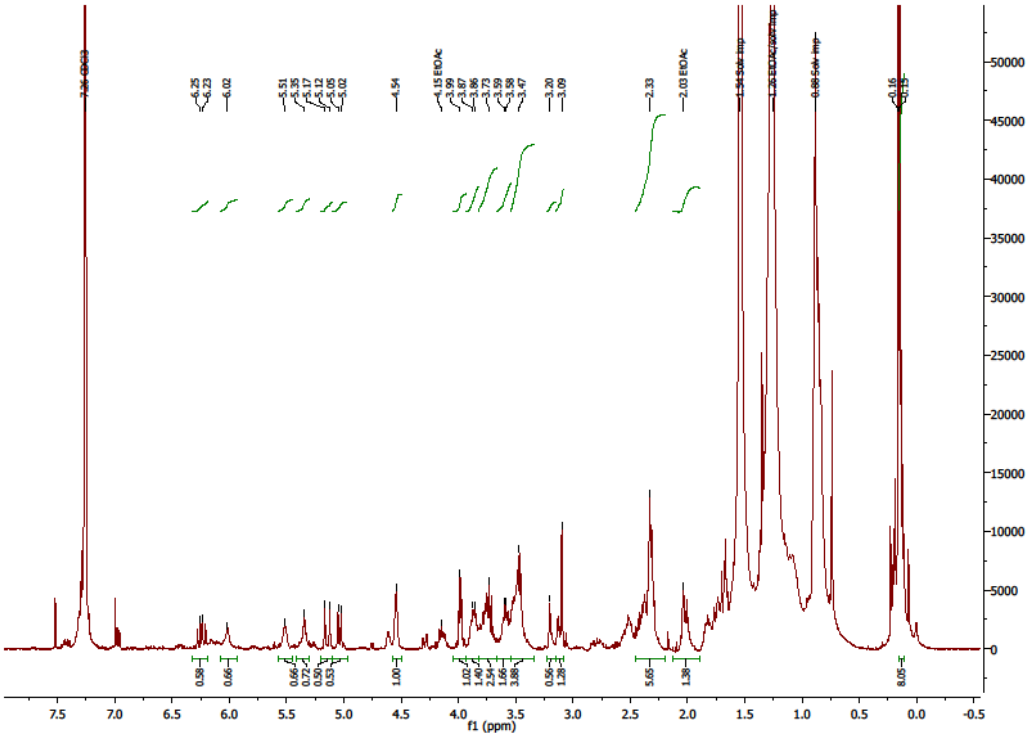

Figure 8: Spectrum of the reduction of compound 5 using zinc. Possible alkene-signals visible in the 5-6.5 ppm region.

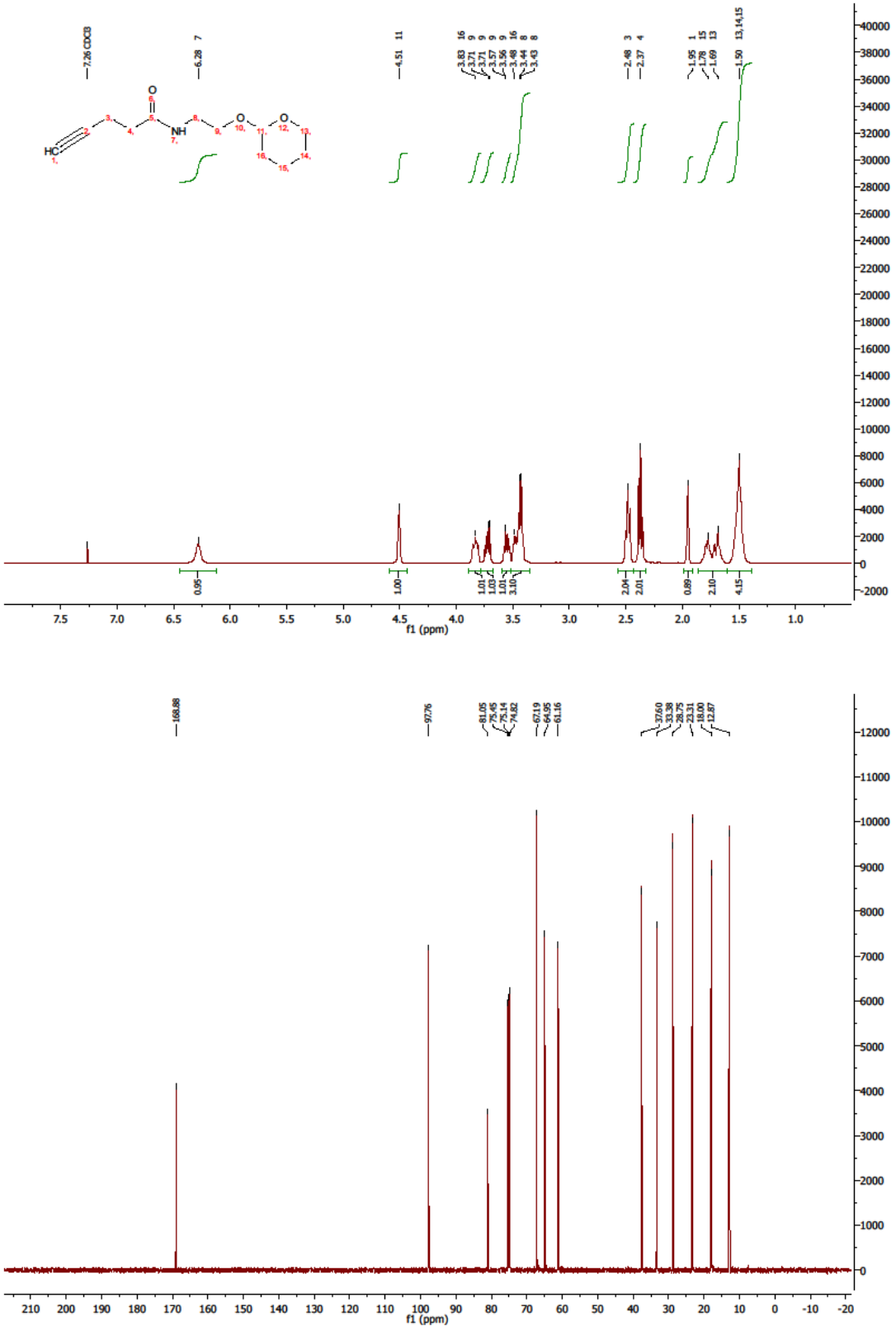

Figure 9: ^{1}H and ^{13}C spectra of compound 1. In the upper spectrum (^{1}H) the peaks are assigned.

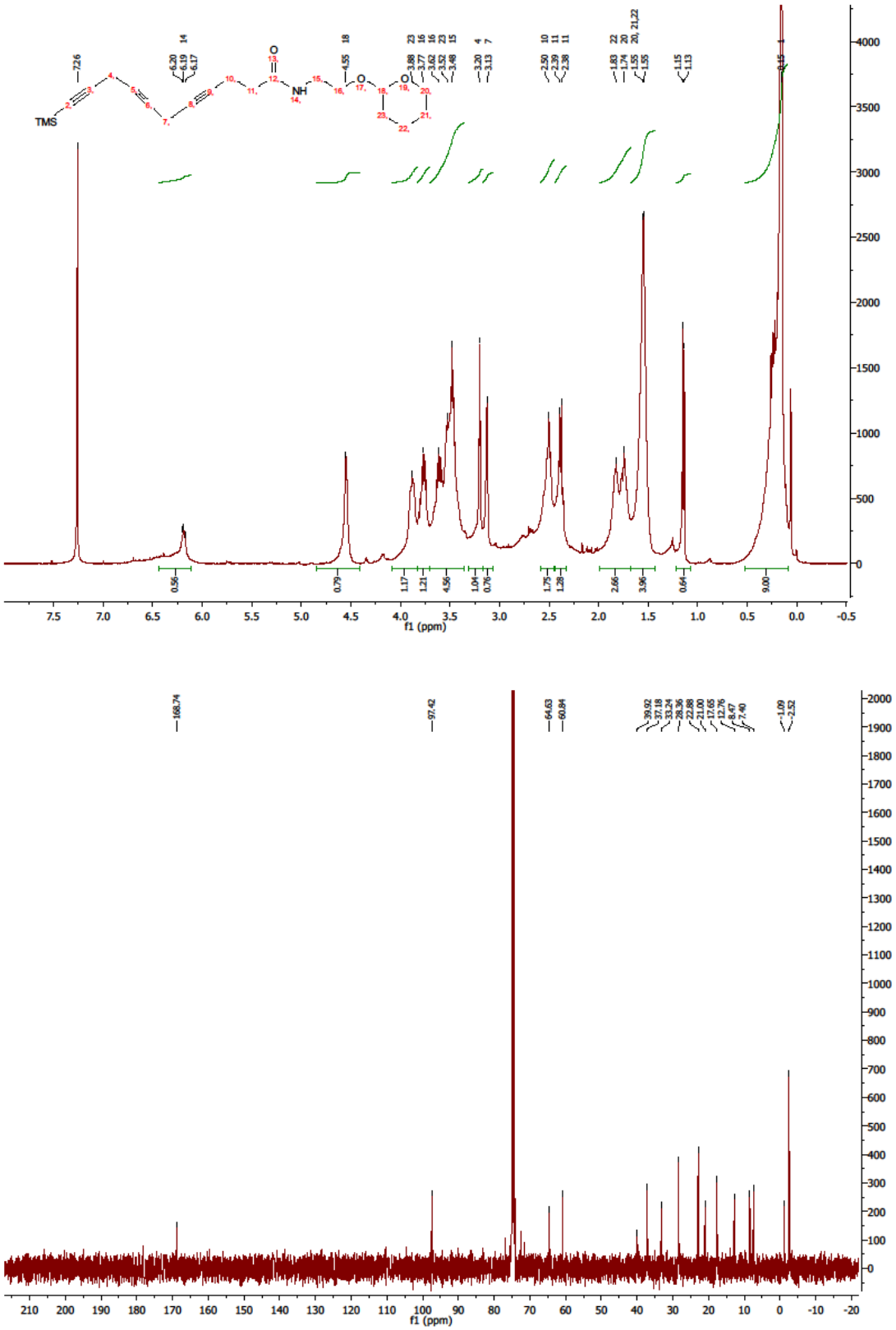

Figure 10: ^{1}H and $_{13}C$ spectra of compound 5. In the upper spectrum (^{1}H) the peaks are assigned.

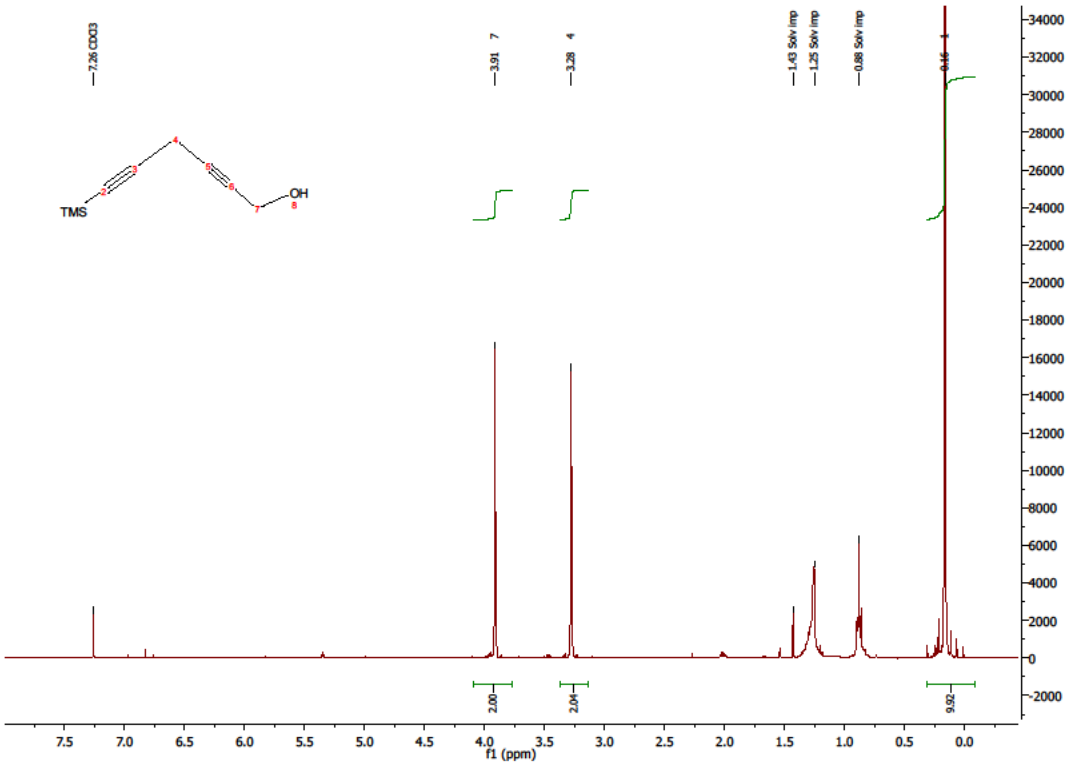

Figure 11 1H spectrum of compound 2 with assignments. Some major solvent impurities are visible (solv imp in spectrum).

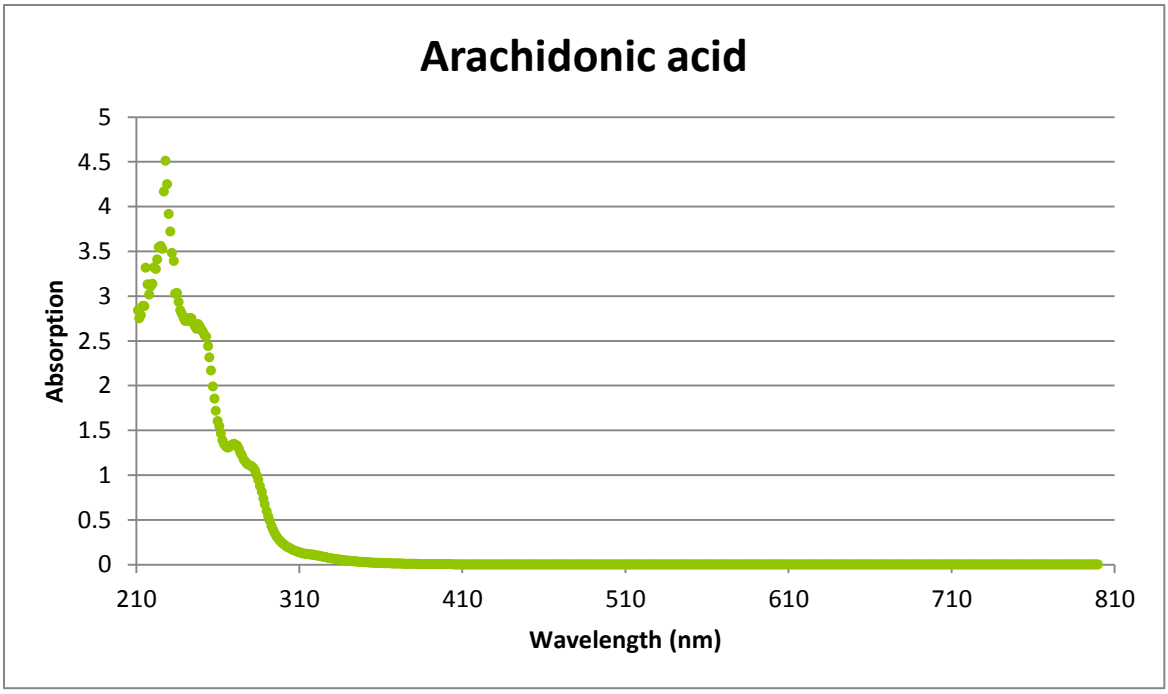

Figure 12: Absorption spectrum of a solution of approximately 6,5 mM arachidonic acid (ARA)