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Treball Final de Grau

Preparation of 3-azidocoumarins and alkyne-analogues of fatty acids for click chemistry.

Preparació de 3-azidocumarines i derivats alquínics d'àcids grassos per reaccions "click".

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Campus d'Excel·lència Internacional B:KC Barcelona Knowledge Campus

Els grans coneixements engendren els grans dubtes.

Aristòtil

Primerament voldria agrair a en Jordi García, tutor d'aquest treball, tant la seva dedicació com el bon tracte que m'ha donat durant aquests mesos, fet que m'ha motivat a posar tot el meu esforç en aquest projecte.

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Per últim agrair als meus amics, tant de la universitat com de fora, els seus ànims i paraules d'alè, així com els bons moments que em donen i que em fan tan feliç, estat anímic que es tradueix en optimisme i motivació per seguir treballant i tirar endavant.

Aquest treball se'l vull dedicar a la meva àvia materna, per no rendir-se, lluitar i aconseguir sortir endavant, i per lo molt que l'estimo.

REPORT

CONTENTS

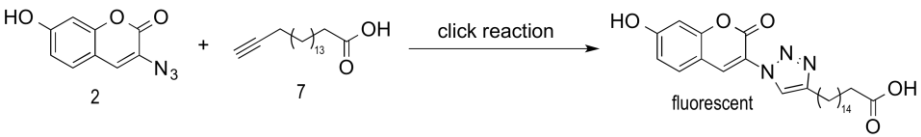
1. SUMMARY

Click chemistry is evolving as a powerful tool in biological applications because it allows the sensitive and specific detection of compounds with alkyne or azido groups. Particularly, this click-chemistry-based method has been applied to the tracing of fatty acid metabolism. Fatty acids are abundant constituents of all biological systems, and their metabolism is important for normal function at all levels of an organism. Changes in fatty acid metabolism can cause severe diseases and have become a focus of recurrent research.

The present work deals with the synthesis and characterization of two known compounds: the fluorogenic dye 3-azido-7-hydroxycoumarin bearing an azido group and an alkyne-analogue of a fatty acid. Those compounds could be useful for the study of fatty acids metabolism because they allow to form "in situ" a triazole derivate with fluorescence activity which can be detected and quantified.

Introduction of a small terminal triple bond does not significantly disturb the chain structure and it allows to trace the fatty acid by a click reaction with the azido coumarin. Thus, a fluorescent compound is formed containing a triazole moiety which can be detected and quantified once the cell has been exposed to the alkyne-analogue and metabolites have reacted with the profluorophore.

Both products have been sent to the biochemical research group leaded by Dra. Casals in the Universitat Internacional de Catalunya (UIC) to study the activity of carnitine palmitoyltransferase I enzyme (CPT1) by this method involving click reaction.



Keywords: click chemistry, coumarin, fatty acids, synthesis, metabolism, CPT1.

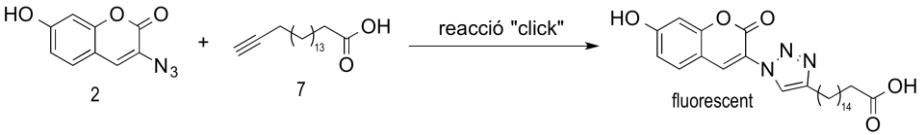
2. RESUM

La anomenada “click chemistry” està evolucionant com una poderosa eina en aplicacions biològiques degut a que permet la detecció específica i sensible de compostos amb grups alquí o azido. Particularment, aquest mètode basat en la química “click” ha estat aplicat en el seguiment del metabolisme d’àcids grassos. Els àcids grassos són constituents abundants de tots els sistemes biològics, i el seu metabolisme és important per a un funcionament normal a tots els nivells d’un organisme. Canvis en aquest metabolisme poden provocar malalties severes i ha esdevingut un focus en la investigació actual.

El treball que es presenta tracta la síntesi i la caracterització de dos compostos coneguts: la 3-azido-7-hidroxicumarina (un colorant fluorogènic), que conté un grup azido, i un derivat d’àcid gras amb un triple enllaç terminal. Aquests compostos poden ser útils en l’estudi del metabolisme dels àcids grassos degut a que permeten formar “in situ” un derivat fluorescent amb un grup triazole que pot ser detectat i quantificat.

La introducció d’un triple enllaç terminal petit pràcticament no pertorba l’estructura de la cadena i permet marcar l’àcid gras mitjançant una reacció “click” amb la azidocumarina. D’aquesta manera es forma un compost fluorescent que conté un triazole i que pot ser detectat i quantificat un cop la cèl·lula ha estat exposada al derivat alquínic i s’han fet reaccionar els metabòlits amb el profluoròfor.

Ambdós productes han estat enviats al grup de investigació bioquímica de la Universitat Internacional de Catalunya (UIC) liderat per la Dra. Casals per tal d’estudiar l’activitat de l’enzim carnitina palmitoiltransferasa I (CPT1) mitjançant aquest mètode que implica la reacció “click” .



Paraules clau: click chemistry, cumarina, àcids grassos, síntesi, metabolisme, CPT1.

3. INTRODUCTION

Fatty acids are ubiquitous metabolites in living organisms with a linear hydrocarbon chain structure bearing a terminal carboxylic group. They are essential compounds, and an incorrect metabolism of them is associated with pathological states and metabolic overload diseases.

Because of this, the study of metabolism of those substances has become an important focus on biomedical research. In the past, radioisotopes were used to tracing fatty acid metabolism[1], typically ^{3}H and ^{14}C for oleate and palmitate because they represented the most abundant in mammalian cells and circulation. This technique with radioisotopes has limited sensitivity and higher cost, and also requires special laboratories. For this reason, discovery of click chemistry's application in this field has been very useful.

CPT1 enzyme (carnitine palmitoyltransferase I) is a mitochondrial enzyme related to metabolism of those compounds. It is responsible for acyl carnitine formation. It is placed into the mitochondrial outer membrane and it is the first component of the carnitine palmitoyltransferase system, catalysing the transfer of the acyl group from coenzyme A to carnitine, forming acylcarnitine.

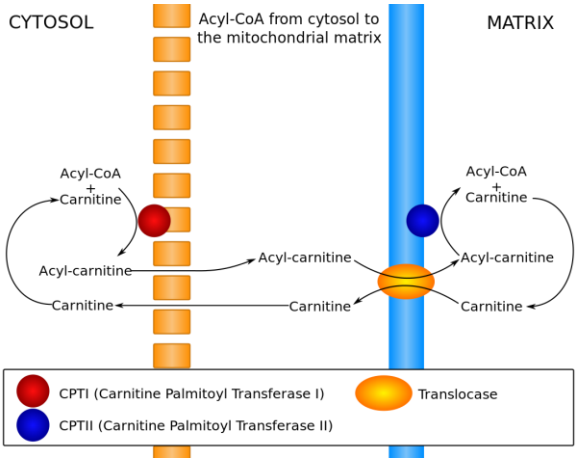


Figure 1. The carnitine palmitoyltransferase system.

(Slagt, 24/05/15 via Wikimedia Commons, Creative Commons Attribution)

Long-chain fatty acids cannot enter the mitochondria by diffusion; they need to be activated first. On the outer mitochondrial membrane they are transformed onto long-chain fatty acyl-CoA and then imported into the mitochondria by CPT1 and CPT2[2].

CPT1 disorders are associated with diseases as type 2 diabetes and insulin resistance, which cause high free fatty acid level in humans and decrease the ability of muscles to oxidize fatty acids. Increase of malonyl-CoA inhibits CPT1, causing less transport of long fatty acids into mitochondria[3].

Another interesting compound is 3-azido-7-hydroxycoumarin, a derivate from coumarin (*2H*-chromen-2-one, Figure 2). Coumarins are substances of biological relevance due to their biological activities. Thus, similarly to flavonoids, coumarins can reduce inflammation and affect the formation of reactive oxygen species, which can damage some cell structures. Besides this anti-inflammatory and antioxidant capacity, they can also reduce tissue edema[4,5]. What it is relevant for this work, however, are their properties as a fluorogenic dye.

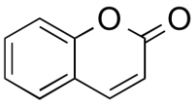


Figure 2. Coumarin.

3.1. CLICK CHEMISTRY

The "click chemistry" term (CC) is applied to the formation of new compounds through heteroatom links using spring-loaded reactants. As defined by Sharpless *et al*.[6], click chemistry is based on reactions with high selectivity and reliability, modular, wide in scope, with very high yields that generate only inoffensive by-products easy to remove. They are stereospecific, but not necessarily enantioselective, which include simple reaction conditions and benign solvent, and are ruled by kinetic control.

Observing molecules created by nature, there is a preference for carbon-heteroatom bonds. Nevertheless, click reactions also include non-aldol carbonyl chemistry or additions to carbon-carbon multiple bonds, not only carbon-heteroatom bonds. Many of those reactions proceed well in water, apart from in organic solvents.

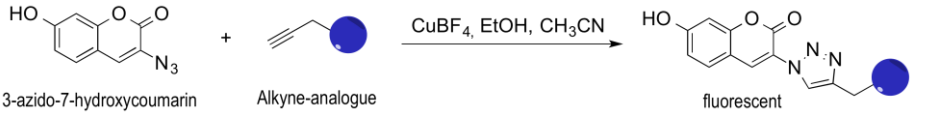
3.1.1. Click chemistry in fatty acids metabolism

Tracing fatty acids metabolism can be done with radioisotopes, but this approach is expensive and time demanding, and has a low detection limit for tritium. Moreover, tritium manipulation needs special equipment and safety requirements. In contrast, the sensitivity of click-labelling is high and the linear signal generated allows direct quantification[1] without special safety cares.

The most popular click reaction used for this purpose is the Huisgen 1,3-dipolar cycloaddition between azides and terminal alkynes. This reaction does not require protecting groups and gives a di-substituted triazole. To avoid the formation of a mixture of the 1,4 and 1,5 regioisomers, the reaction is performed in presence of copper(I) as a catalyst. Whether the reaction is performed this way, only 1,4-disubstituted 1,2,3-triazole is formed[7]. This process constitutes the premier example of click chemistry, and is often referred to as "the click reaction".

Alkyne and azide group are small in size and have been employed as a pair of orthogonal linkers for chemoselective ligations[8]. Coumarin is small, biocompatible and easy to manipulate synthetically, and its reaction with de alkyne-analogue gives a strong fluorescent product. Typically, analogues of fatty acids containing terminal alkyne groups are supplemented to cells in culture to follow the lipid metabolism. They can also be supplemented to mouse brain slices or injected *in vivo* to mice. These alkyne-acids supplies are used by cells for lipid synthesis or modification, and after a defined time the tagged compounds and their metabolic products are extracted and exposed to 7-hydroxy-3-azidocoumarin in presence of copper(I) for click-labelling. Thanks to fluorescence is possible to know where the fatty acids are in cells if the reaction is carried out before lipid extraction. The lipid extracts are then loaded onto a TLC plate and visualized with LED excitation.

Neurons do not burn fatty acids, only glucose, so one of the biochemist's main objectives related to this work is know what happens to fatty acids when they arrive at hypothalamus. They had two species of mouse: normal ones, and "knockout". The last ones are modified mice without the CPT1 gene, and they inject alkyne-analogues to both species. After some days, mice are sacrificed and the study is carried out.



Scheme 1. Reaction of the coumarin and the lipid extract carried out by UIC biochemists.

Azides and alkynes are introduced into organic compounds easily, and some bioconjugation reactions can be carried out without affecting living tissues[9]. Numerous biomolecules as proteins, oligosaccharides or DNA can be labelled, but click chemistry has more uses in bioconjugation. Chaikof *et al.*[10] expose the immobilization of proteins and carbohydrates onto solid surfaces *via* click chemistry, forming a triazole between a glass slide derivatized with alkyne bearing substituents. This kind of immobilization does not give unwanted products.

4. OBJECTIVES

The main objective of this work is the preparation of two compounds:

3-azido-7-hydroxycoumarin, a fluorogenic dye, starting from 2,4-dihydroxy benzaldehyde and *N*-acetylglycine.

17-octadecyn-1-oic acid, an alkyne-analogue produced in a five-steps method, starting from oleic acid and transforming the double bond into triple bond before the migration of it.

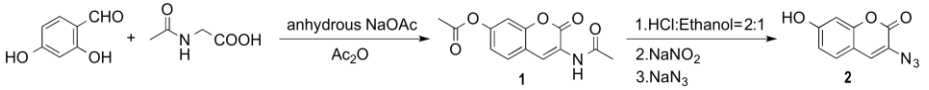
5. RESULTS AND DISCUSSION

The reactions carried out and the corresponding results are treated in this section. Some reactions deserve a detailed explanation, but more of them are usual transformations that do not require further comments.

It should be pointed out that only product **7** required purification by column chromatography, one of the target compounds of this work. Thus, the intermediates obtained and characterised in each step (described in section 6) could contain certain amount of impurities.

5.1. PREPARATION OF 3-AZIDO-7-HYDROXYCOUMARIN

The synthesis was carried out in two steps as described in the literature[8] starting from 2,4-dihydroxy benzaldehyde as shown in Scheme 2.



Scheme 2. The two-step reaction to afford 3-azido-7-hydroxycoumarin.

In the first step *N*-acetylglycine reacts with acetic anhydride forming a cyclic intermediate (an azlactone). This intermediate suffers the enolization and the two fragments are assembled via aldol reaction when the intermediate (the nucleophile) and the aldehyde (electrophile) react. The process also involves two esterification reactions (with the 2,4-dihydroxyl groups) and finally the dehydration of the alcohol formed in the aldol reaction, giving the α,β-unsatured lactone. In addition, the conjugation of the double bond with the carbonyl and the aromatic ring favours the process.

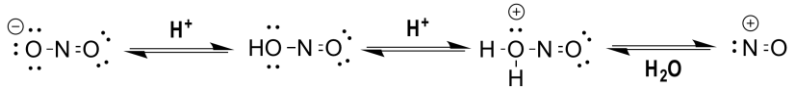
When TLC (hexane/ethyl acetate 50:50) of the filtered solution was carried out, it was not clear if the desired product remained in solution because in the TLC appeared a lot of spots; so, the TLC analysis was not conclusive. The amount of product **1** obtained was very low (0.550 g, 11%), so the solution was distilled in order to try recovering product.

About half of the solvent was removed by distillation. The concentrated solution was allowed to cool down. A brown solid was recovered by filtration next day (0.655 g). However, TLC

analysis (hexane/ethyl acetate 50:50) showed that it was not the same as the desired yellow product, so that compound was discarded.

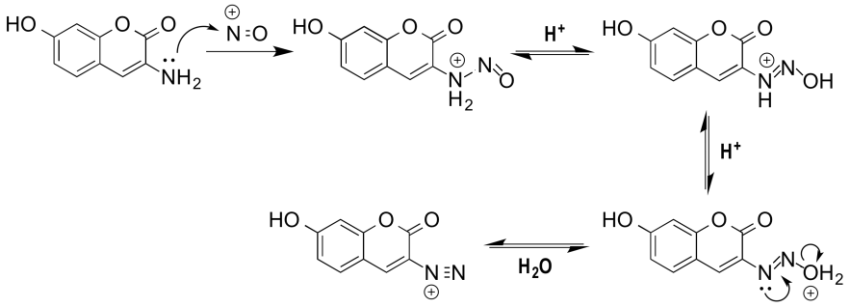
^{1}H NMR of the product **1** showed that the reaction took place. It was possible to see the signals of hydrogen linked to conjugated double bonds between 7 and 9 ppm, and the proton of the amide group at 9.74 ppm.

This product was refluxed in acid medium to hydrolyse both acetate and amide group. Amines react with $NaNO_2$ in acid medium to form diazonium salts (diazotization). Nitrous acid is generated "in situ" from sodium nitrite and hydrochloric acid, and forms the reactive specie NO^+.



Scheme 3. Acid-base equilibria that form NO^+.

The free electron pair of the amine can attack this specie, and after some acid-base balances the diazonium salt was formed (Scheme 4), stabilized by resonance. The azide was then added and nucleophilic substitution took place to form 3-azido-7-hydroxycoumarin (compound **2**, 90%).



Scheme 4. Sequence of the diazonium intermediate formation.

^{1}H NMR spectra prove the correct formation of the azidocoumarin. Thus, a characteristic wide band at 10.56 ppm that match the hydroxyl group was observed, and the rest of signals were also assigned in base of their multiplicity. ^{13}C NMR spectrum has 9 signals as expected, and carbonyl, hydroxyl and azido corresponding peaks were identified in IR spectrum. This information matches the reference data[8]. However, melting point could not be properly measured. The overall yield of the synthesis was 10%.

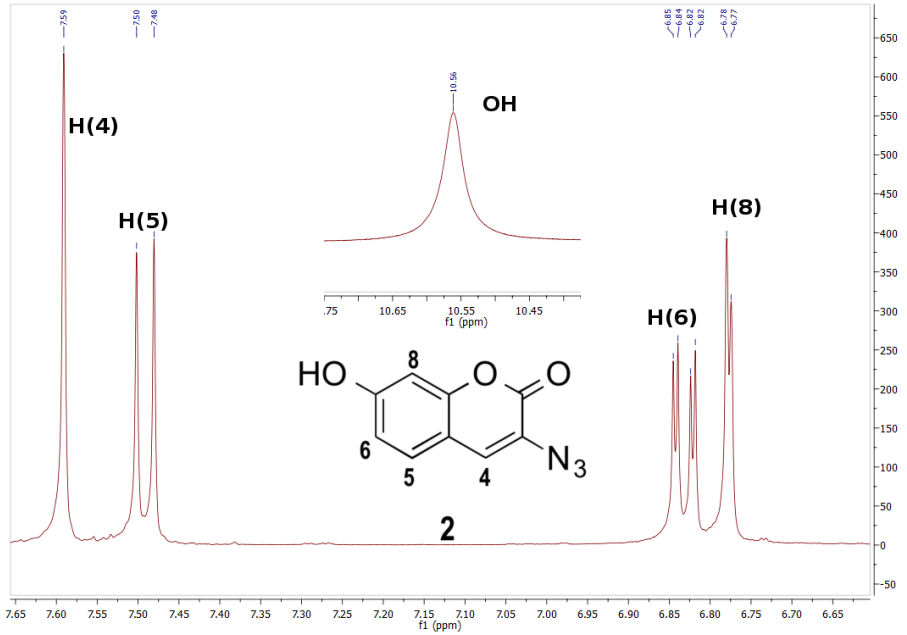


Figure 3. ^{1}H NMR spectrum of 3-azido-7-hydroxycoumarin.

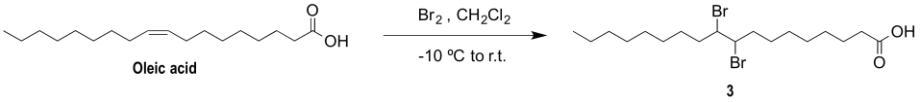
5.2. PREPARATION OF 17-OCTADECYN-1-OIC ACID

The product **7** was synthetized in 5 steps. The starting reagent was commercially available (9*Z*)-octadec-9-enoic acid (oleic acid), a monounsatured fatty acid.

The first objective was the transformation of the double bond into triple. For this purpose, the first step was the addition of bromine to the double bond for further elimination of hydrobromic acid in a second step to get the triple bond. Thus, bromine was carefully added dropwise to oleic acid. It should be noted that bromine is a corrosive and toxic reagent that should be carefully manipulated.

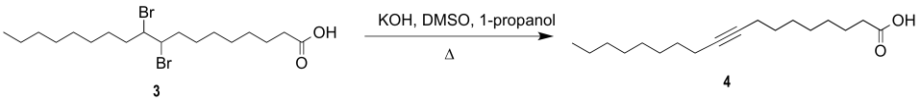
After the excess bromine was eliminated and extractions were carried out, 40.371 g of 9,10-dibromostearic acid were obtained (110%) despite the theoretical amount of dibrominated product were only 36.798 g. The reason was given by the ^{1}H NMR spectrum since it showed the presence of residual solvent which could not be entirely removed. Not signals from starting oleic acid were visible indicating that the crude did not contain starting reagent. The ^{13}C NMR spectrum contains 17 signals instead of 18, the number of carbon atoms in the molecule; the

missing peak corresponds to carbonyl present in the carboxylic group. Moreover, IR showed a carbonyl signal.



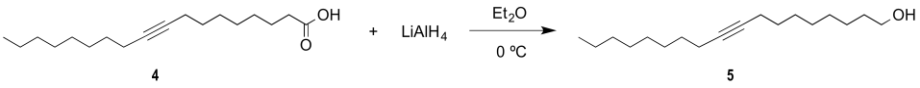
Scheme 5. First step of the alkyne-analogue synthesis: dibromination.

The second step deals with the dehydrobromination of the 9,10-dibromoacid. For this reaction, the authors described that DMSO/propanol mixture is an effective medium that could enhance the reaction rate at relatively low temperatures[11]. The mixture was allowed to reflux for one hour to ensure the complete elimination and the formation of the triple bond. The ^{1}H NMR spectrum of the crude obtained showed that the desired 9-stearolic acid (4.693 g, 49%) slightly impurified was correctly formed.



Scheme 6. Dehydrobromination to form stearolic acid.

Extractions (AcOEt, 4 x 20 mL) of the remaining filtrated solution were performed and the combined organic extracts were dried over $MgSO_4$. The volatiles were removed to obtain a yellow oil. The ^{1}H NMR spectrum of the oil showed that the amount of impurities was much higher than in the solid product, so this oil was not used for further reactions.



Entry	Stearolic acid [g]	$LiAlH_4$ [g]	Total Et_2O [ml]	Prod. [g]	Yield [%]
1	1.014	0.277	20	0.707	73
2	2.580	0.699	50	1.703	70

Table 1. Quantities and results of the acid reduction.

For the migration of the triple bond to form the terminal alkyne-analogue the protection of the carboxylic group was necessary. The best option was the reduction of the carboxylic group to alcohol with $LiAlH_4$[12]. At this point of the synthetic sequence, almost 4 grams of compound **4** were available. However, in the first attempt only one was used. The reaction is sensible to

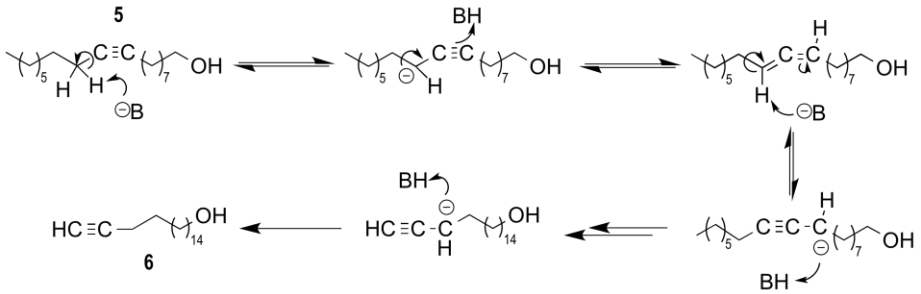
humidity, so it must be carried out in anhydrous conditions, under N_2 atmosphere. The system and reagents were always purged to ensure the appropriate performance of the reaction.

When the alkyne was added dropwise by a dropping funnel, part of the product remained on flask's wall and did not react. In the first attempt (Table **1**, entry **1**) after 4 hours reacting at ice-bath temperature the stirring remained overnight at r.t. The TLC (CH_2Cl_2/MeOH 95:5) of the isolated alcohol showed that the product did not contain unreacted acid, although ^{1}H NMR spectrum revealed that some impurities were present.

In a second run (Table **1**, entry **2**), 2.210 g of oil were obtained. TLC (CH_2Cl_2/MeOH 95:5) and NMR showed that some unreacted acid was present. The extraction with NaOH (10 mL, 0.02 N) allowed us to remove must of the acid. Then, 1.703 g of yellow oil were obtained after drying the organic layer. TLC showed that the amount of acid decreased but the product still was not pure.

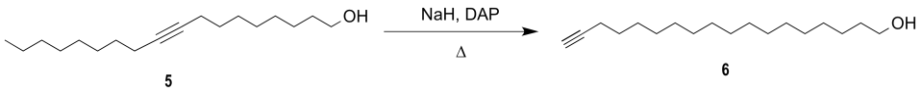
The NMR spectrum allowed ensuring that the product was the alcohol and not the acid. Proton NMR of the acid had a triplet at 2.34 ppm while the alcohol spectrum showed a triplet at 3.62 ppm. ^{13}C NMR supported these conclusions due to the absence of the carbonyl carbon signal at 180.23.

Once the carboxylic group had been protected the alkyne migration could be carried out. This step involved some troubles the first time it was tried, but after some corrections the desired product was obtained. As described in the literature[13], NaH and 1,3-propandiamine (DAP) were used for this reaction. Since commercial NaH was 60% in mineral oil, it should be washed with hexane to remove the mineral oil. Anhydrous conditions were required as in the previous reaction, and DAP must be poured dropwise over NaH under vigorous stirring to minimize foaming. NaH reacts with DAP, which later reacts with the alkyne in a sequence of protonation-deprotonation reactions to form the terminal triple bond. When the triple bond migrates to the terminal location is stabilized so the reaction does not come back.



Scheme 7. Part of the triple bond migration mechanism. B^- represents deprotonated DAP.

The reaction was carried out three times. In the first run, reaction did not work so it was repeated twice, the last time to obtain more product.



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Entry	9-Octadecynol [g]	NaH (60%) [g]	DAP [ml]	Prod. [g]	Yield [%]
1	0.666	0.379	10	-	-
2	0.520	0.393	12	0.114	22
3	1.703	1.422	35	1.368	80

Table 2. Alkyne migration reaction and its results.

In the first run (Table **2**, Entry **1**) part of anhydrous hexane used for washing NaH remained in the flask. To introduce the alkyne reagent (compound **5**) little amount of anhydrous hexane was used because it was difficult to transfer all the oil to the flask. TLC (CH_2Cl_2/MeOH 98:2) comparing the obtained yellow solid and pure 9-octadecyn-1-ol showed spots with the same Rf and also the same colour when revealed with *p*-anisaldehyde. ^1H NMR spectra confirmed that most of the crude was unreacted starting reagent (presence of the $–CH_3$ triplet and absence of the triplet corresponding to C≡CH, see Figure 4).

Some changes and corrections of the procedure were done the second time. 5 equivalents of NaH were used without residual hexane from washing. More care was taken with the anhydrous medium, purging all reagents before using them. To transfer all the reagent (an oil when warmed in a bath) 2 extra mL of DAP were used instead of anhydrous hexane as the first time. These changes made possible the achievement of the desired product. TLC (CH_2Cl_2/MeOH 98:2) was different this time (a red spot instead of grey) and proton spectrum

demonstrated that the reaction occurred. However, due to experimental losses only 0.114 g of 17-octadecyn-1-ol were recovered.

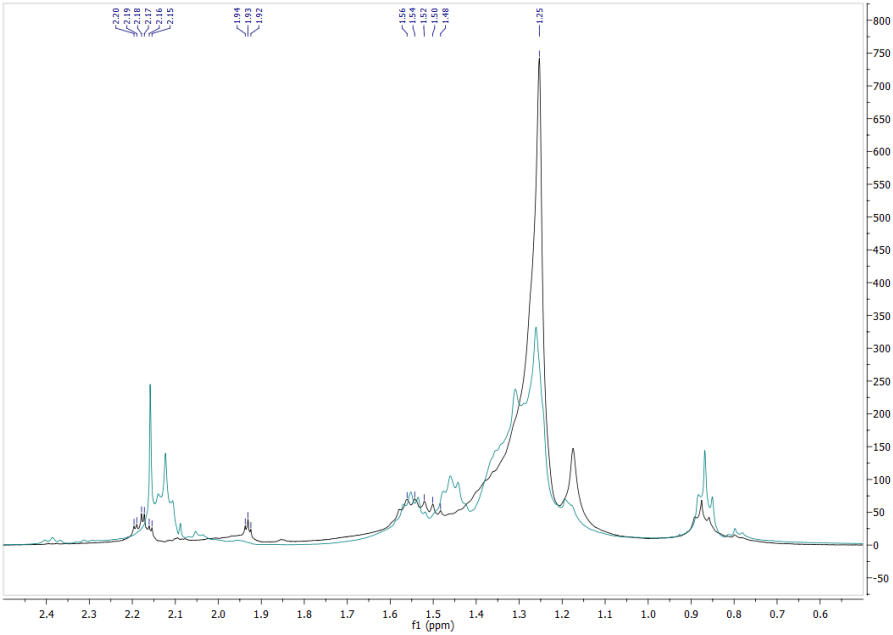
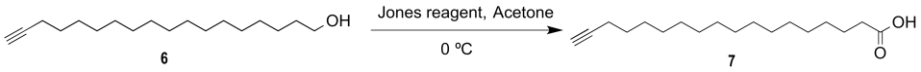


Figure 4. Comparison between ^{1}H spectrum of 9-octadecynol (in blue) and 17-octadecynol (black).

The third attempt was carried out in similar way to the second, purging all reagents and transferring the oil with 5 mL of DAP. The obtained product was an orange solid instead of the yellow expected due to the impurities, in higher amount. But both TLC and ^{1}H NMR confirmed that the reaction was successfully acomplished.

Finally only one step is necessary to reach the objective: the oxidation to carboxylic acid of the hydroxyl group.

Jones reagent is an easy way to oxidize alcohol to carbonyl compounds. For primary alcohols, the conversion does not stop in aldehyde but the formation of carboxylic acid due to the presence of water. This reagent is a mixture of CrO_3 in water and concentrated H_2SO_4, acting Cr(VI) as the oxidant agent. The reaction was performed with no difficulties; there was no need of special cares as in other steps. Jones reagent was added dropwise until no colour change and then allowed to react. The reaction was repeated twice.



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Entry	Alcohol [g]	Acetone [mL]	Reaction time [min]	Crude [g]	Yield [%]
1	0.105	4	10	0.092	82
2	1.358	50	50	1.043	73

Table 3. Oxidation of 17-octadecyn-1-ol to carboxylic acid.

When the reaction was performed, TLC (CH_2Cl_2/MeOH 98:2) of the crude showed that apparently all alcohol became acid. The product was purified by column chromatography (0.075 g, hexane/AcOEt 60:40, total column yield of 95%) obtaining two fraction sets of 58 and 13 mg respectively. ^{1}H NMR spectra of both fractions showed a roughly similar composition (the carboxylic acid) although first set contained little amount of impurities. This set was used for recording the IR and ^{13}C NMR spectra and the pure one for melting point measurement. The collected characterization data agrees with bibliographic data[14,15]. Melting point was the same and both NMR spectrums were different from the obtained in the previous step. IR and ^{13}C NMR showed the presence of the carbonyl group.

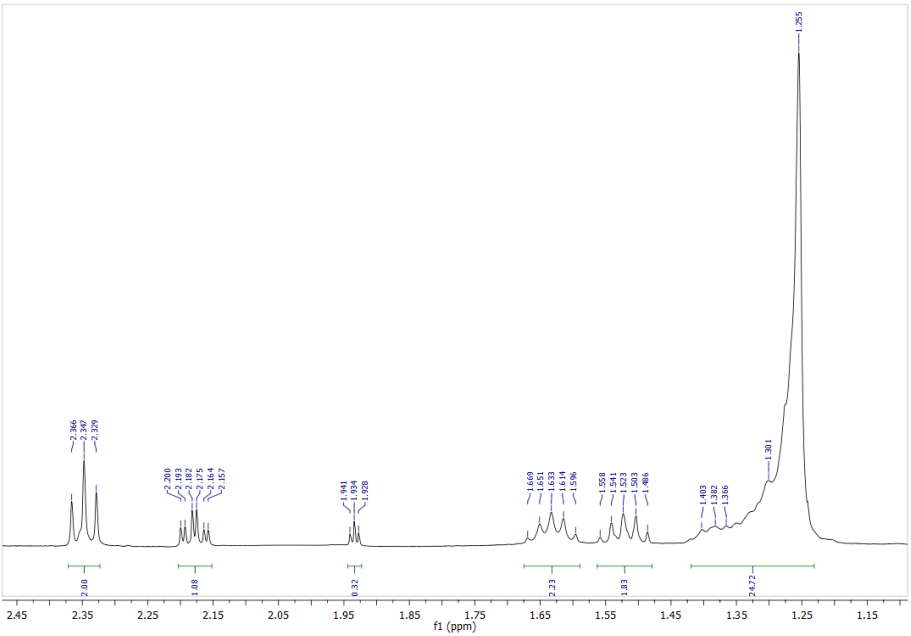


Figure 5. ^{1}H NMR spectrum of 17-octadecyn-1-oic acid.

The second attempt was carried out with a less pure starting material. After 10 minutes, TLC (CH_2Cl_2/MeOH 98:2) showed that the product contained unreacted alcohol, so more Jones reagent was added and the mixture was allowed to react for further 40 minutes. 1.043 g of green solid were obtained, still containing some unreacted octadecyn-1-ol.

Colum chromatography of this solid was performed to purify it (CH_2Cl_2/MeOH 98:2). Results of this process are shown in Table 4. Taking into account the obtained weights of each fractions set the yield of column chromatography purification was 73%.

Entry	Fractions	Prod. Weight [g]	Appearance
1	8-12	0.115	Oily yellow solid
2	13-27	0.378	Yellowish solid
3	28-38	0.157	White solid
4	39-57	0.110	Yellow solid

Table 4. Results of the purification column.

Fractions from 13 to 27 contained both unreacted alcohol (as minority component) and compound **7**. This fact was obvious when TLC (CH_2Cl_2/MeOH 98:2) and NMR proton spectrum were done (the triplet at 3.63 ppm corresponding to C*H*$_2$OH was visible in the NMR data). The pale yellow colour also confirmed the presence of undesired substances.

The white solid obtained from fractions 28 to 38 (entry **3**) was considered pure enough, so it was delivered to the biochemical research group after drying it so they could carry out the study of fatty acid metabolism.

Only those almost pure grams of acid were included when the step yield was calculated, the main reason because its low value (11%).

Regarding the overall yield of the synthesis of 17-octadecyn-1-oic acid (**7**), if we consider the higher yield of each optimized step, the result is 20%. In similar way, the calculation can be done taking the lowest results. This time the result is 0,9%.

In general, the moderate low yields obtained could be attributed to experimental losses or the low purity of some reagents or synthetic intermediates involved.

6. EXPERIMENTAL SECTION

Firstly, a list of reagents and solvents and the methods used for the description of all compounds is shown in this section, then preparation of them is described together with the characterization of the obtained products.

6.1. MATERIALS AND METHODS

6.1.1. Reagents and solvents

Reagent/solvent	Supplier
2,4-dihydroxy benzaldehyde	Aldrich
N-acetylglycine	Aldrich
Anhydrous sodium acetate	Aldrich
Acetic anhydride	Aldrich
HCl (conc.)	Scharlau
Sodium nitrite	Aldrich
Sodium azide	Aldrich
Anhydrous $MgSO_4$	J. Escuder
Oleic acid	Aldrich
Dichloromethane	Scharlau
Bromine	Panreac
Na_2SO_3	Panreac
Dimethyl sulfoxide	Aldrich
Potassium hydroxide	J. Escuder
1-propanol	Aldrich
$LiAlH_4$	Aldrich
Diethyl ether	Aldrich
Sulfuric acid (conc.)	Scharlau

Sodium hydride	Aldrich
1,3-diaminopropane	Aldrich
Hexane	Scharlau
Chromium trioxide	Acros Organics
Acetone	Scharlau
Methanol	Panreac
Ethanol	Scharlau
Ethyl acetate	Scharlau
Chloroform-d	Aldrich
Dimethyl sulfoxide-d_6	Aldrich

Table 5. List of used reagents and solvents.

Preparation of the *p*-anisaldehyde solution (developer): 25 mL of *p*-anisaldehyde, 35 mL of concentrated H_2SO_4 and 10 mL of glacial acetic acid are dissolved in 930 mL of ethanol.

Preparation of the $KMnO_4$ developer solution: 3 g of $KMnO_4$ and 20 g of K_2CO_3 are dissolved in 300 mL of water. Then 5 mL of NaOH 5% are added.

Preparation of Jones reagent: 18.18 g of CrO_3 are dissolved in water (25 mL) and cooled to ice-bath temperature. Then a mixture of concentrated sulfuric acid (17 mL) and water (50 mL) is added slowly under stirring.

6.1.2. Methods and instrumentation

6.1.2.1. Nuclear magnetic resonance (NMR)

^{1}H and ^{13}C spectra data was collected with a Varian Mercury 400 spectrometer at 25 ºC. Tetramethylsilane was the intern reference compound (0 ppm). Chemical shift (δ) is always in ppm and coupling constants (*J*) in Hz. Solvent is indicated in each characterization.

6.1.2.2. Infrared spectroscopy (IR)

IR spectrum of some products was collected by placing the sample on the instrument (both solid and liquid compounds). The instrument is a FT-IR Nicolet 6700. In the characterization of substances only the most relevant peaks (in cm^{-1}) are described.

6.1.2.3. Melting point

For the measures of the melting point, a SMP 10 instrument was used by placing the sample on a capillary tube.

6.1.2.4. Thin layer chromatography (TLC)

For all reactions this method was used when they finished, and sometimes for the control of the reaction advance. Analytical TLC Silica gel 60 F_{254} was used (Merck, 0.2 mm of thickness). Solutions of *p*-anisaldehyde, potassium permanganate or light (UV: 254 nm and 365 nm) were used as developers.

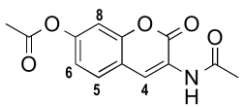
6.1.2.5. Column chromatography

This method was used to purify substances. Silica gel was used as stationary phase, and the elution was favored with pressure air. Eluent is specified in each case.

6.2. SYNTHESIS OF 3-AZIDO-7-HYDROXYCOUMARIN

6.2.1. Synthesis of 1

2,4-dihydroxy benzaldehyde (2.761 g, 2.20 mmol), *N*-acetylglycine (2.357 g, 20 mmol) and anhydrous sodium acetate (4.922 g, 60 mmol) on acetic anhydride (100 mL) were refluxed under stirring for 4 hours. The mixture was cooled to room temperature and then poured onto ice, giving a yellow precipitate. The solid was filtered and washed with ice water to afford 3-acetamido-2-oxo-2*H*-chromen-7-yl acetate (0.550 g, 11%), which was used in the next step without further purification.



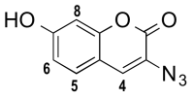
1

Yellow solid. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.74 (s, 1H, N*H*), 8.61 (s, 1H, *H*(4)), 7.74 (d, *J* = 8.5, 1H, *H*(5)), 7.26 (d, *J* = 2.3, 1H, *H*(8)), 7.13 (dd, *J* = 8.5, 2.2, 1H, *H*(6)), 2.29 (s, 3H, OCOC*H*$_3$), 2.16 (s, 3H,NCOC*H*$_3$).

6.2.2. Synthesis of 2

Compound **1** (0.550 g, 2.11 mmol) was refluxed in a solution of concentrated HCl and ethanol 2:1 (30 mL) for 1 hour. Then ice water was added (40 mL) and the solution was cooled in an ice bath, and $NaNO_2$ (2.769 g, 40.13 mmol) was added. The mixture was stirred for 10

minutes before the addition of NaN_3 (3.900 g, 60 mmol) in portions. After stirring for another 15 minutes the brown precipitate was filtered off and washed with water. The solid was dissolved in ethyl acetate, dried with anhydrous $MgSO_4$ and filtered off. Evaporation of the solvent provided 3-azido-7-hydroxycoumarin (0.388 g, 90 %).



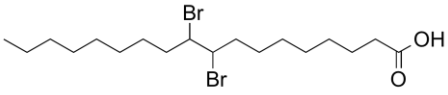
2

Brown solid. IR: 3288 (OH), 3050 (C=CH), 2105 (N_3), 1678 (C=O), 1620 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.56 (s, 1H, O*H*), 7.59 (s, 1H, *H*(4)), 7.49 (d, *J* = 8.5, 1H, *H*(5)), 6.83 (dd, *J* = 8.5, 2.3, 1H, *H*(6)), 6.78 (d, *J* = 2.2, 1H, *H*(8)). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 161.2, 158.2, 153.6, 130.0, 128.7, 122.0, 114.7, 112.2, 102.9.

6.3. SYNTHESIS OF 17-OCTADECYN-1-OIC ACID

6.3.1. Synthesis of 3

Oleic acid (26.101 g, 83.2 mmol) was dissolved on dichloromethane (120 ml). Br_2 (5 mL, 99.8 mmol) was added dropwise under stirring while the mixture was at ice bath temperature. After addition, the solution was warmed to room temperature and stirring was kept for 30 minutes. $NaHSO_3$ was used to reduce bromine excess (dropwise until no colour change). The solution was washed with water (3 x 50 mL), dried over anhydrous $MgSO_4$, filtered and concentrated by evaporation, providing 9,10-dibromostearic acid (40.371 g, 109%, residual solvent detected by NMR).



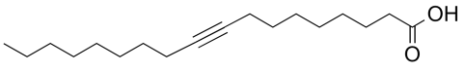
3

Pale yellow oil. IR: 2921 (CH), 2852 (CH), 1705 (C=O), 1460 (C-OH). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.21 (dm, 2H, C*H*BrC*H*Br), 2.36 (t, *J* = 7.5, 2H, CH_2CO$_2$H), 2.10-1.99 (m, 2H, CH_2), 1.89-1.78 (m, 2H, CH_2), 1.69-1.53 (m, 4H, 2 x CH_2), 1.42-1.24 (m, 18H, 9 x CH_2), 0.88 (t, *J* = 6.9, 3H, CH$_2$CH_3). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 61.9, 61.8, 37.0, 36.9, 36.0, 33.9, 31.5, 31.3, 31.1, 31.0, 30.9, 30.7, 29.9, 29.8, 26.7, 24.8, 16.2.

6.3.2. Synthesis of 4

9,10-dibromostearic acid (compound **3**, 14.998 g, 33.9 mmol), KOH (8.406 g, 149.8 mmol) and DMSO (7.5 mL, 105.59 mmol) in 1-propanol (80 mL) were refluxed for 1 hour. The mixture was cooled to room temperature before being poured onto ice-cold HCl (100 mL, 2N). The solid

was filtered and washed with cold HCl 2N. Stearolic acid (9-octadecyn-1-oic acid, 4.693 g, 49%) was obtained.



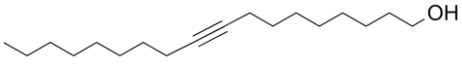
4

Pale yellow solid. Melting point: 43-45 ºC. IR: 2920 (C-H), 2851 (C-H), 1686 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.34 (t, *J* = 6.8, 2H, C*H*$_2$CO$_2$H), 2.13 (t, *J* = 6.5, 4H, C*H*$_2$C≡CC*H*$_2$), 1.63 (qu, *J* = 7.2, 2H, C*H*$_2$CH$_2$CO$_2$H), 1.57-1.22 (m, 20H, 10 x C*H*$_2$), 0.88 (t, *J* = 5.9, 3H, CH$_2$C*H*$_3$). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 180.23, 80.50, 80.20, 34.17, 32.00, 29.37, 29.31, 29.28, 29.20, 29.10, 29.02, 28.91, 28.76, 24.77, 22.81, 18.90, 18.87, 14.25.

6.3.3. Synthesis of 5

$LiAlH_4$ (0.277 g, 7.30 mmol) was purged with N_2 and cooled in an ice bath before the addition of anhydrous diethyl ether (10 mL). To the cooled suspension, compound **4** (1.014 g, 3.62 mmol, purged with N_2) in anhydrous diethyl ether (10 mL) was added dropwise. After 4 hours at ice-bath temperature, unreacted $LiAlH_4$ (grey) was destroyed with ice and cold water (carefully) until it became a white solid. Then the suspension was acidified with H_2SO_4 until total dilution. The solution was extracted with diethyl ether (4 x 20 mL), and the extracts were dried ($MgSO_4$) and filtered. Solvent was evaporated to afford 9-octadecyn-1-ol (compound **5**, 0.707 g, 73%).

When the synthesis was repeated with 2.58 g of 9-octadecyn-1-oic acid (9.21 mmol) and 0.699 g of $LiAlH_4$ (18.42 mmol) in 25 mL of anhydrous diethyl ether respectively, 1.703 g of **5** were obtained (70%).



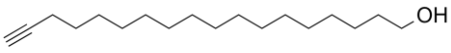
5

Pale yellow solid. Melting point: 29-31 ºC. IR: 3276 (OH), 2918 (C-H), 2847 (C-H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.62 (t, *J* = 6.6, 2H, C*H*$_2$OH), 2.12 (t, *J* = 6.8, 4H, C*H*$_2$C≡CC*H*$_2$), 1.55 (qu, *J* = 6.8, 2H, C*H*$_2$CH$_2$OH), 1.46 (qu, *J* = 6.4, 4H, C*H*$_2$CH$_2$C≡C), 1.40-1.24 (m, 18H, 9 x C*H*$_2$), 0.87 (t, *J* = 6.5, 3H, CH$_2$C*H*$_3$). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 80.45, 80.31, 63.23, 32.95, 32.00, 29.48, 29.38, 29.33, 29.29, 29.29, 29.28, 29.03, 28.94, 25.85, 22.82, 18.92, 18.91, 14.26.

6.3.4. Synthesis of 6

NaH (0.393 g, 60% in mineral oil, 9.75 mmol) was washed of oil with anhydrous hexane (3 x 5 mL) and cooled with an ice bath. 1,3-diaminopropane (10 mL, 119.80 mmol) was added under stirring after the system was purged with N_2. The suspension was heated to 80 ºC and allowed to react for 2 hours at this temperature. The reaction was then cooled to ice-bath temperature and **5** (0.520 g, 1.95 mmol) was added dropwise over 5 minutes. The mixture was heated to 100 ºC over a period of 40 minutes. After 120 minutes under stirring, the mixture was cooled to 10 ºC and poured into ice (20 mL of cold water were used to rinse the flask). The solution was extracted with hexane (4 x 20 mL), and organic layer was washed with water (4 x 10 mL), saturated aqueous $NaHCO_3$ (10 mL) and saturated NaCl (10 mL) and dried with $MgSO_4$. Filtration and evaporation of solvent provided 17-octadecyn-1-ol (0.114 g, 22%).

After repeating the procedure with 1.422 g NaH (35.55 mmol), 1.703 g of **5** (6.39 mmol) and 35 mL of DAP (419.26 mmol), doing extractions and washing with 20 mL, 1.368 g of **6** were obtained (80%).



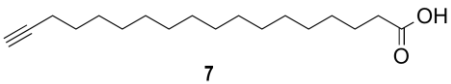
6

Yellow solid. Melting point: 54-55 ºC. IR: 3286 (C≡C-H), 2916 (C-H), 2848 (C-H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.63 (t, *J* = 6.6, 2H, C*H*$_2$OH), 2.18 (td, *J* = 7.1, 2.7, 2H, C*H*$_2$C≡CH), 1.93 (t, *J* = 2.7, 1H, C≡C*H*), 1.60-1.48 (m, 4H, C*H*$_2$(2)+C*H*$_2$(15)), 1.40-1.24 (m, 24H, 12 x C*H*$_2$). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 84.94, 68.16, 63.17, 32.95, 32.02, 29.84, 29.80, 29.76, 29.74, 29.64, 29.58, 29.25, 28.91, 28.64, 25.89, 22.83, 18.54, 14.25.

6.3.5. Synthesis of 7

17-octadecyn-1-ol (0.105 g, 0.4 mmol) in acetone (4 mL) was cooled (0 ºC). Jones reagent (8 N) was added dropwise under stirring until the solution remained orange. After 10 minutes, 40 mL of water were added and the aqueous layer was extracted with hexane (4 x 30 mL). The combined extracts were washed with HCl (0.1 N, 50 mL), dried over anhydrous MgSO4 and the solvent was evaporated to provide 0.092 g. When the crude was purified by column chromatography (hexane/ethyl acetate 60:40), pure 17-octadyn-1-oic acid was obtained (0.071 g, 63%).

The synthesis was repeated with 1.358 g of **6** (5.1 mmol, impure) in 50 mL of acetone. The mixture was allowed to react for 1 hour, and provided 1.043 g of a green solid. 0.157 of purified acid (11%) were obtained after doing a column chromatography (CH_2Cl_2/MeOH 98:2).



7

White solid. Melting point: 66-67 ºC. IR: 3282 (C≡C-H), 3044 (OH), 2914 (C-H), 2848 (C-H), 1692 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.35 (t, *J* = 7.5, 2H, C*H*$_2$COOH), 2.18 (td, *J* = 7.1, 2.6, 2H, C*H*$_2$C≡CH), 1.93 (t, *J* = 2.5, 1H, C≡C*H*), 1.63 (qu, , *J* = 7.6, 2H, C*H*$_2$CH$_2$COOH), 1.52 (qu, , *J* = 7.2, 2H, C*H*$_2$CH$_2$C≡C-H), 1.41-1.24 (m, 22H, 11 x C*H*$_2$). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 179.65, 84.85, 68.04, 34.01, 29.67, 29.65, 29.63, 29.61, 29.53, 29.46, 29.27, 29.14, 29.09, 28.80, 28.53, 24.71, 19.43.

7. CONCLUSIONS

As expected, all compounds were obtained without many problems and the alkyne-analogue of the fatty acid could be delivered to the biochemical research group from UIC.

All reactions are robust and could be carried out in mild conditions, without high temperature and at atmospheric pressure. They also could be performed in presence of undesired by-products or impurities although they affected the yield (as shown in results).

The importance of the absence of water in the reacting media was reflected on steps 3 and 4 of the preparation of 17-octadecyn-1-oic acid. Those reactions involved sensible reagents ($LiALH_4$ and NaH) so they had to be carried out with the appropriate care, under N_2 atmosphere as they were performed in this work. Thus they were successful and desired product was obtained in both reactions.

This work includes many well-established reactions as aldol reaction, dehydrobromination or the oxidation of alcohols. Performance of known reactions allows us to understand better what happens in each case and the required conditions. But also a less common reaction was carried out: the alkyne migration. This reaction seems to be a useful tool to form terminal alkyne analogues, with advantages as the no need of solvent or its high reliability to form the terminal triple bond.

Due to the planned applications of the prepared compounds, this project will be useful to study the lipid metabolism by using click chemistry. This kind of reaction seems to be very useful in biochemical research and with a wide range of applications.

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9. ACRONYMS

Abbreviation	Meaning
UIC	Universitat Internacional de Catalunya
CPT	Carnitine Palmitoyltransferase
CC	Click Chemistry
LED	Light-emitting Diode
EtOH	Ethanol
DNA	Deoxyribonucleic acid
NaOAc	Sodium Acetate
Ac_2O	Acetic Anhydride
TLC	Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance
IR	Infrared
r.t.	Room Temperature
DMSO	Dimethyl Sulfoxide
AcOEt	Ethyl Acetate
Et_2O	Diethyl Ether
MeOH	Methanol
DAP	1,3-Diaminopropane

Table 6. List of used acronyms and abbreviations.