MASTER'S THESIS 2019

Antitumor effects of selected intermediates in synthesis of unsaturated acyl glucoside. *In vitro* experiments on cancer cell lines.

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Trondheim, August 30th, 2019

Acknowledgements

I wish to thank both of my supervisors, Nebojša Simić and Odrun Arna Gederaas for giving me the chance to take part in this interesting project, and for all the help they gave me during past two years. I also would like to thank Sondre Nervik, the PhD candidate in this project, for his patience in work with me and all the advices given, and Kristin Grendstad for introducing me to the world of cell culture in practice.

Lastly I wish to thank my family and friends, who were supporting me for all this time.

Declaration

I hereby declare that the work presented in this master's thesis has been conducted in accordance of the rules and regulations for the MsChem (Master in Chemistry 2 year master's program) at the Norwegian University of Science and Technology. The work presented has been done individually under the supervision of Nebojša Simić, Odrun Arna Gederaas and Sondre Nervik.

The practical work has been performed in the period between September 2017 and July 2019.

Trondheim, August 30th, 2019

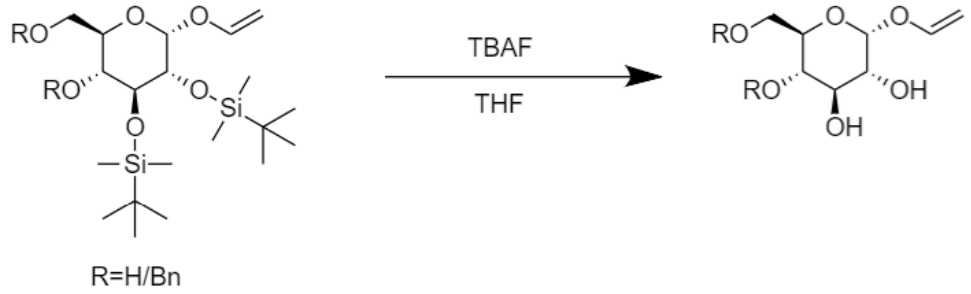
Daniel Gandawski

Abstract

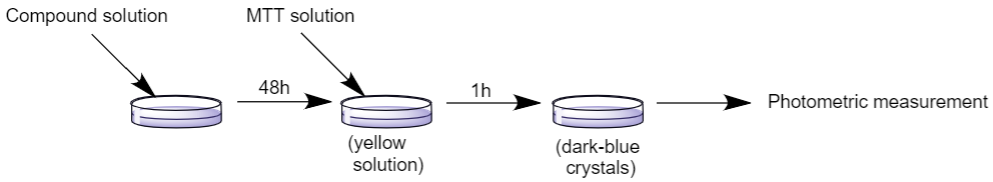
In connection to an ongoing total synthesis project at NTNU, biological evaluation of intermediates and potential fatty acid substituents as anticancer agents were conducted by MTT assay. In addition, the synthesis of two novel glycopyranoses by fluorine-mediated cleavage of silyl ether groups was performed for comparison with aforementioned intermediates. In total, twelve compounds were tested on rat glioma F98 cell line using cell survival experiment (MTT assay). It was shown that the intermediates possess the cytotoxicity against F98 cancer cells, but the free fatty acids did not affect cell viability.

Graphical Abstract

Synthesis overview



MTT assay scheme



Abbreviations

DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
IC	inhibitory concentration
LD	lethal dose
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
SQDG	sulfoquinovosyldiacylglicerol
TBAF	tetra-n-butylammonium fluoride
THF	tetrahydrofuran

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1. Introduction

This project is part of a larger ongoing total sythesis project towards the compound shown in Fig. 1.

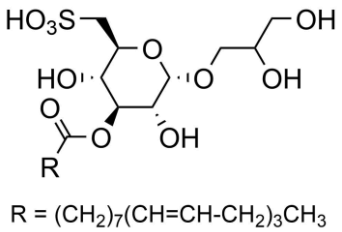
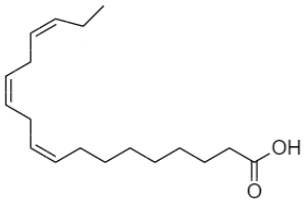
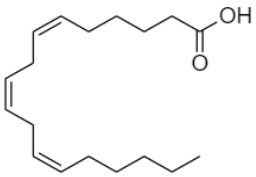
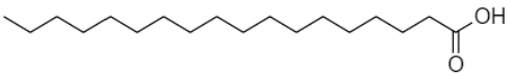
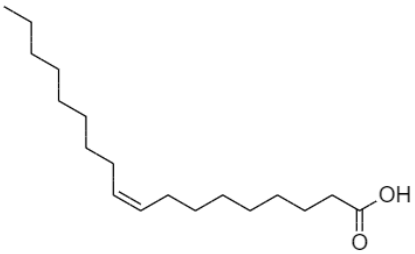
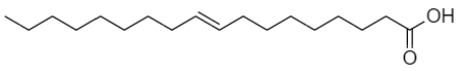
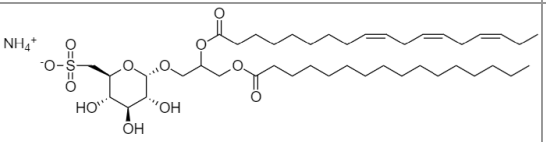
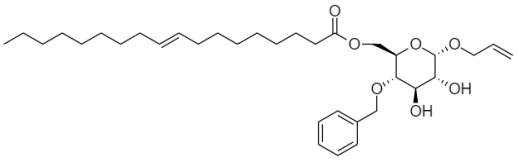


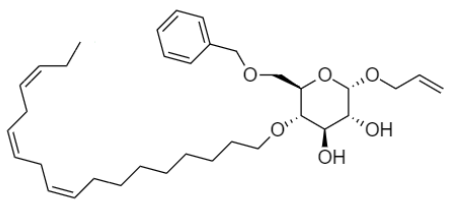
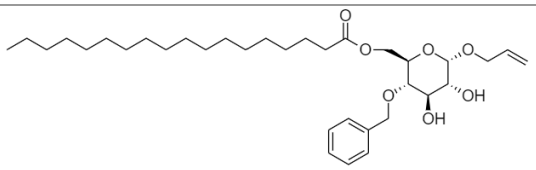
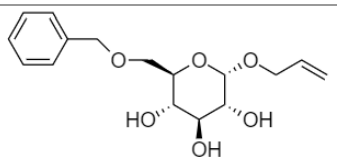
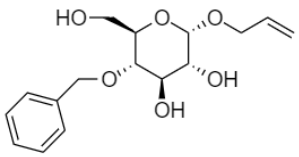
Fig. 1. Chemical structure of the target molecule.

The main purpose of this study was to evaluate the bioactivity of the synthesized intermediates against a selected cell line. The bioactivities were compared to a reference compound, sulfoquinovosyl diacylglycerol (SQDG), which is similar in structure (**2a,** Table 1), and to moieties found in the intermediates such as fatty acids (**1a-e**, Table 1) and unesterified glucopyranoses (**4a**, **4b** in Table 1). In addition, synthesis of the **4a** and **4b** was performed.

Full list of tested compounds is shown in Table 1.

Table 1. List of tested compounds with names, chemical structures, and solvent (solvents) used for preparation of the stock solutions.

No.	Name	Structure	Solvent (stock solution)	Cell survival figure
1a	α-linolenic acid		Ethanol, DMSO	Fig. 3., Fig. 10.
1b	γ-linolenic acid		Ethanol	Fig. 4.
1c	Stearic acid		Ethanol, DMSO	Fig. 5., Fig. 11.
1d	Oleic acid		Ethanol	Fig. 6.
1e	Elaidic acid		Ethanol, DMSO	Fig. 7., Fig. 12.
2a	Sufloquinovo-syldiacyl-glicerol SQDG		DMSO	Fig. 13.
3a	1-O-allyl-4-O-benzyl-6-O-elaidate-α-D-glucopyranoside		DMSO	Fig. 14.

3b	1-O-allyl-4-O-α-linolenate-6-O-benzyl-α-D-glucopyranoside		DMSO	Fig. 15.
3c	1-O-allyl-4-O-benzyl-6-O-stearate-α-D-glucopyranoside		DMSO	-
4a	1-O-allyl-6-O-benzyl- α-D-glucopyranoside		DMSO	Fig. 17.
4b	1-O-allyl-4-O-benzyl- α-D-glucopyranoside		DMSO	Fig. 18.

Intermediates selected for testing were labeled **3a**, **3b**, **3c**, **4a**, and **4b**, and for my synthesis **4a**, and **4b**. Along with the selected intermediates five fatty acids, and the reference SQDG was tested.

1.1. Cell culture

Cell culture, in general, is the process in which cells are grown outside their natural environment [1]. They need to be kept in a sterile environment with carefully controlled conditions (temperature, humidity, atmosphere) in special culture vessels, in medium containing nutrients necessary for their survival (amino acids, carbohydrates, vitamins, minerals) and in balanced pH [2]. There are two types of cell cultures due to the way of cultivation: adherent (or monolayer) culture, in which cells require special surface or artificial substrate for growth, and suspension culture, in which cells are suspended in medium [2].

Cell culture is a great technique which can be used for studies in: physiology and developmental biology, pathology, medicine, and pharmacology [3].

In order to keep the cultured cells alive and in good condition, some manipulations have to be done. Most common manipulations on cell cultures are *medium change* and *subculturing*.

Medium change is the process of replacing old medium with fresh. It ensures that the pH and amount of nutrients are at appropriate level. In adherent cell culture (which was the type used in this project), this process involves washing cells with a buffer (i.e. phosphate buffered saline, PBS), in order to remove dead cells and traces of metabolites before addition of fresh medium.

Subculturing (also known as splitting cells) is the process of transferring of a small number of cells into the new vessel along with the fresh medium. In adherent cell culture this process involves usage of buffer for washing and enzyme trypsin for detaching cells from the surface.

Both processes have to be conducted in sterile environment to decrease the risk of contamination. As a further precaution against contamination, the medium can be supplemented with antibiotics.

Cell culture requires the presence of the appropriate medium, which is the mixture of the nutrients necessary for the cells to live. Different cell lines require different mediums. The F98 cell line requires Dulbecos' Modified Eagle Medium supplemented with L-glutamine, and fetal bovine serum (FBS).

1.2. The MTT assay

The MTT assay is a colorimetric method for measuring survival or proliferation of the cells. Cells showing metabolic activity are able to transform yellow, water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a dark blue, water-insoluble formazan crystals (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan). Formazan crystals are highly soluble in organic solvents, e.g. alcohols, and such solutions can be used for photometric measurements. The amount of produced formazan is directly related to the absorbance of its solution [4]. This assay is also very sensitive, and gives reliable results even for very small amounts of living cells. As the method relies on the metabolic activity of the cells, dead cells are not measured.

1.3. Glycolipids

The target molecule in this total synthesis project (Fig. 1.) belongs to the group of glycolipids found and isolated from *Sclerochloa dura* extract [5]. This extract was investigated by Bukhari et al. [6] in order to evaluate its' anti-inflamatory and antioxidant activities. As shown in [7] there is conection between the inflammation and development of cancer. For that reason the biological activity assessment was introduced into this project. Since the target molecule has not yet been reached, it was decided to evaluate potential cytotoxicity of the chosen intermediates in this synthesis.

2. Materials and methods

2.1. F98 cell line

The F98 glioma is chemically induced tumor from the Fisher rat [8, 9]. It is classified as an undifferetiated malignant glioma. This cell line is suitable for both *in vivo* and *in vitro* studies of rat brain tumor, but since it simulates human glioblastoma multiforme to a great extent, it can be also used for testing therapies suitable for humans.

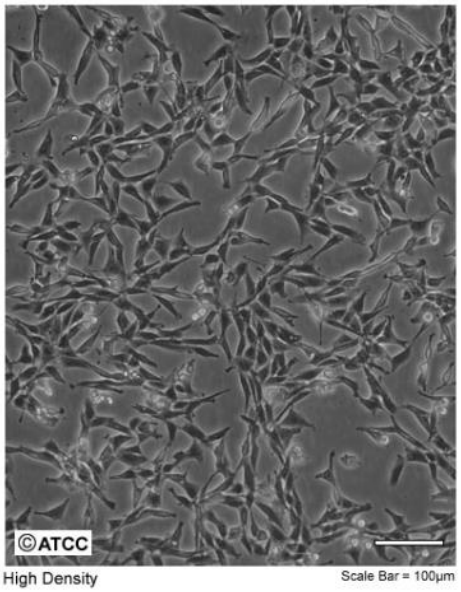


Fig. 2. Light microscopy photograph of the rat glioma F98 cell line [10].

The F98 cell line was ordered from ATCC® catalogue in 2012.

2.2. Cultivation of the F98 cells

The F98 cells were cultured in sterile culture flasks (75 mL, Corning), and incubated in modified, humidified atmosphere (95% air, 5% CO_2), in 37 ºC. As a growth medium, Dulbecos' Modified Eagle Medium with addition of FBS (10% v/v), L-glutamine(0.34% w/v), and penicillin/streptomycin (1% w/v), was used.

2.2.1. Subculture of the F98 cells

The cells were subcultured twice a week using the following procedure:

- The cells were observed under inverted microscope in order to check the level of confluence, the cells' condition, and to detect possible culture contaminations.
- In the sterile bench the old growth medium was removed, the cells were washed (PBS, 7 mL/wash, 37 ºC, twice) to remove traces of old medium and dead cells.
- Trypsin was added (3 mL, 37 ºC) and the flask was placed in the incubator. Cells were incubated until proper detachement (single cells floating in the trypsin solution, 5-7 min). It was confirmed using the microscope.
- Cell suspension (0.5 mL) was transfered with strile pipet to new flasks along with fresh medium (10 mL/flask, 37 ºC)

Last subculturing before seeding out cells for the cell survival experiment (MTT assay) was performed using three new culture flasks (75mL).

2.2.2. Medium renewal

The producer and supplier, ATCC®, recommends medium renewal every 2-3 days.

It was done as follows:

- The condition of the cells was checked in the microscope.
- The old medium was removed, and the cells were washed with PBS (7 mL, 37 ºC, once).

- Fresh medium (10 mL/flask) was added, and the flask was placed in the incubator until next subculturing.

2.2.3. Cell counting

Before seeding out, the cells had to be counted. It was performed manually using Bürker chamber as follows:

- Trypsinated cells were transferred into 50 mL sterile centrifuge tube and fresh medium (37 °C) was added to total volume of 20 mL. The tube was centrifuged (1500 rpm, 5 min).
- Supernatant was carefully removed and medium (1 mL, 37 °C) was added. Cell pellet was carefully resuspended in the medium using autopipet in order to preapre homogenous suspension. More medium was added (19 mL, 37 °C) and the suspension was mixed.
- Cell suspension was placed on the prepared chamber, and the cells in 3 squares were counted in the microscope.

The number of cells in 1ml of the suspension were calculated as follows:

$$NoC = average\ number\ of\ cells\ in\ one\ square \times 10^4$$

where:

NoC – number of cells

2.3. MTT assay protocol

Day 1. Seeding out cells.

- All three nearly confluent 75 cm^3 flasks were carefully checked in the microscope, and two of them containing cells in best condition were taken for further processing. Remaining flask was discarded.
- On the sterile bench, the old medium was removed, the cells were washed with PBS (7 ml/wash, 37 °C, twice), and the trypsin was added (3 ml/flask, 37 °C). Cells were incubated for 5-7 minutes in order to detach them.

- Trypsinated cells' suspension was used for cell counting as described in section 2.2.3.
- Calculated volume of cell suspension was transferred to the sterile flask along with the fresh medium (37 °C) to the total volume of 66 cm^3
- Cells were seeded out into sterile petri dishes (20 dishes, ⌀ = 6cm, $0.25x10^6$ cells/dish).

Remaining cell suspension was used to start new cell culture.

Day 3 Treatment

- Following the plan of the experiment, the right volume (2.5 mL – x µL/dish, see the Appendix B) of the medium was transfered into the 15 mL tubes, followed by the calculated volume of the stock solution of the tested compound.
- The cells were checked in the microscope. The old medium was removed and cells were washed with PBS (3 mL/dish, 37 °C, once).
- Three dishes were serving as a control dishes and 2.5 mL of fresh medium was added into them. Rest of the dishes were filled with solutions of the tested compounds (2.5 mL/dish, 37 °C) as planned (see the Appendix B).
- Plates (⌀ = 15cm) with dishes were covered with aluminium foil and placed in the incubator. Incubation time: 48 h, 37 °C.

Day 5. MTT assay

- The working solution of MTT (0.5 mg/mL) was prepared by mixing 5 mL of MTT stock solution (5 mg/mL) with 45 mL of fresh medium. The tube with solution was covered with aluminium foil, and placed in the water bath.
- The culture dishes (⌀ = 6cm) were checked in the microscope in order to see the cells' condition. The old medium was removed, and the cells were washed with PBS (2.5 mL/dish, 37 °C, once).
- Working MTT solution was added (2 mL/dish, 37 °C) and dishes were placed in the incubator covered with aluminium foil for 1 hour.
- MTT solution was removed, and isopropanol was added (2 mL/dish). Dishes were placed on the orbital shaker (80 rpm, 30 min).
- Cell solutions from the dishes were carfully transferred into seperate centrifuge tubes and centrifuged (1500 rpm, 5 min).
- The centrifuged solutions (50 µL) were transferred into seperate cuvettes designed for photometric measurements, along with isopropanol (950 µL/cuvette).
- The spectrophotometer was set on 0 using pure isopropanol, and the absorbance of the cell supernatants were measured at 595 nm.

Exemplary plan for the MTT assay can be found in Appendix B

2.4. Preparation of benzylated compounds

All solvents and reagents used in the synthesis of **4a** and **4b** were purchased from Sigma-Aldrich and used without further purification. An exception from this is the silylated starting materials which were procured by an other research group member.

Column flash chromatography was performed using silica gel 60 Å (40- 64 nm) as the stationary phase. Mobile phase composition is mentioned for each synthesised compound. Nitrogen was used for pressure.

High Performance Liquid Chromatography was executed using an Agilent UHPLC system, consisting of the following: Agilent 1290 Infinity binary pump VL, G4220B, Agilent 1290 Infinity, G4226A auto sampler, Agilent 1260 TCAA, G1316A degasser, and a 1260 DAD, G4212-60007 Diode Array Detector.

A Zorbax Bonus-RP 250 x 4.6 mm column with a Zorbax Bonus-RP 12.5 x 4.6 mm guard column was used with C18 as the stationary phase.

Analytical method: Gradient elution starting at 80:20 ACN:H_2O to 100% ACN over 50 min, isocratic at 100% ACN for 10 min. Flowrate: 1 mL/min.

Allyl 2,3-di-O-*tert*-butyldimethylsilyl-6-O-benzyl-*α*-glucopyranose (106 mg, 0.20 mmol) was dissolved in THF (4 ml). TBAF (1M in THF, 0.59 ml, 0.60 mmol) was added and the mixture was stirred at room temperature for 16 h. Evaporation under reduced pressure and purification by silica gel column chromatography R_f = 0.34 (2:1, *n*-pentane:EtOAc) yielded compound **4a** (35 mg, 0.11mmol, 55%) as a white solid.

t_R (analytical HPLC) = 7.012 min. **^{1}H-NMR** (600 MHz, $CDCl_3$) δ: 7.30-7.35 (m, 4H, Ar-CH), 7.25-7.29 (m, 1H, *p*-Ar-CH), 5.89 (m, 1H, CH_2-C<u>H</u>=CH_2), 5.28 (d(b), 1H, J = 17.2 Hz, CH_2-CH=C<u>H_2</u>), 5.17 (d(b), 1H, J = 10.30 Hz, CH_2-CH=C<u>H_2</u>), 4.90 (d, 1H, J = 3.81 Hz, H-1), 4.57 (q, 2H, J = 12.20 Hz, PhC<u>H_2</u>), 4.31 (s(b), 1H, 4-OH), 4.19 (ddt, 1H, J = 12.90 Hz, 5.30 Hz, 1.40 Hz, C<u>H_2</u>-CH=CH_2), 4.02 (ddt, 1H, J = 12.90 Hz, 6.30 Hz, 1.26 Hz, C<u>H_2</u>-CH=CH_2), 3.67-3.80 (m, 5H, H-3, H-4, H-5, H-$6_{a/b}$), 3.54 (t, J = 9.22 Hz, H-2), 3.29 (s(b), 1H, 2-OH), 2.18 (s(b), 1H, 3-OH). HRMS (ESI+) m/z: 333.1311 $[M+Na]^+$ (Calc. for $C_{30}H_{34}O_6SNa$: 333.1314)

Allyl 2,3-di-O-*tert*-butyldimethylsilyl-4-O-benzyl-*α*-glucopyranose (496 mg, 0.92 mmol) was dissolved in THF (7 ml). TBAF (1M in THF, 2.76 ml, 2.76 mmol) was added and the mixture was stirred at room temperature for 16 h. Evaporation under reduced pressure and purification by silica gel column chromatography R_f = 0.40 (2:1, *n*-pentane:EtOAc) yielding compound **4a** (267 mg, 0.86 mmol, 93%) as a white solid.

t_R (analytical HPLC) = 8.787 min. **^{1}H-NMR** (600 MHz, $CDCl_3$) δ: 7.34-7.38 (m, 4H, Ar-CH), 7.29-7.33 (m, 1H, *p*-Ar-CH), 5.89 (m, 1H, CH_2-C<u>H</u>=CH_2), 5.29 (d(b), 1H, J = 17.2 Hz, CH_2-CH=C<u>H_2</u>), 5.21 (d(b), 1H, J = 10.41 Hz, CH_2-CH=C<u>H_2</u>), 4.91 (d, 1H, J = 3.93 Hz, H-1), 4.88 (d, 1H, J = 11.43 Hz, $PhCH_2$), 4.73 (d, 1H, J = 11.43 Hz, $PhCH_2$), 4.20 (ddt, 1H, J = 12.80 Hz, 5.30 Hz, 1.42 Hz, C<u>H_2</u>-CH=CH_2), 4.02 (ddt, 1H, J = 12.80 Hz, 6.20 Hz, 1.22 Hz, C<u>H_2</u>-CH=CH_2), 3.90 (t, 1H, J = 9.2 Hz, H-3), 3.74-3.84 (m, 2H, H-$6_{a/b}$), 3.69 (dt, 1H, J = 9.81 Hz, 3.31 Hz, H-4), 3.50 (s(b), 1H, H-2), 3.47 (t, 1H, J = 9.42 Hz, H-5), 2.66 (s(b), 1H, 3-OH), 2.17 (d(b), 1H, J = 7.42 Hz, 2-OH), 1.81 (t(b), 1H, J = 6.24 Hz, 6-OH). **^{13}C-NMR**(150MHz,$CDCl_3$) δ: 138.2 (1C, Ph-Cq), 133.4 (1C, CH_2-<u>C</u>H=CH_2), 128.6 (2C, Ar-C), 128.1 (2C, Ar-C), 128.0 (1C, *p*-Ar-C), 118.1 (1C, CH_2-CH=<u>C</u>H_2), 97.2 (1C, C-1), 77.1 (1C, C-5), 75.2 (1C, C-3), 74.7 (2C, Ph<u>C</u>H_2), 72.7 (1C, C-2), 68.6 (1C, <u>C</u>H_2-CH=CH_2), 61.9 (1C, C-6). HRMS (ESI+) m/z: 333.1308 $[M+Na]^+$ (Calc. for $C_{16}H_{22}O_6Na$ 333.1314).

Spectra for compounds **4a** and **4b** can be found in Appendices A1-A9.

3. Results

In presented thesis 12 compounds were tested in order to determine whether they are cytotoxic against the F98 cell line or not, and which part of synthesized molecules can be responsible for the cytotoxicity. Full list of tested compounds is shown in Table. 1. in Chapter 1.

Compounds **1a**, **1b**, **1c**, **1d**, **1e**, and **2a** were purchased in Sigma-Aldrich. Compounds **3a**, **3b**, **3c**, **4a**, **4b**, are novel compounds synthesized in the present project.

3.1. Evaluation of the fatty acids cytotoxicity

Intermediates synthesized in the present project have a fatty acid moieties. It was important to test, if they show any cytotoxicity against the rat glioma F98 cell line. In first experiments, the stock solutions were prepared in ethanol (concentration 2mM). The results obtained are shown in Fig. 3-7.

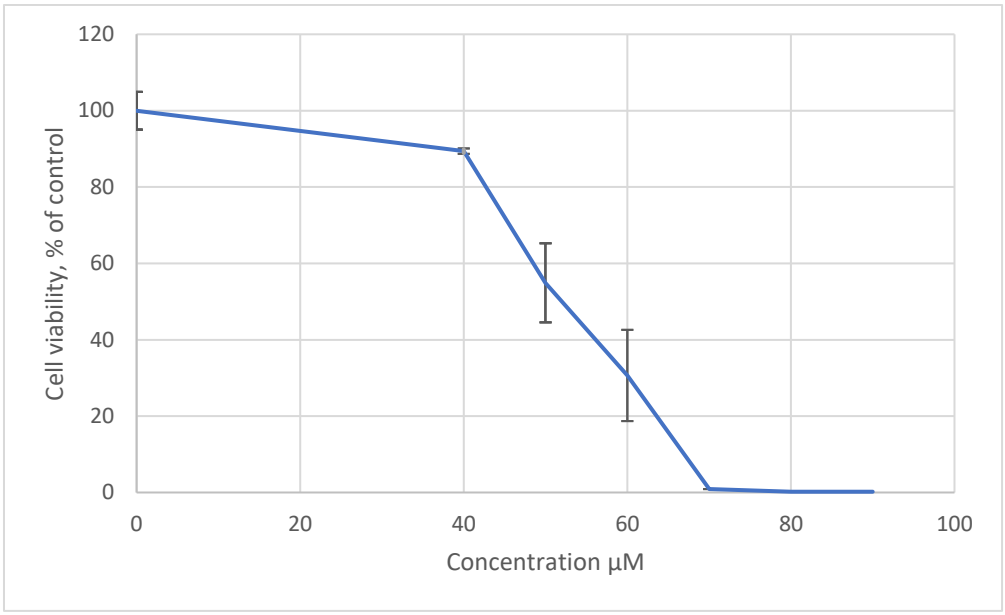


Fig. 3. Effects of α-linolenic acid (0-90 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

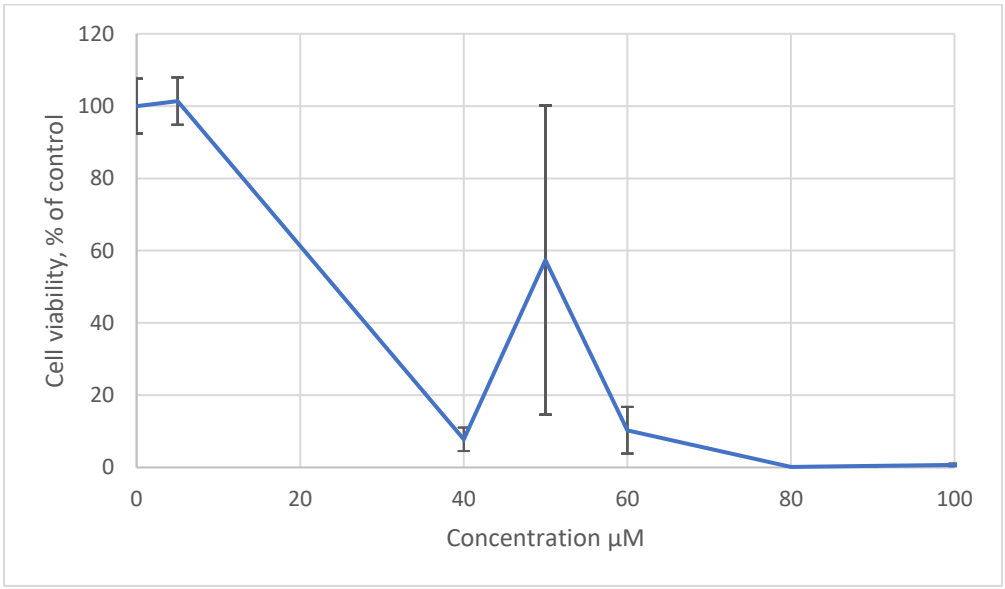


Fig. 4. Effects of γ-linolenic acid (0-100 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

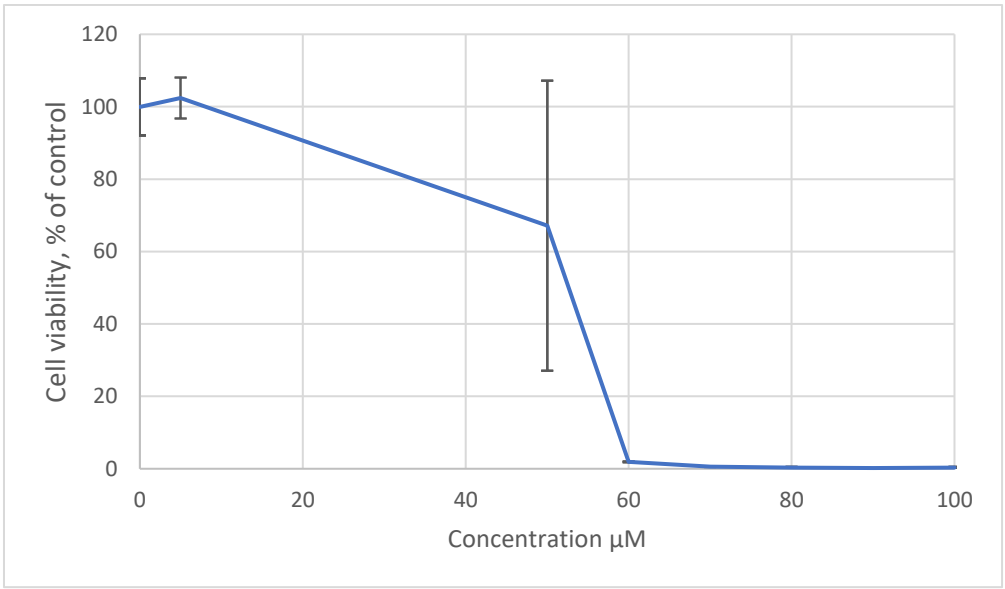


Fig. 5. Effects of stearic acid (0-100 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

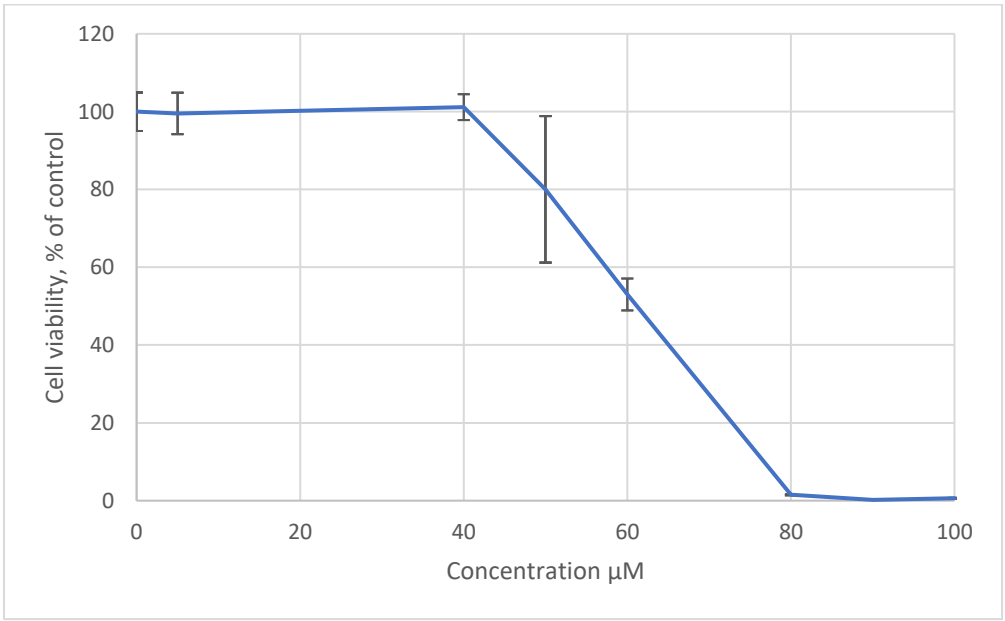


Fig. 6. Effects of oleic acid (0-100 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

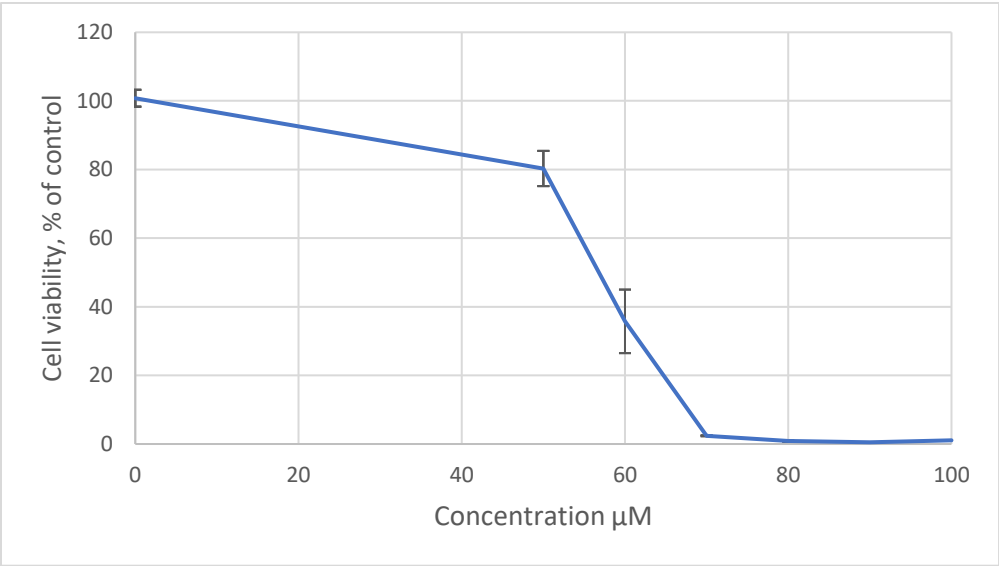


Fig. 7. Effects of elaidic acid (0-100 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

All tested fatty acids named **1a**-**1e** were showing suprisingly high cytotoxicity. Low concentrations of these compounds (60-80 µM) were able to kill all cells in the dishes and calculated LD_{50} values for the compounds are shown in Table 2.

Table 2. Calculated LD_{50} values for the fatty acids.

Compound	**1a**	**1b**	**1c**	**1d**	**1e**
LD_{50} (µM)	52.02	-	52.63	61.16	56.80

LD_{50} for compound **1b** was not calculated since the results show two concentrations with 50% cell survival.

3.2. Evaluation of the solvents' toxicity

Data gained from the tests of fatty acids, dissolved in ethanol were showing high cytotoxicity in low concentrations. This led us to belive, that there is a cytotoxic effect caused by the solvent. For that reason the effect of pure ethanol on cell viability was tested by MTT assay (Fig. 8).

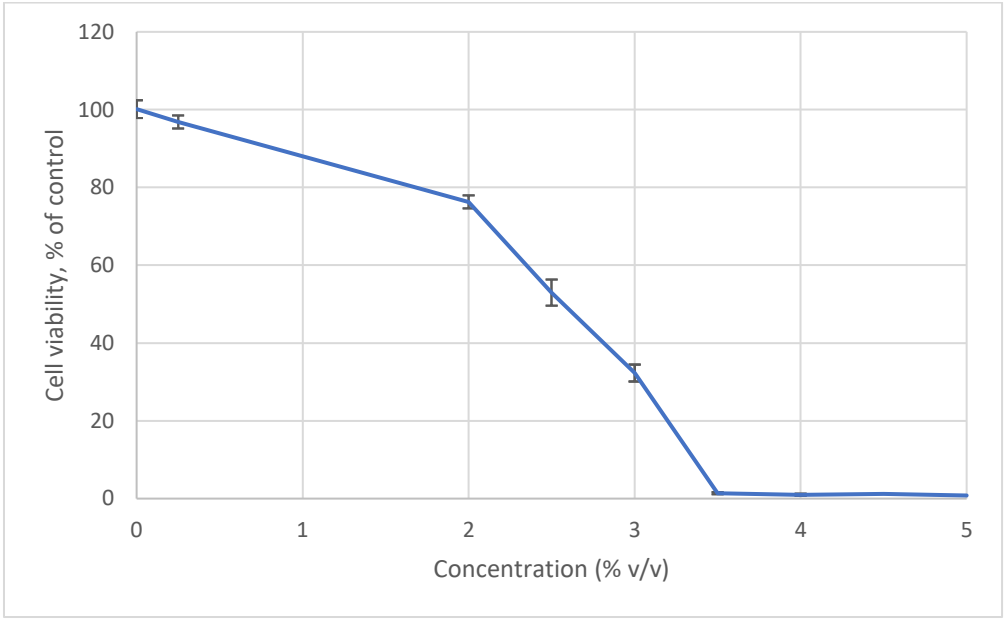


Fig. 8. Effects of ethanol (0.25-5% v/v, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

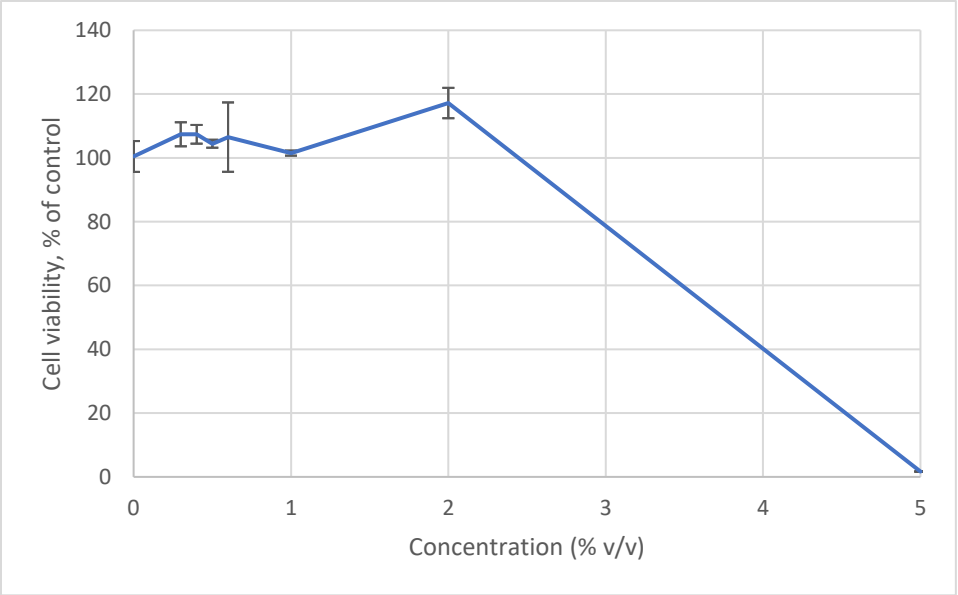


Fig. 9. Effects of DMSO (0.3-5% v/v, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

The chosen solvent for the next experiments was dimethyl sulfoxide (DMSO), and it was decided to test its' influence on the F98 cell line as well. Results obtained from this experiment are shown in Fig. 9.

The concentration range of the solvents in medium was the same as the concentration range in the experiments with the tested compounds. The relations between concentrations are shown in Table 3., and Table 4.

Table 3. Relation between the volume of the solvent, its' concentration (% v/v) and concentration of the tested compound for ethanol.

Volume of the solvent (µL in 2.5 mL mixture with medium)	Concetration of the solvent (% v/v)	Concentration of the tested compound (µM)
6.25	0.25	5
50	2	40
62.5	2.5	50
75	3	60
87.5	3.5	70
100	4	80
112.5	4.5	90
125	5	100

Table 4. Relation between the volume of the solvent, its' concentration (%v/v) and concentration of the tested compound for DMSO.

Volume of the solvent (µL in 2.5 mL mixture with medium)	Concetration of the solvent (% v/v)	Concentration of the tested compound (µM)
7.5	0.3	30
10	0.4	40
12.5	0.5	50
15	0.6	60
25	1	100
50	2	200
125	5	500

The results obtained from the ethanol test shown that the viability of the cells decreases in every concentration of ethanol. The calculated LD_{50} is 64.29 µL of total 2.5 mL which reffers to 2.57% of the ethanol and 51.34 µM of the tested compounds. For the DMSO calculated LD_{50} is 93.63 µL, which reffers to 3.74% of the solvent and 374 µM of the tested compounds.

3.3. Evaluation of the cytotoxicity of the fatty acids dissolved in DMSO

Three of the previously tested fatty acids (α-linolenic acid, stearic acid, and elaidic acid), which were supposed to be used in synthesis of the intermediates of interest, were tested again using DMSO as a solvent to preapre the stock solutions. Concentration of the stock solutions in DMSO was 10mM. Results are shown in Fig. 10-12.

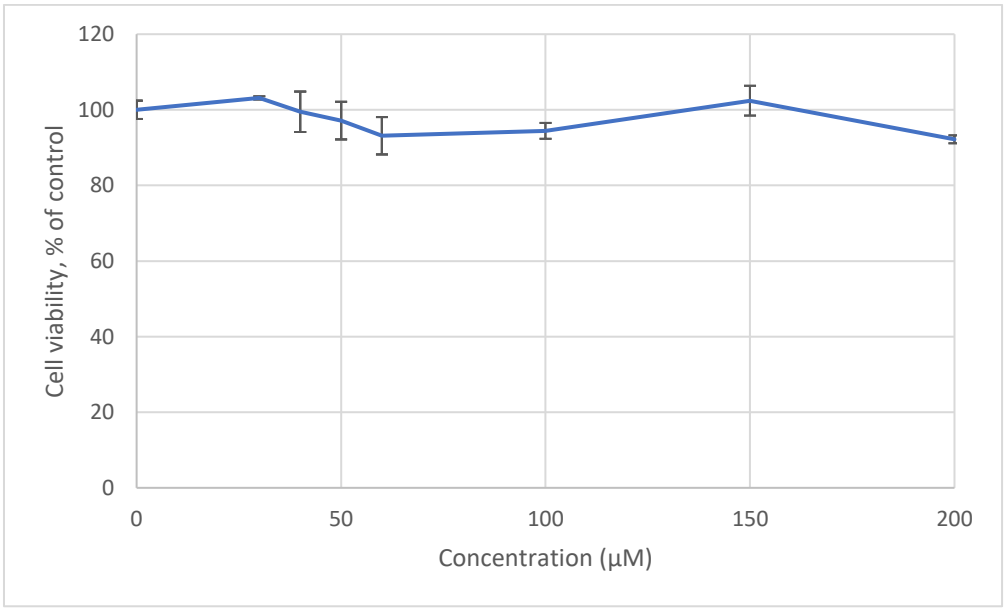


Fig. 10. Effects of α-linolenic acid (0-200 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

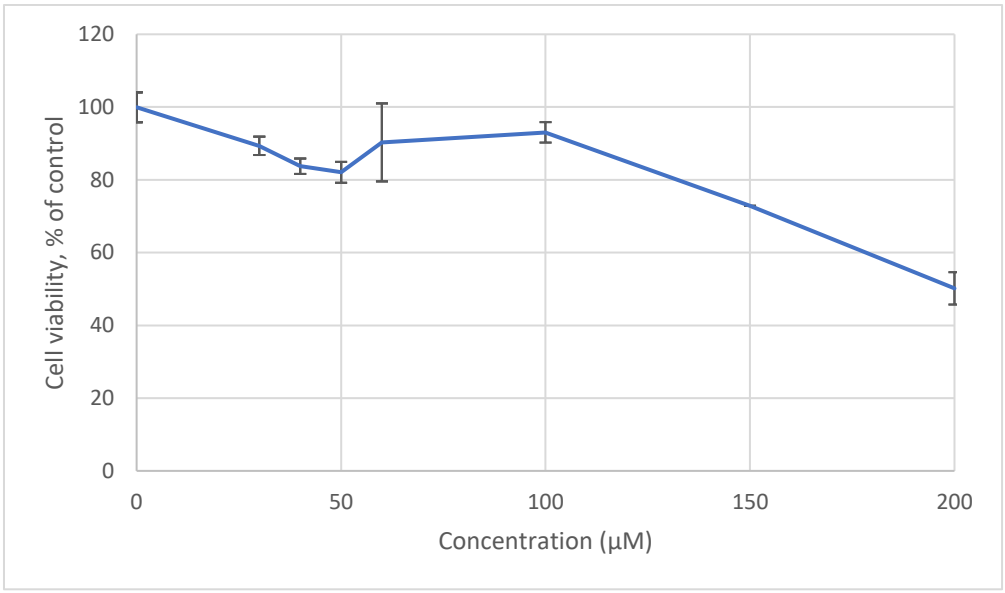


Fig. 11. *Effects of stearic acid (0-200 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.*

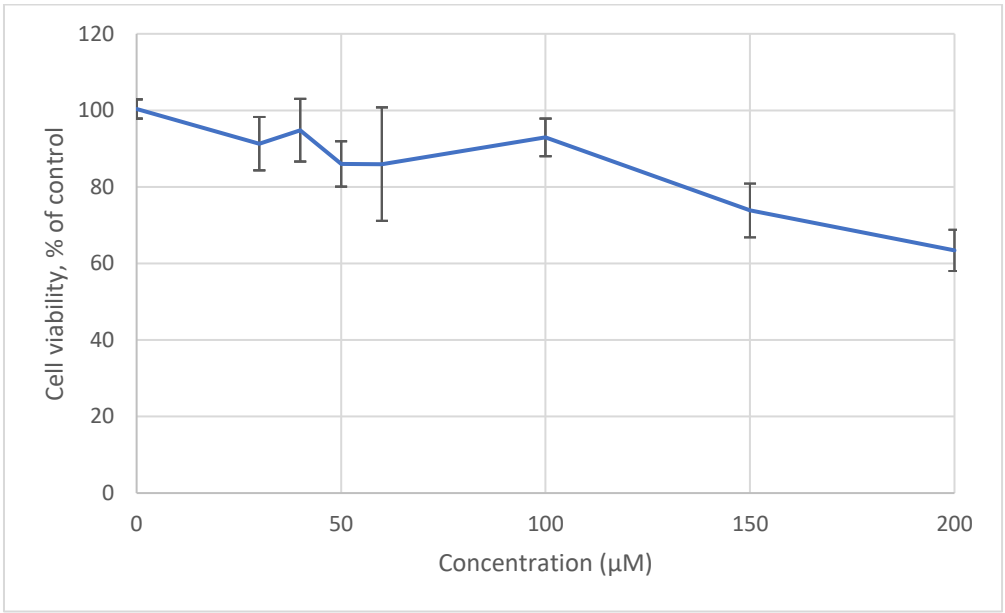


Fig. 12. Effects of elaidic acid (0-200 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

After the change of the solvent, fatty acids tested are not showing toxicity in lower consentrations. Toxicity of these fatty acids is simillar to the toxicity of the solvent.

The cell viability on the F98 cell line after incubation with α-linolenic, stearic, and elaidic acid was tested on concentration from 0-200 µM. For that reason LD_{50} values are not shown.

3.4. Evaluation of the cytotoxicity of the sulfoquinovosyldiacylglicerol (SQDG)

It was interesting to check the effect of SQDG on the cell line chosen for this project, especially that there are reports that sulfoglycolipids possess and antitumor activity [11].

Results obtained in this project are shown in Fig. 13.

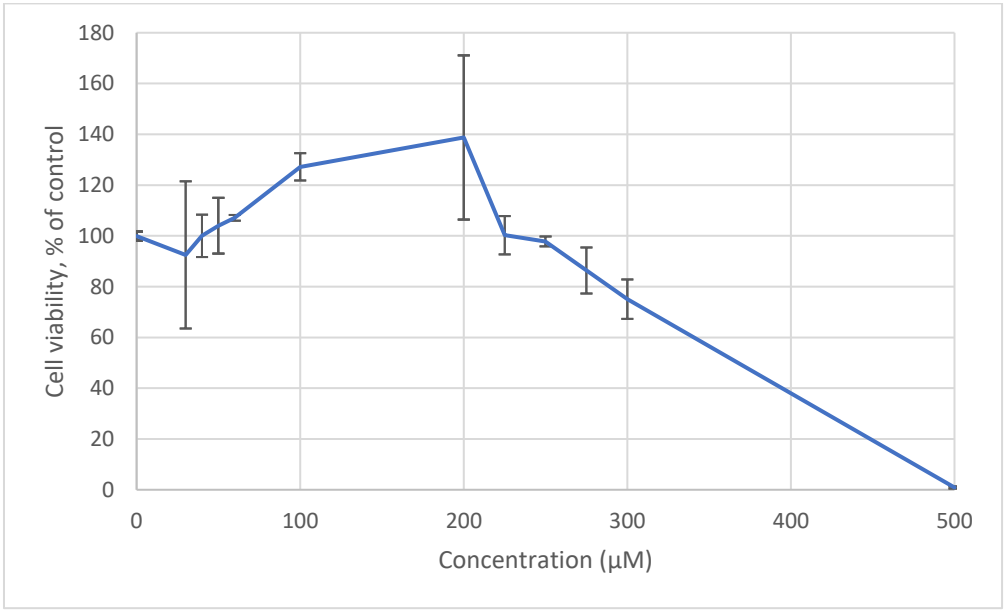


Fig. 13. Effects of sulphoquinovosyldiacylglicerol (0-500 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

Three tests in different concentration of sulphoquinovosyl diacyl glicerol were performed. First, the MTT experiment with final concentration of 60 µM did not show any effect on the cells. The broader range of concentrations were tested in second experiment. This experiment shown opposite effect to the expected one. At 200 µM viability of cells increased up to 157% of the control. Third test was made then to check the viability of the cells in concentrations from 200 µM to 300 µM, but the results were surprising again, since at 200µM concentration there was no effect (100% viability), and the viability was dropping slowly with increasing concentration.

Calculated LD_{50} for this compound is 367.59 µM and is similar to the LD_{50} value for the DMSO. The accuracy of this value is questionable due to the few data points in the 300-500 µM range.

3.5. Evaluation of the cytotoxicity of the synthesized intermediates

Five intermediates from the total synthesis project were subject for biological evaluation. Of these, three were synthesized by a fellow student in the research group. All of the intermediates are, to the best of our knowledge, novel. At first, the tests of intermediates (**3a**, **3b**, and **3c**) were conducted. Results are shown in Fig.14., and Fig. 15.

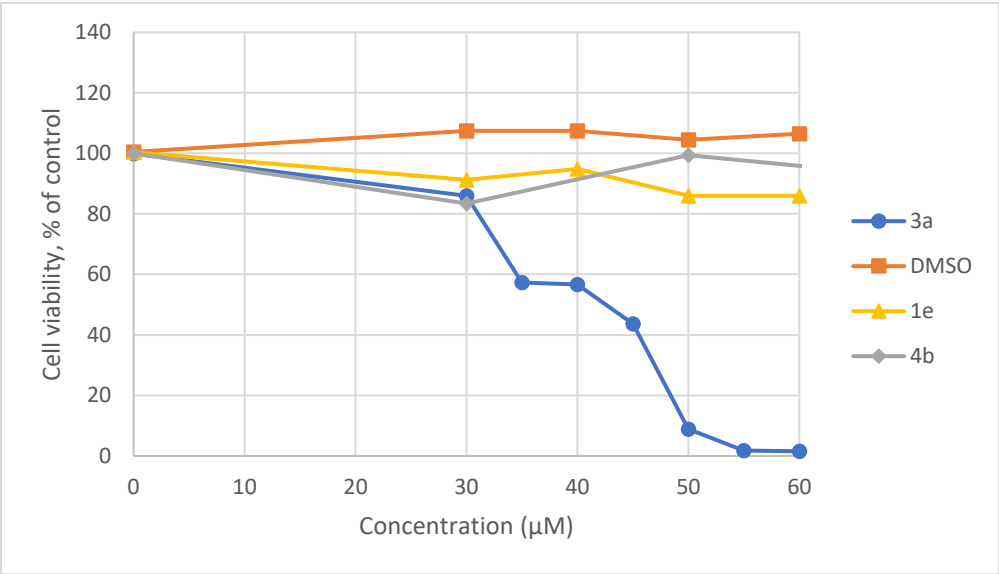


Fig. 14. Effects of 1-O-allyl-4-O-benzyl-6-O-elaidate-α-D-glucopyranoside (0-60 µM, 48 h) on cell survival performer on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are combined with data obtained in tests of elaidic acid, DMSO and 1-O-allyl-4-O-benzyl- α-D-glucopyranoside.

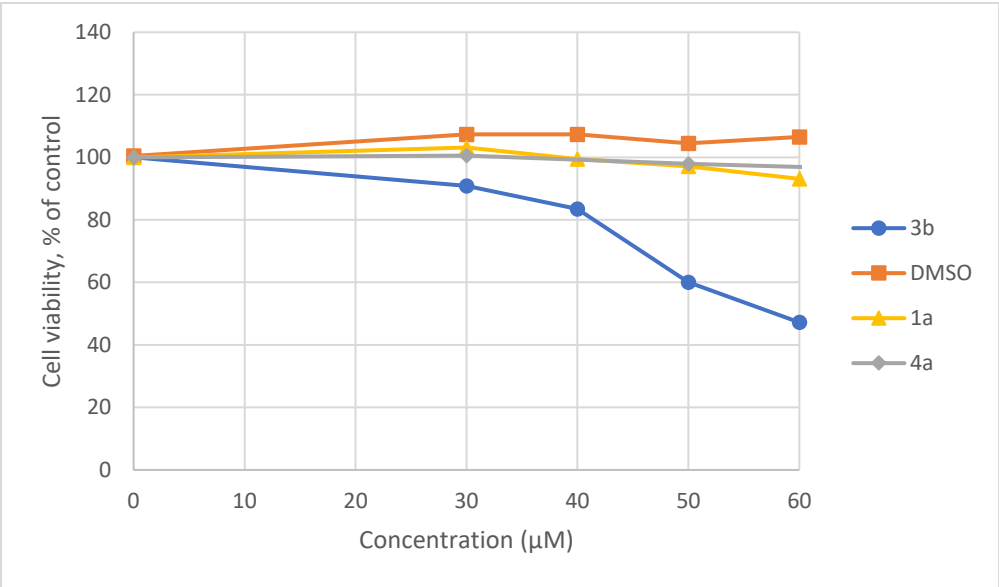


Fig. 15. Effects of 1-O-allyl-4-O-α-linolenate-6-O-benzyl-α-D-glucopyranoside (0-60 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are combined with data obtained in tests of α-linolenic acid, DMSO and 1-O-allyl-6-O-benzyl- α-D-glucopyranoside.

Results were combined with the results from the MTT assays of fatty acids present in the molecule, the solvent used to prepare the stock solution, and the intermediate without fatty acid moiety. It can be observed that the toxicity of the **3a**, and **3b** intermediates is much higher compared to other previously tested compounds. Calculated LD_{50} value for **3a** was 42.56 µM, and for **3b** was 57.82 µM

The cell survival analysis of 1-O-allyl-4-O-benzyl-6-O-stearate-α-D-glucopyranoside (compound **3c** in Table 1) was not successful (results not shown). The cells did not survive even in the lowest concentrations. The stock solution was then analyzed using NMR. This analysis shown that the stock solution is not pure, probably due to the decomposition of the compound (Fig. 16.)

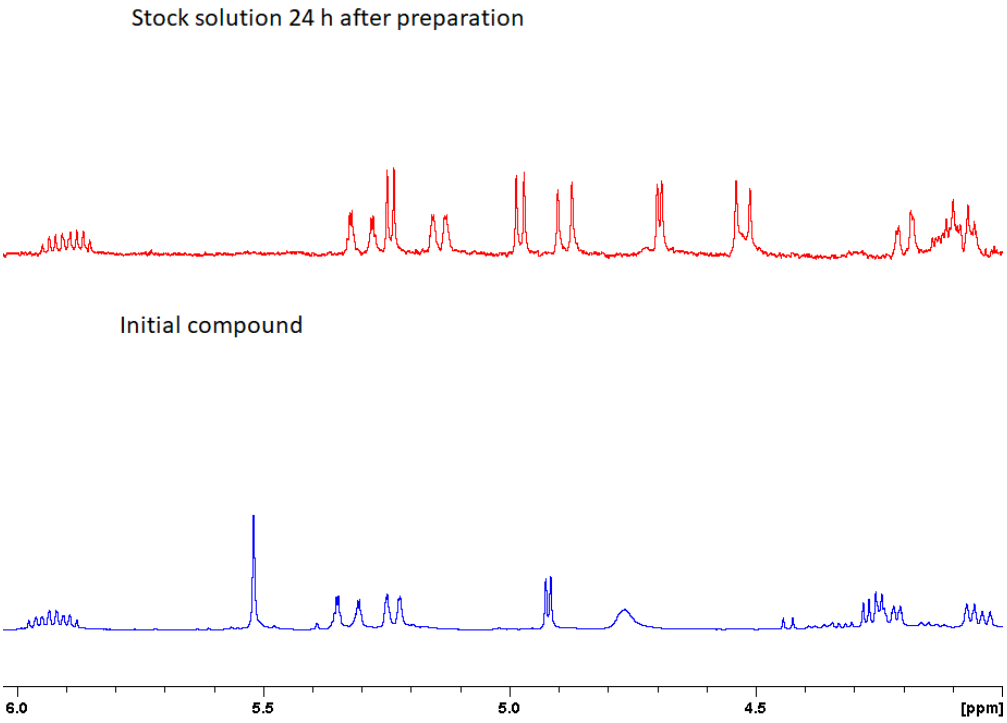


*Fig. 16. The comparison of the NMR spectra of compound **3c** before and after preparation of stock solution.*

It was believed that the observed cytotoxicity of the intermediates **3a** and **3b** may come from the presence of the benzyl group. Intermediates **4a** and **4b** were synthesized then in order to check this hypothesis. Results are shown together with **3a** and **3b** in Fig. 14., adn Fig. 15.

It can be seen that the removal of fatty acid group from the molecule changed the cytotoxicity significantly. In the analyzed concentrations (0-60 µM) the effect of intermediates **4a** and **4b** is slightly higher than this of solvent and fatty acid present in the full intermediates. Even broader range of concentrations, for which results are shown in Fig. 17. and Fig. 18., were not showing high effect (cell viability at 150 µM was around 80% in both cases)

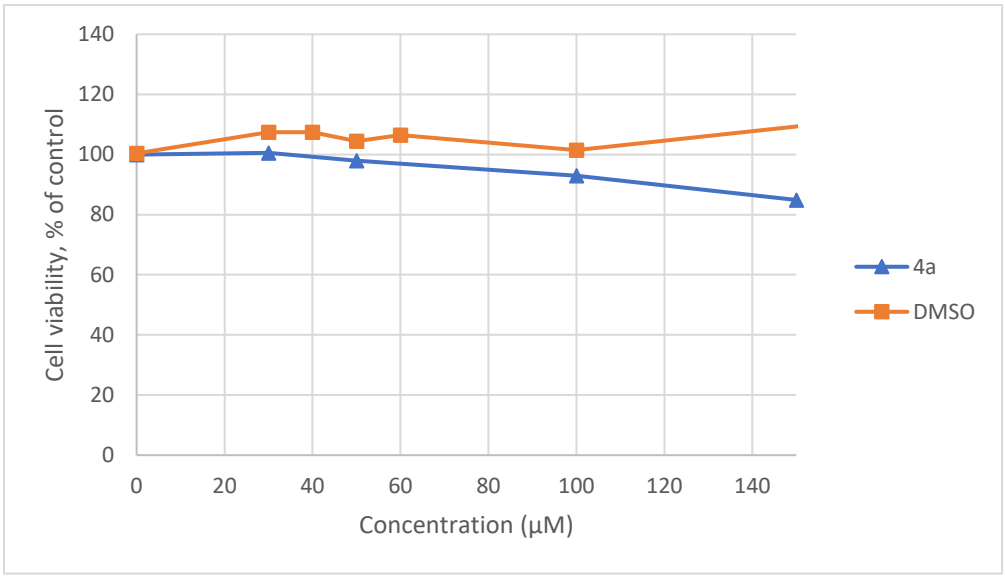


Fig. 17. Effects of 1-O-allyl-6-O-benzyl- α-D-glucopyranoside (0-150 µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are combined with data obtained in tests of DMSO.

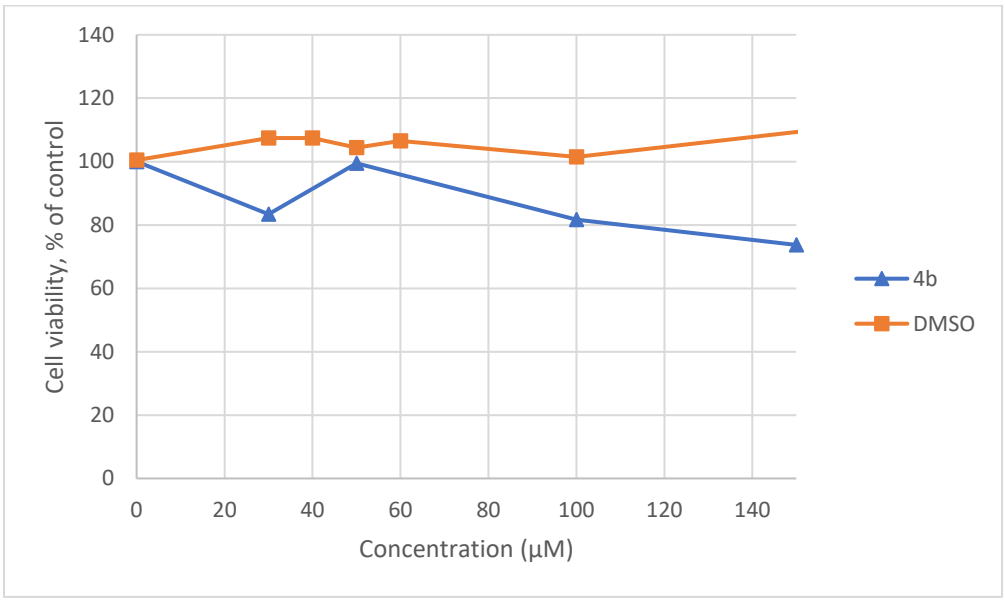


Fig. 18. Effects of 1-O-allyl-4-O-benzyl- α-D-glucopyranoside (0-150 µM, 48 h) on cell survival performer on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are combined with data obtained in tests of DMSO.

3.6 Synthesis of intermediates

From two intermediates prepared by other research group member were selected for deprotection in order to have compounds without fatty acid substituents for comparison with **3a** and **3b**. The reaction scheme is given in Fig. 19.

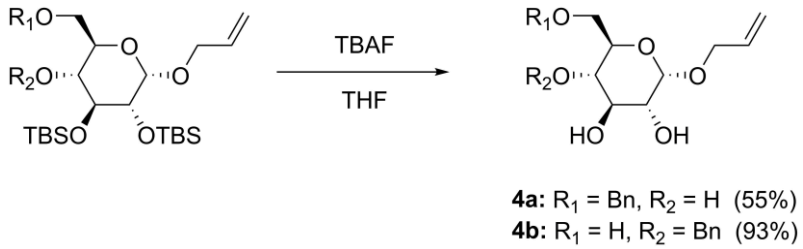


Fig. 19. TBAF in THF (3 eqv), THF, rt, 16 h.

The products were directly purified on silica gel flash chromatography giving decent purity and yields.

4. Discussion

Results obtained in the first tests of fattty acids (Fig. 3-7.) were surprising, because they were showing high cytotoxicity against the F98 cancer cells. There is report [12] showing that compound **1b** (γ-linolenic acid) shows cytotoxicity against human myelogenous leukemia K562 cell line (IC_{50}=101.59 μM, 24 h). It was also observed that pacitaxel, drug used against human breast carcinoma MDA-MB-231 cells, was showing synergistic interactions when added concurrently with γ-linolenic acid [13]. Oleic acid (**1d**) was showing showing similar, but less significant effect on that cell line. Tanaka et al. retoprted cytotoxic effect of **1a** (α-linolenic acid) and **1b** (γ-linolenic acid) on ovarian KF28 cancer cells, and no effect of the **1d** (oleic acid) [14]. Stearic acid (**1c**) was also showing cytotoxic effect on KF28 cells. In report by Kuwata et al., it was shown that oleic acid was increasing the survival of ovarian HNOA cancer cells [15]. Elaidic acid was reported to decrease viability of SH-SY5Y neuroblastoma cells [16]. As it can be seen fatty acids tested in present project possess cytotoxicity against some cell line, or are increasing cytotoxic effect of other drugs. Some of them can also increase the viability of the cancer cells (oleic acid on HNOA cancer cells). However, the results presented in Chapter 3.1. were showing that F98 cell line is very susceptible for the influence of these compounds (see Table 2 in Chapter 3.1. for calculated LD_{50} values).

The experiment with pure ethanol had to be conducted to check the influence of the solvent. The results of these experiments confirmed, that the high cytotoxicity of the tested fatty acids (LD_{50} values shown in Table 2 in Chapter 3.1.) was a result of the presence of the ethanol. Neuman et al. reported that the viability of Hep G2 human liver cancer cells drops to 68.5% at 0.47% ethanol concentration after 24 h incubation [17]. Susceptibility to ethanol varies between different cell lines, as reported by Tapani et al. [18]. F98 cell line seems to be susceptible for the influence of this solvent even at low concentrations, since at 87.5 μL/dish (total volume 2.5 mL) which corresponds to 3.5% concentration, there were no survived cells, but less susceptible than mentioned Hep G2 cell line.

The calculated LD_{50} for ethanol was corresponding with 51.43 μM concentration of the tested fatty acids. This value is simillar to the values obtained for compound **1a** and **1c** (52.02 and 52.63 μM respectively, Table 2. in Chapter 3.1.), and slightly higher then for the rest of the compounds (**1d** 61.16 μM, **1e** 56.80 μM, **1f** 55.08 μM, Table 2. in Chapter 3.1). These results confirm that the decrease in the viability of the cells was not due to the presence of the tested compounds, but to the presence of ethanol. It can be observed as well, that the presence of the

compounds **1d**, **1e** and **1f** slightly increased the cells' viability in the presence of ethanol.

As ethanol proved inadequate for our purposes, it was decided to investigate whether DMSO was a suitable solvent. Furthermore, it was decided to prepare stock solutions in stronger concentrations in order to avoid the interference of cytotoxicity of DMSO on the tested compounds. The results were showing, that DMSO does not possess cytotoxicity in the concentration range used in further experiments, and increases the viability of the F98 cells in the concentrations up to 2% (Fig. 2. in Chapter 3.2.).

Since the solvent was changed it was reasonable to re-test the fatty acids (which were selected to be used in the synthesis of the intermediates) for comparison. Compounds named **1a**, **1c**, and **1e** in Table 1 were chosen to be used in the synthesis, and those compounds were tested again.

Compound **1a** (Fig. 11.) was not showing any bioactivity, and the cell viability was close to the 100% for whole the concentration range, and was slightly lower than the viability in pure DMSO (Fig. 10.). Compound **1c** showed higher cytotoxicity and the viability of the cells droped down to 50.16% at 200 µM (Fig. 12.). Compound **1e** also showed higher cytotoxicity than **1a**, but the viability droped down to 63.41% at 200 µM (Fig. 13.). It can be assumed, that compounds **1c** and **1e** possess cytotoxicity against the F98 cell line, but it is the case just for the higher concentrations of the compound.

The results obtained from the tests of the compound **2a** were also surprising, as shown in Fig. 14. Results shown in this figure are combined results from three different experiments. Results from each experiment are attached in Appendix C. As shown in Fig. 20. in Appendix C, in first experiment the concentration range was 0-60 µM, and the viability of the cells was from 92.50% up to 107.10%. This level of viability was similar to this in the test of pure DMSO. For the second experiment (Fig. 21. in Appendix C) higher concentrations (100 µM, 200 µM, and 500 µM) were tested. Results gained in this experiment were also surprising, since there was significant increase in viability at 100 µM up to 127.19%, and up to 157.01% at 200 µM. The viability at 500 µM was 0%. The third test was conducted then, to see, how the viability of the cells changes between 200 µM and 300 µM, and if it would be possible to find LD_{50} value in this concentration range. But the results (Fig. 22. in Appendix C) were surprising again, since the viability at 200 µM was 102.23%, which was significantly lower, than the value from the second experiment (157.01%; Fig. 21., Appendix C). The average viability at 200 µM was 138.75%. The previous studies by Sahara et al. [11], were showing, that sulfoglicolipids are potent antitumor compounds. They shown that compound **2a** was not cytotoxic against

W14 and A-549 cell lines (*in vitro*), but it's monoacyl derivative inhibits the growth of these cells. Also in *in vivo* experiments in mice the inhibition of the tumor growth was observed. It has been speculated that the difference between SQDGs and SQMGs might be related to differing cell permeabilities [19]. The research group has not generated any data to corrobate this explaination, but a similar effect was seen when compairing the bioacivity of SQDG to those of the monoacetylated compounds (**3a**, **3b**) on the F98 cell line.

After experiments on SQDG, compounds **3a**, **3b**, **3c**, **4a**, and **4b** were tested. These molecules were intermediates in the synthesis of the target molecule (Fig. 1.) in present project. As it was shown in the Fig. 15. and Fig. 16. compounds **3a** and **3b** are cytotoxic against the F98 cell line with LD_{50} of 42.56 µM and 57.82 µM, respectively. It is observed that the viability of cells treated with these compounds is much lower than the viability measured with pure solvent, fatty acids, or compounds **4a** and **4b**. These results indicate, that this group of compounds contains potent antitumor agents which should be further evaluated.

The test of compounds **4a** and **4b** were conducted in order to check, if there will be change in cytotoxicity when the fatty acid moiety is removed. The results shown (Fig. 14., and Fig. 15.), that there is significant change in the cytotoxicity between the pairs of the compounds (**3a** and **4b**, **3b** and **4a**), and the cytotoxicity comes from whole molecules **4a** and **4b**, and not from their parts. Even when broader range of concentrations was tested (results shown in Fig. 17. and Fig. 18. in Chapter 3.5.) the viability of F98 cells was decreasing slowly and at 150 µM was 84.88% for **4a** and 73.69% for **4b**.

The yields for my synthesized intermediates were decent, however, the low purity of the starting material for compound **4a**, as well as the low available amount necessitated the sacrifice of some of the product during column chromatography for purity concerns.

Due to time constraints, a full set of NMR spectra could not be obtained for compound **4a**. The shift assignment were performed by 1H NMR and COSY, and must therefore be considered incomplete. Furthermore, there are ambiguities regarding the hydroxy groups as the chemical shifts were found consideraby higher than in **4b**. Particularly the broad signal at 4.30 ppm is quite not worthy considering that the solvent used was chloroform.

5. Conclusion

Cell survival experiments conducted in this project has shown, that the intermediates in the total synthesis project are potential antitumor agents. The free fatty acids and the reported DNA polymerase inhibitor, SQDG, did not exhibit antitumor acivity. However, without fatty acid substituents present in the molecule, the bioactivity was drastically reduced, indicating that the sugar-fatty acid ester functionality is crucial for antitumor potency.

The synthesis of intermediates by deprotection of silyl ethers were easily carried out using standard fluorine reagents and gave moderate to excelent yieds (55-93%). The structures were confirmed by NMR and high resolution MS.

6. Future work

One could regard this research project as a structure-activity relationship study in its infancy. While it has been established that the carbohydrate fatty acid ester functionality is essencial for the antitumor activity for the the compounds tested. However, how the nature of the fatty acid and the carbohydrate moiety affects the activity remains unclear as very few fatty acids were screened, and only glycopyranoses were investigated. Furthermore, the effects of the 1-O-allylic and 4/6-O-benzylic substituents are unknown. Therefore, in continuation of this work a broader array of fatty acids and carbohydrate backbones should be investigated. Additionally, derivatives without benzylic substituents and with allyl ether removed or oxidized to glycerol should be procured and tested.

7. Literature

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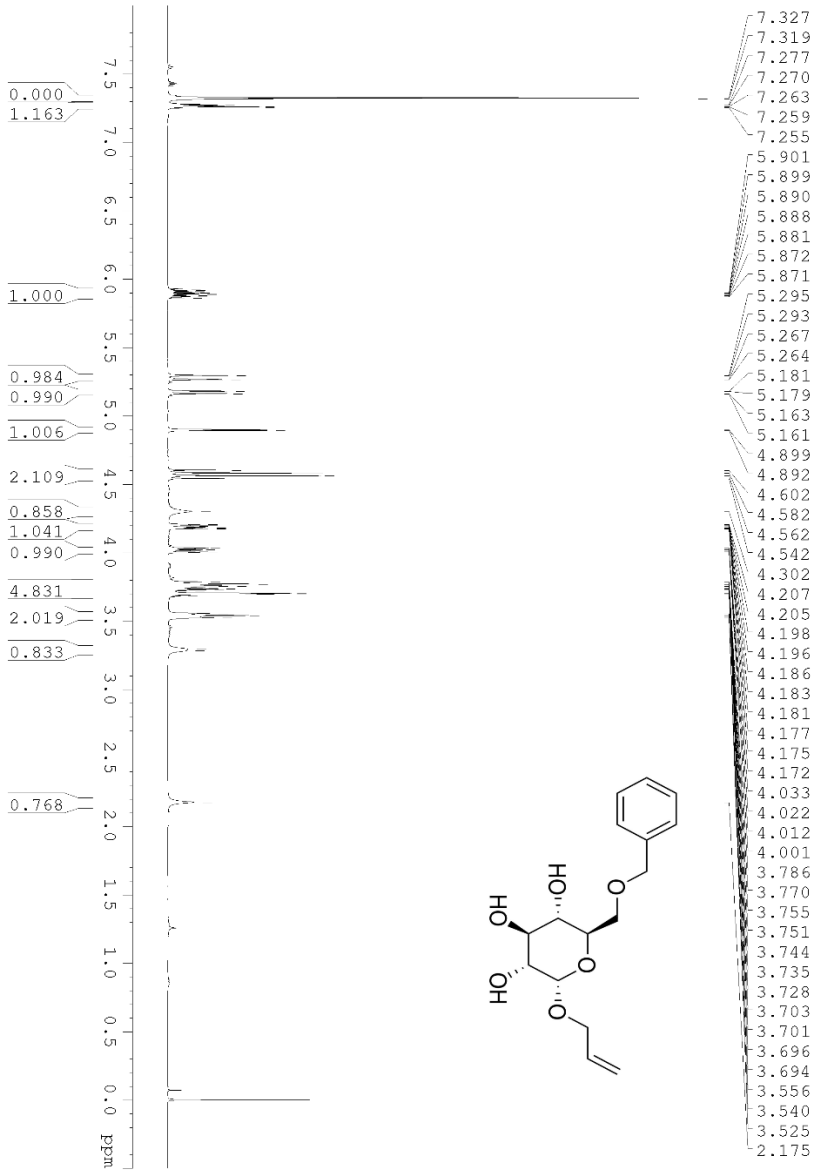
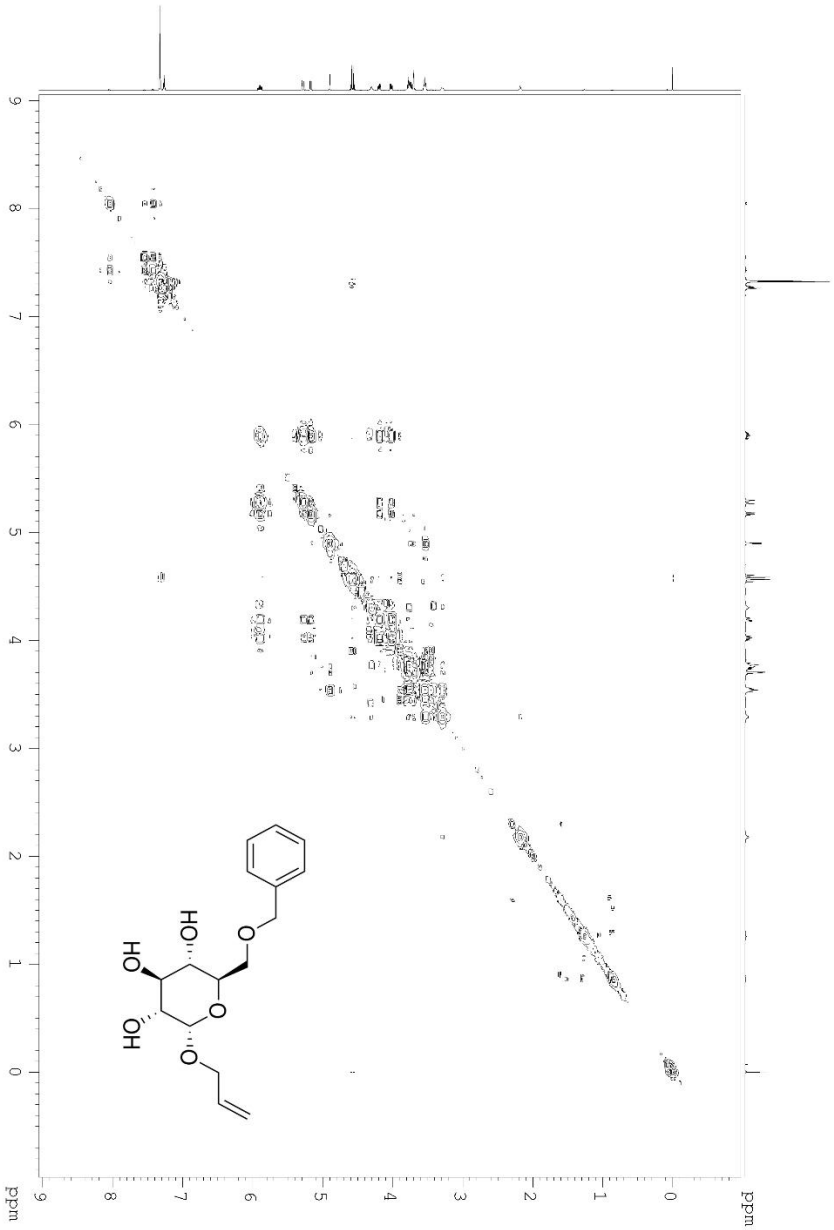
Appendix A1

1H NMR spectrum for compound **4a.**



Appendix A2

COSY spectrum for compound **4a**.

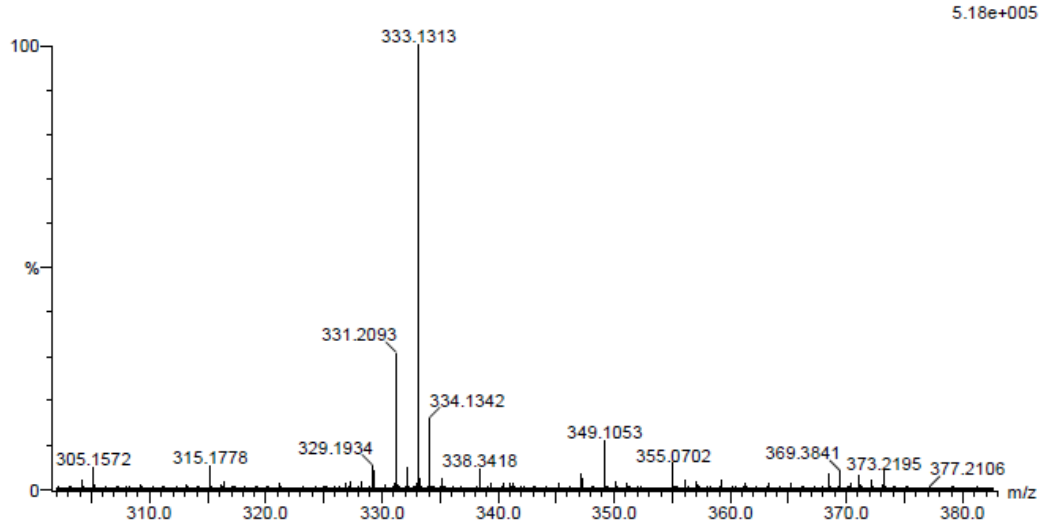


Appendix A3

MS spectrum for compound **4a**.

Single Mass Analysis
Tolerance = 2.0 PPM / DBE: min = -50.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

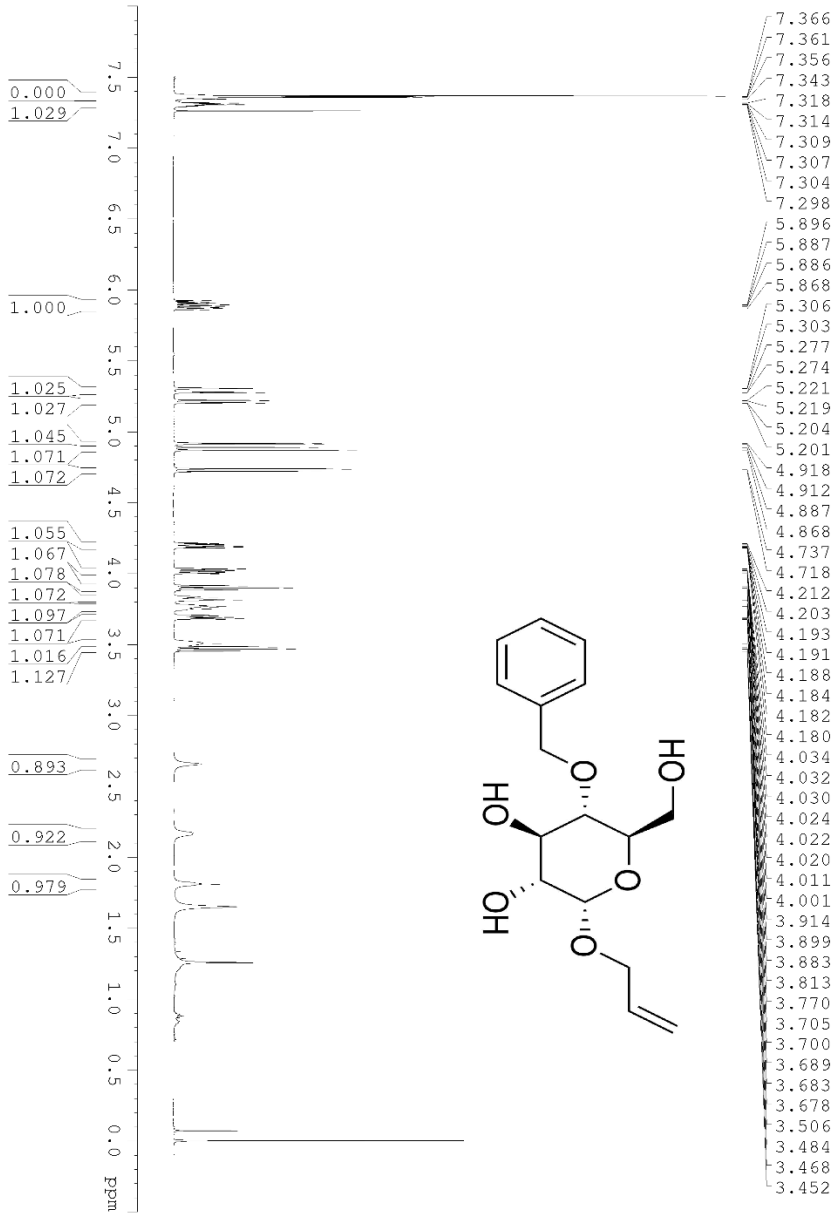
Monoisotopic Mass, Even Electron Ions
2374 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-100 H: 0-150 N: 0-10 O: 0-10 Na: 0-1
2019-643sondre 81 (0.768) AM2 (Ar,35000.0,0.00,0.00); Cm (62:82)
1: TOF MS ES+



Minimum:				-50.0				
Maximum:		5.0	2.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
333.1313	333.1314	-0.1	-0.3	5.5	1162.8	0.262	76.94	C16 H22 O6 Na
	333.1311	0.2	0.6	9.5	1164.0	1.467	23.06	C14 H17 N6 O4

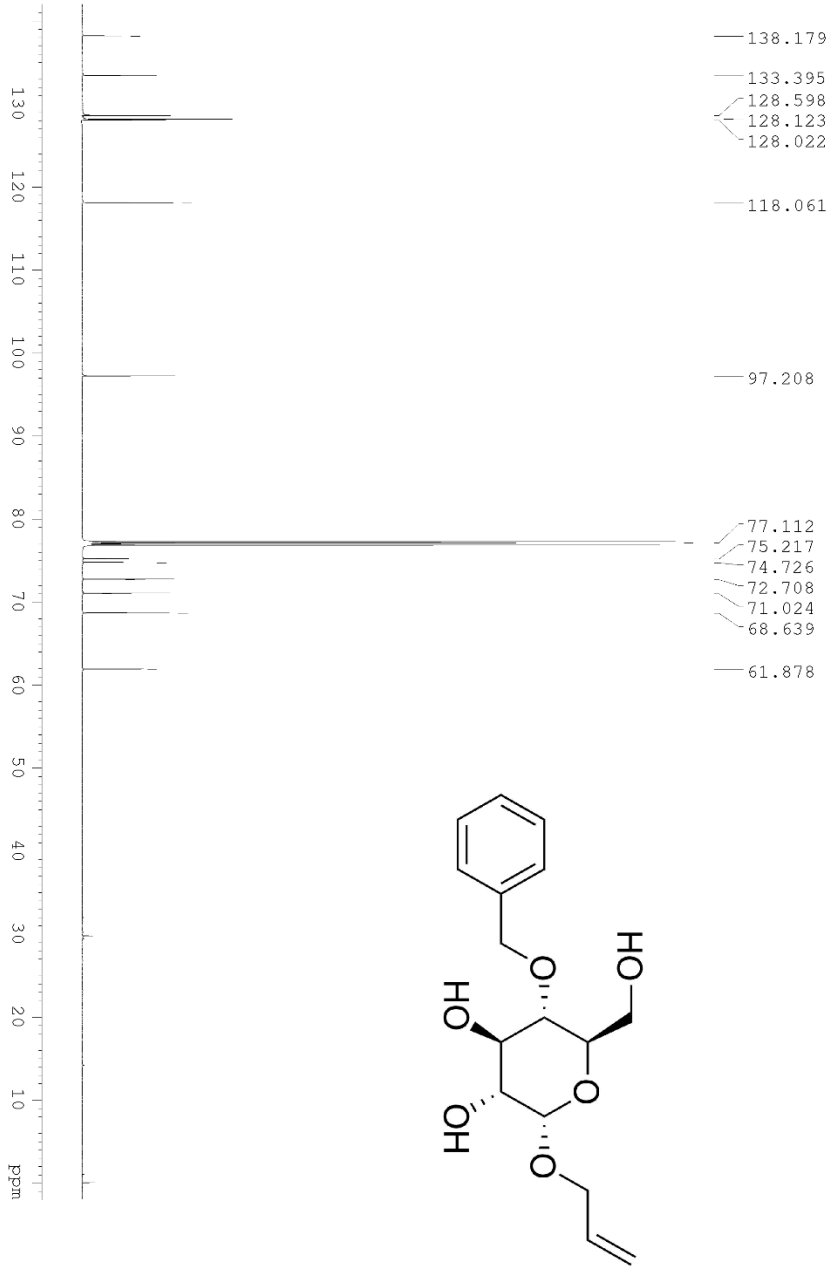
Appendix A4

1H NMR spectrum for compound **4b**.



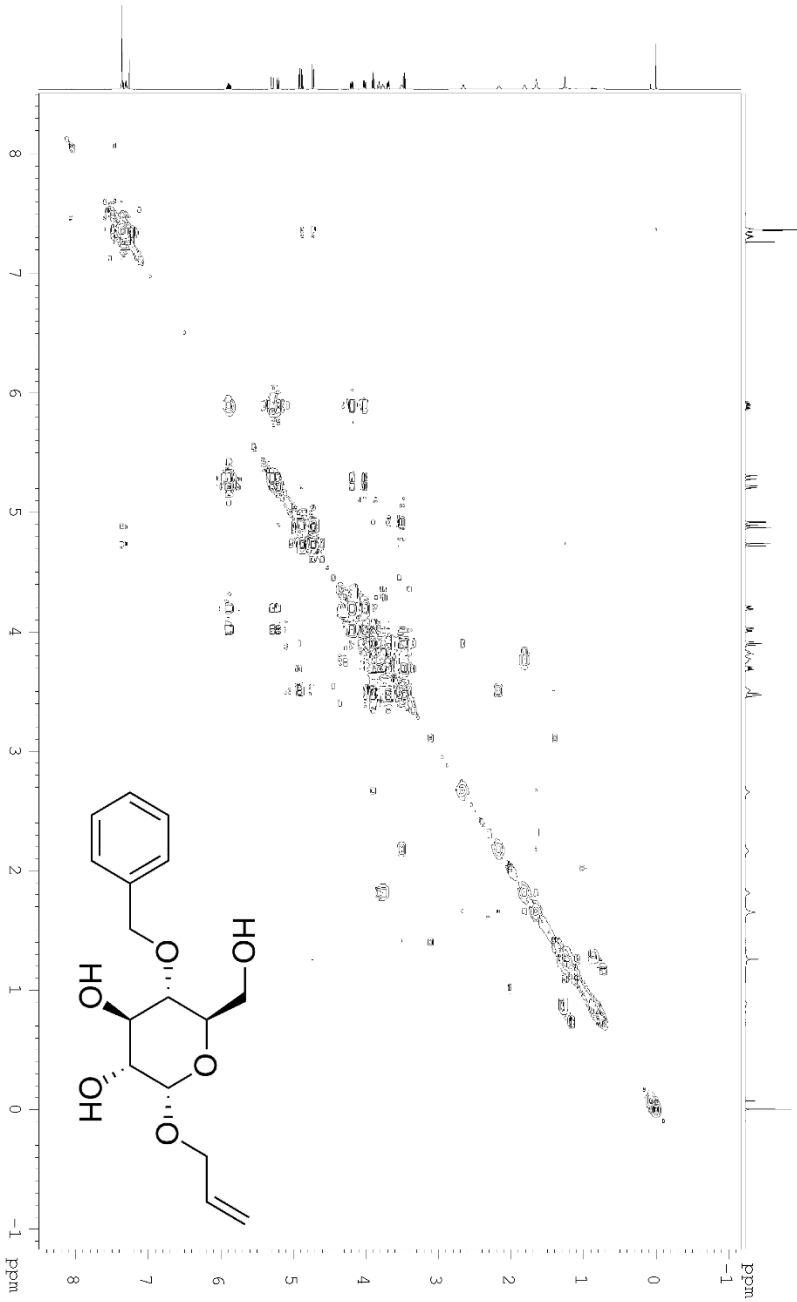
Appendix A5

13C NMR spectrum for compound **4b**.



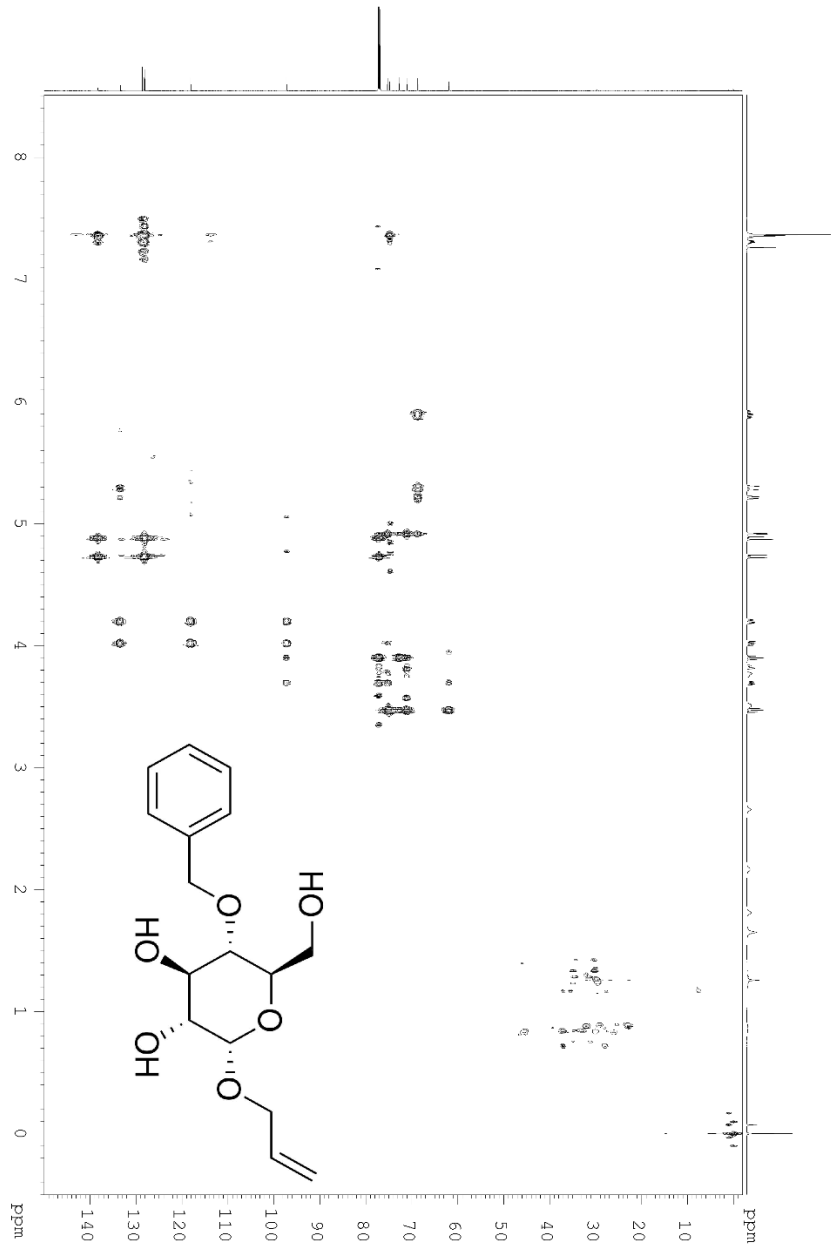
Appendix A6

COSY spectrum for compound **4b**.



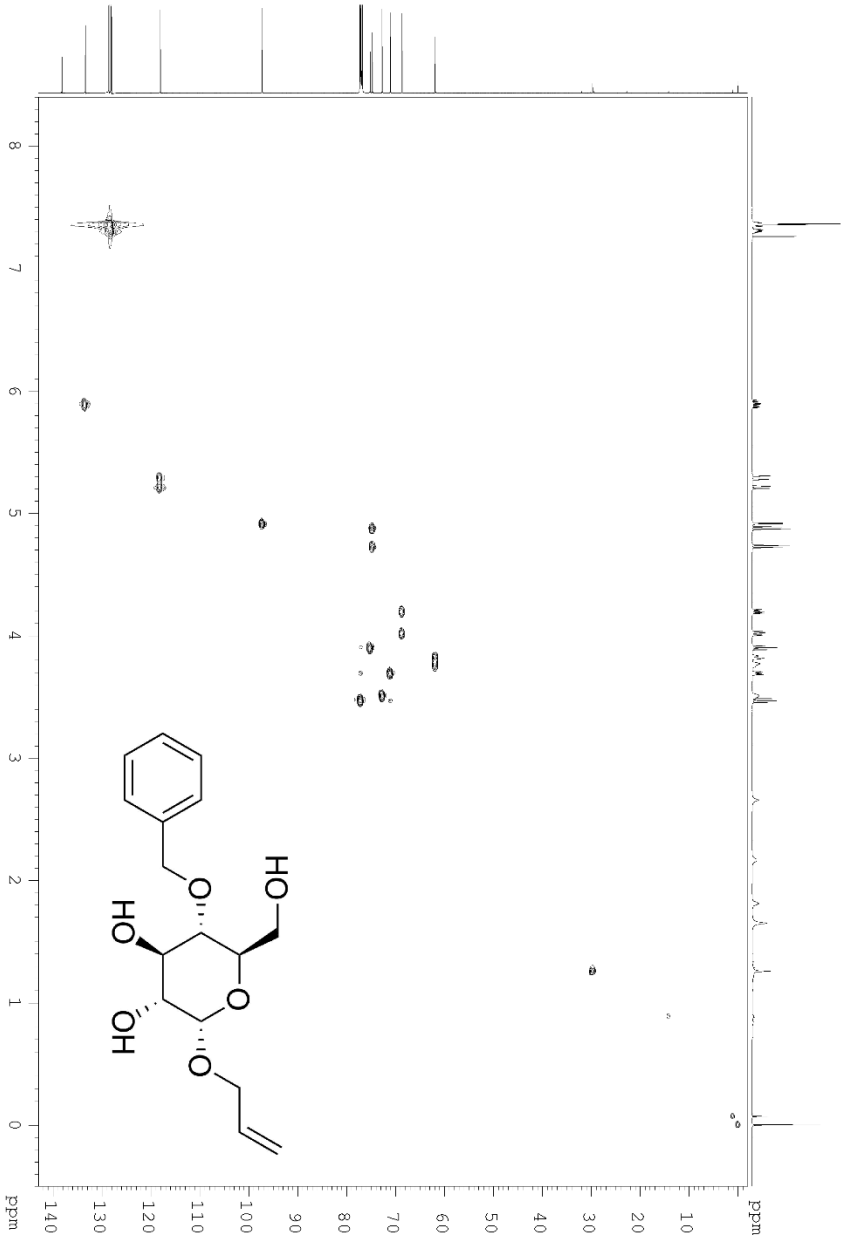
Appendix A7

HMBC spectrum for compound **4b**.



Appendix A8

HSQC spectrum for compound **4b**.

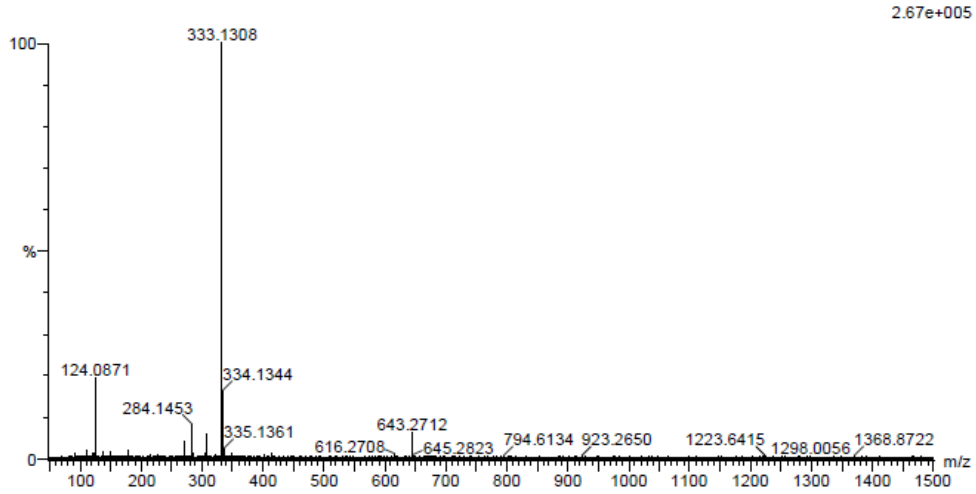


Appendix A9

MS spectrum for compound **4b**.

Single Mass Analysis
Tolerance = 2.0 PPM / DBE: min = -50.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
2374 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-100 H: 0-150 N: 0-10 O: 0-10 Na: 0-1
2019-642_sondre 145 (1.363) AM2 (Ar,35000.0,0.00,0.00); Cm (134:146)
1: TOF MS ES+



Minimum:				-50.0				
Maximum:		5.0	2.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
333.1308	333.1311	-0.3	-0.9	9.5	933.0	0.281	75.50	C14 H17 N6 O4
	333.1314	-0.6	-1.8	5.5	934.1	1.407	24.50	C16 H22 O6 Na

Appendix B

Exemplary plan for the MTT assay

No.	Concentration (µM)		Volume of medium (mL)	x (Volume of stock solution, µL)
	2a	**3a**		
1	-	-	2.5	-
2	-	-	2.5	-
3	-	-	2.5	-
4	30		2.5-x	7.5
5	30		2.5-x	7.5
6	40		2.5-x	10
7	40		2.5-x	10
8	50		2.5-x	12.5
9	50		2.5-x	12.5
10	50		2.5-x	12.5
11	60		2.5-x	15
12	60		2.5-x	15
13		30	2.5-x	7.5
14		30	2.5-x	7.5
15		40	2.5-x	10
16		40	2.5-x	10
17		50	2.5-x	12.5
18		50	2.5-x	12.5
19		60	2.5-x	15
20		60	2.5-x	15

Appendix C

Results obtained in tests of SQDG

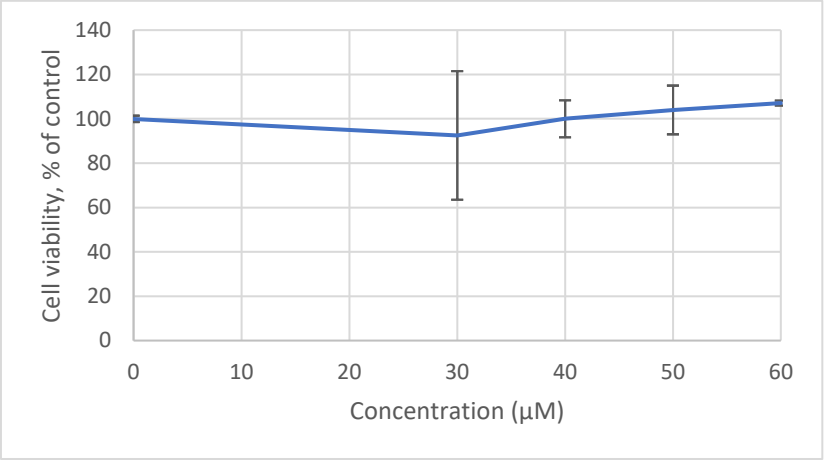


Fig. 20. Effects of sulphoquinovosyldiacylglicerol (0-60µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.

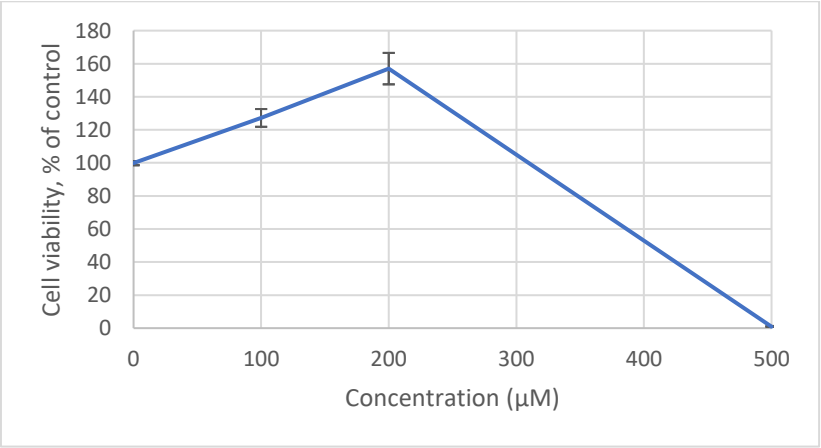


Fig. 21. *Effects of sulphoquinovosyldiacylglicerol (0-500µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.*

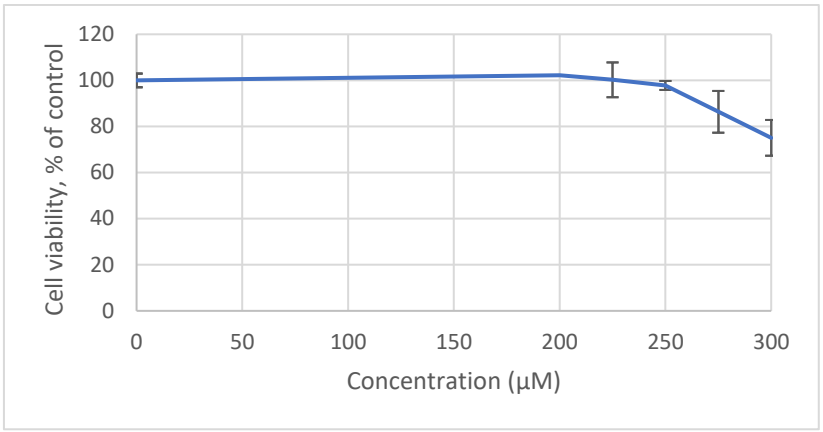


Fig. 22. *Effects of sulphoquinovosyldiacylglicerol (200-300µM, 48 h) on cell survival performed on F98 rat glioma cells. The cell survival was measured by the MTT assay. Data are the mean value +/- SD.*