

Investigations on the Enzymatic Synthesis of Novel Glycolipids

Establishment and optimization of the synthesis including product characterization

zur Erlangung des akademischen Grades eines

DOKTORS DER INGENIEURWISSENSCHAFTEN (Dr.-Ing.)

der Fakultät für Chemieingenieurwesen und Verfahrenstechnik des

Karlsruher Institut für Technologie (KIT)

genehmigte

DISSERTATION

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Tag der mündlichen Prüfung: 29.9.2014

Auch die längste Reise beginnt mit dem ersten Schritt.

(frei nach Laotse, 6.Jhd. v. C.)

Acknowledgements

I would like to gratefully thank the following persons for their help and support during my doctoral thesis:

- Professor Dr. Christoph Syldatk for giving me the opportunity to carry out my doctoral thesis in his working group. Thanks for all your guidance and support and for enabling me to visit South Africa and Namibia during my time at your institute.
- Professor Dr. Rudolf Hausmann for his supervision and the kind acceptance of the co-referee. Thank you for all the controversial discussions to keep me focused and motivated.
- All my students: Christin, Saskia, Tim, Robin, Jonas, Olga, Marius, Steffi, Julia, Jan, Yannic and Adrian. Thank you for all your hard work during your internships or bachelor- and master theses!
- All my dear colleagues from the technical biology for an excellent and friendly atmosphere in the lab and in the office. Thank you for making this three years so much fun and Karlsruhe my second home.
- Dr. Lars Wiemann and Dr. Tobias Gärtner from the BioCat group of the IGB Fraunhofer in Straubing for the friendly help with structure elucidation using NMR.
- Dipl. Ing. Frank Kirschhöfer, Dipl. Ing. Michael Nusser and Dr. Gerald Brenner-Weiß from the Institute of Functional Interfaces (KIT) for the kind help performing mass spectrometry.
- Dipl. Ing. Bastian Kannengiesser and Professor Dr. Andreas Liese from the Institute of Technical Biocatalysis, Technical University Hamburg-Harburg for the nice cooperation regarding the work with deep eutectic solvents.
- Professor Dr. Donald Cowan from the University of Pretoria, Professor Dr. Marla Tuffin, Dr. Heide Goodmann both from the University of the Western Cape and Dr. Marilize Le Roes-Hill from the Cape Peninsula University of Technology and all co-workers in their institutes for an unforgettable time in South Africa and Namibia!
- Funding from the partly BMBF funded ERA-IB project *BioSurf* was gratefully acknowledged.
- My family for their love and support throughout the years!
- Last but surely not least: Anne!

Preamble

This thesis consists of a compilation of manuscripts describing completed and ongoing works regarding the enzymatic synthesis of glycolipids. All manuscripts originated between July 2011 and May 2014 and are already published, submitted for publication or are under preparation therefore. All manuscripts are identical in content to published or submitted articles, but not in layout and style.

Chapter I provides a general introduction to surfactants and glycolipids in particular, focusing on their enzymatic synthesis in organic solvents. Hence basic fundamentals about surfactants and enzymes are complemented with an overview describing the state of knowledge about important factors affecting the enzymatic synthesis of glycolipids.

In chapter II research proposals are presented.

The results and discussion section in Chapter III is based on manuscripts for publication and divided into four subchapters. Subchapter A deals with the development of a microtiter plate-based assay to detect the synthetic activity of enzymes in organic solvents, thus, facilitating the screening for enzyme-solvent combinations suitable for the enzymatic synthesis of glycolipids.

The second subchapter describes the lipase-catalyzed synthesis of novel glycolipids, namely the amino sugar fatty acid esters N-acetyl glucosamin-6-O-hexanoate and N-butyryl glucosamine-6-O-hexanoate. This chapter is based on the publication: Enzymatic synthesis of amino sugar fatty acid esters, *Eur. J. Lipid. Sci. Technol.* 2014, 116, 423 – 428; doi:101002/ejilt.201300380

In subchapter C the characterization and optimization of the enzymatic synthesis of amino sugar fatty acid esters is presented, discussing the impact of the substrate ratio, the initial water activity, the fatty acid chain length and the reaction temperature on the synthesis.

Subchapter D depicts methods for the enzymatic synthesis of glucose-hexanoate esters in deep eutectic solvents. In addition, techniques for extracting glycolipids from deep eutectic solvents and for mass determination of glycolipids are presented.

Chapter IV sums up this work by giving concluding remarks and an outlook.

Publications and presentations

Original papers

- 2014** Martin Pöhnlein, Christin Slomka, Olga Kukhareenko, Tobias Gärtner, Lars O. Wiemann, Volker Sieber, Christoph Syldatk and Rudolf Hausmann
“Enzymatic synthesis of amino sugar fatty acid esters”
European Journal of Lipid Science and Technology 116; (4) 423 – 428
doi:101002/ejilt.201300380

Under preparation for submission for publication:

- 2014** Martin Pöhnlein, Tim Finkbeiner, Christoph Syldatk and Rudolf Hausmann
“Development of a microtiter plate-based assay for the detection of synthetic lipase activity in organic solvents”
accepted for publication in Biotechnology Letters, in press
- 2014** Martin Pöhnlein, Jonas Ulrich, Frank Kirschhöfer, Michael Nusser, Bastian Kannengiesser, Gerald Brenner-Weiß, Andreas Liese, Christoph Syldatk and Rudolf Hausmann
“Lipase-catalyzed glycolipid synthesis in deep eutectic solvents”
submitted to: European Journal of Lipid Science and Technology
- 2014** Martin Pöhnlein, Rudolf Hausmann, Siegmund Lang, Christoph Syldatk
Review: “Enzymatic Synthesis and Modification of Glycolipids”
submitted to: European Journal of Lipid Science and Technology

Talks and presentations at conferences

09 / 2013

Talk:

Martin Pöhnlein

“Enzymatic Synthesis of Glycolipids”

BioNoCo Summer School, Aachen, Germany

05 / 2013

Talk:

Martin Pöhnlein

“Enzymatic Synthesis of Glycolipids”

Biosurfactants – Challenges and perspectives, DECHEMA, Frankfurt a.M., Germany

05 / 2013

Poster presentation:

Johannes Kügler*, Martin Pöhnlein*, Yannic Irtel von Brenndorff, Adrian Sanden,

Marla Tuffin, Don Cowan and Christoph Syldatk

“Who eats all that seal fat? - a study on soil communities found underneath seal carcasses in Antarctica”

Biosurfactants – Challenges and perspectives, DECHEMA, Frankfurt a.M., Germany

* contributed equally to this presentation

09 / 2012

Poster presentation:

Martin Pöhnlein, Christin Slomka, Christoph Syldatk and Rudolf Hausmann

“Chemo-enzymatic Synthesis of Glycolipids”

Biocatalysis in Lipid Modification; European Federation for the Science and Technology of Lipids, Greifswald, Germany

09 / 2012

Poster presentation:

Martin Pöhnlein, Christin Slomka, Christoph Syldatk und Rudolf Hausmann

“Chemo-enzymatische Synthese von Glycolipiden“

30. DECHEMA Jahrestagung, Karlsruhe, Germany

08 / 2011

Poster presentation:

Martin Pöhnlein, Melanie Gerlitzki, Christoph Syldatk and Rudolf Hausmann

“Enzymatic Synthesis of New Surfactants”

DECHEMA Summer School: Biotransformations, Bad Herrenalb, Germany

Zusammenfassung

Das Bestreben der chemischen Industrie sich mehr und mehr von petrochemischen Prozessen zu lösen und stattdessen bestimmte Produkte auf nachhaltige und umweltverträglichere Art und Weise zu synthetisieren, hat seit der Jahrtausendwende stetig zugenommen. Tenside biologischen Ursprungs, insbesondere Glycolipide, bieten schon heute eine Alternative zu chemisch hergestellten Tensiden, da sie auf Basis nachwachsender Rohstoffe und unter milden Reaktionsbedingungen hergestellt werden können, z.B. durch enzymatische Prozesse.

Die Entdeckung der Stabilität und der synthetischen Aktivität von Lipasen in organischen Lösemitteln in den 1980ern führte zu zahlreichen Versuchen hinsichtlich der enzymatischen Acylierung von Zuckern mit Fettsäuren oder Fettalkoholen mit dem Ziel neuartige Glycolipide zu synthetisieren. Diese enzymatische Synthese von Glycolipiden kann zahlreiche Glycolipidvarianten hervorbringen. Im Gegensatz zu chemischen Reaktionen können durch die enzymatische Synthese regioselektiv gezielt bestimmte Strukturen gewonnen werden, welche sich in ihren tensidischen Eigenschaften unterscheiden können, und somit verschiedenste Anwendungsgebiete abdecken könnten.

Ziel dieser Arbeit, die ein Teil des ERA-IB Projektes „BioSurf – Novel Production Strategies for Biosurfactants“ war, war es selektiv neuartige Glycolipide auf enzymatischem Wege herzustellen. Die Grundbausteine für diese Synthese sollten dabei aus nachwachsenden Rohstoffen stammen, welche im Idealfall nicht in Konkurrenz zur Lebensmittelindustrie stehen.

Dies wurde durch die Auswahl des Aminozuckers N-Acetylglucosamin als Zuckerbaustein der Glycolipide erfüllt. N-Acetylglucosamin ist das Monomer des zweithäufigsten Polymers der Erde, des Chitins und kann daher durch Hydrolyse des Chitins gewonnen werden. Da Chitin als Hauptbestandteil von z.B. dem Exoskelet von Krustentieren, gar nicht bis kaum für Lebensmittelzwecke verwertbar ist, erscheint eine alternative Verwertung sinnvoll. Für eine enzymatische Synthese von Glycolipiden ist N-Acetylglucosamin deshalb sehr interessant, da durch die vorliegende Aminogruppe die Möglichkeit besteht den Zucker sowohl durch eine Esterbindung, als auch durch eine Amidbindung zu acylieren.

Um die Auswahl geeigneter Kombinationen aus Enzym und organischem Lösemittel für eine enzymatische Glycolipidsynthese zu vereinfachen, wurde ein Mikrotiterplatten-basierter Schnelltest entwickelt, was im ersten

Kapitel des Ergebnis- und Diskussionsteils dargestellt ist. Dieser Test beruht auf der Lipase-katalysierten Umesterung zwischen 4-Nitrophenol und einem Fettsäureester, welche mittels einer Farbreaktion direkt nachgewiesen werden kann. Durch Einsatz dieses Tests konnten Kombinationen aus insgesamt sieben verschiedenen Lipasen und sechs organischen Lösemitteln zeitsparend, in kleinen Volumina und in hohem Durchsatz getestet werden. Um die erhaltenen Ergebnisse zu validieren, wurde in analogen Versuchsreihen unter Verwendung der gleichen Enzyme und Lösemittel die mögliche Eignung dieser Enzym-Lösemittel Kombinationen bezüglich einer Glycolipidsynthese nachgewiesen. Durch Vergleich der Ergebnisse des 4-Nitrophenolassays und der Glycolipidsynthese konnte gezeigt werden, dass mit Hilfe des neu entwickelten Mikrotiterplattentests passende Kombinationen aus Enzym und Lösemittel für eine erfolgreiche Glycolipidsynthese ermittelt werden konnten. So konnte gezeigt werden, dass sich die beiden Lösemittel 2-methyl-2-butanol und tert-butanol, sowie die beiden Enzympräparate Novozyme 435[®] und Lipozyme[®] besonders gut für die Synthese von Glykolipiden eignen.

Die so ermittelten Ergebnisse fanden Anwendungen in den im zweiten Kapitel des Ergebnis- und Diskussionsteil beschriebenen Arbeiten. In der Veröffentlichung die diesem Kapitel zu Grunde liegt konnte erstmals die enzymatische Acylierung von N-Acetylglucosamin mit Methylhexanoat gezeigt werden, wodurch das neuartige Glycolipid N-Acetylglucosamin-6-O-hexanoat gewonnen werden konnte. Zudem wurde ein weiteres neuartiges Glycolipid, N-Butyrylglucosamin-6-O-hexanoat, aus dem Aminozucker N-Butyrylglucosamin synthetisiert, sodass insgesamt zwei neuartige Glycolipide beschrieben werden konnten. Für beide Synthesen konnte gezeigt werden, dass eine der größten Herausforderungen der enzymatischen Glycolipidsynthese, die geringe Löslichkeit der Zucker in den organischen Lösemitteln, dadurch verbessert werden kann, indem die Zucker vor Zugabe zum Reaktionsgemisch in Dimethylsulfoxid (DMSO) gelöst werden. Durch die so gewährleistete bessere Löslichkeit im Reaktionsmedium konnte die Glycolipidsynthese entscheidend verbessert werden. Des Weiteren war es möglich den Einfluss der unterschiedlichen Hydrophobizitäten der jeweiligen Aminozucker auf die Synthese zu belegen, mit dem Ergebnis, dass die erhöhte Hydrophobizität des N-Butyrylglucosamin die Synthese deutlich begünstigt, indem die Löslichkeit des Zuckers weiter verbessert wird. Um diese verschiedenen Experimente vergleichen zu können, wurden Analyseverfahren, mittels Dünnschicht- Flüssigkeits- und Gaschromatographie getestet und etabliert. Zudem konnten die tensidischen Eigenschaften der beiden neu gewonnen Glycolipide getestet, sowie ihre chemischen Strukturen mittels Kernspinnresonanzspektroskopie (*engl.* nuclear magnetic resonance spectroscopy, NMR) aufgeklärt werden.

Zur Charakterisierung und Optimierung der enzymatischen Synthese der Aminozyuckerfettsäureester wurden weitere Versuche durchgeführt, um die Einflüsse bestimmter Reaktionsparameter auf die Synthese zu untersuchen, welche im dritten Kapitel des Ergebnis- und Diskussionsteil beschreiben sind. Als kritische Reaktionsparameter sind dabei das Substratverhältnis zwischen Zucker und Fettsäureester, die Wasseraktivität des Reaktionsmediums als Maß des frei im Medium verfügbaren Wassers, sowie die Reaktionstemperatur bekannt. Unterschiedliche Substratverhältnisse können die Synthese dahingehend beeinflussen, dass hohe Fettsäurekonzentrationen die Löslichkeit des Zuckers negativ beeinflussen oder Substratinhibierung des Enzyms hervorrufen können. Die Wasseraktivität des Reaktionsmediums ist von entscheidender Bedeutung für die Umkehr der eigentlichen Hydrolyseaktivität der Enzyme, was nur bei entsprechend niedriger Wasseraktivität möglich ist. Ein zu geringer Wassergehalt hingegen könnte zum Aktivitätsverlust des Enzyms führen. Höhere Reaktionstemperaturen können bis zu einem bestimmten Punkt die Reaktion begünstigen, gegebenenfalls könnte ab bestimmten Temperaturen die thermische Denaturierung des Enzyms eintreten. Mittels Versuchen mit N-Acetylglucosamin und insgesamt drei verschiedenen Fettsäurevinylestern (Vinylhexanoat, Vinylaurat und Vinylpalmitat) konnte der Einfluss aller genannten Reaktionsparameter auf die jeweilige Glycolipidsynthese gezeigt werden. Dabei konnten zusätzlich zwei weitere neuartige Glycolipide, N-Acetylglucosamin-6-O-laurat und N-Acetylglucosamin-6-O-palmitat synthetisiert werden. Die besten getesteten Reaktionsbedingungen lagen jeweils dann vor, wenn zu Beginn der Reaktion ein deutlicher Überschuss an Fettsäureestern und eine möglichst geringe Wasseraktivität im Lösemittel vorlagen. Höhere Wasseraktivitäten führten zu einer deutlich geringeren Ausbeute an Glycolipiden, da bei diesen Bedingungen eine verstärkte Hydrolyseaktivität festgestellt werden konnte. Die optimale Reaktionstemperatur betrug 70°C. Bei dieser Temperatur war die synthetische Aktivität der Lipase am höchsten, bei gleichzeitig geringer Hydrolyseaktivität. Eine Erhöhung der Temperatur führte zu einer Veränderung der Enzymaktivität, was wiederum zu verstärkter Hydrolyse und somit verringerter Glycolipidausbeute führte.

Im letzten Kapitel des Ergebnis- und Diskussionsteils ist die enzymatische Synthese von Glycolipiden in sogenannten deep eutectic solvents (DES) beschrieben, Gemischen aus zwei Feststoffen die sich gegenseitig physikalisch so beeinflussen, dass bereits bei Raumtemperatur eine Flüssigkeit entsteht. Durch Versuche in insgesamt sechs DES konnte erstmals gezeigt werden, dass die verwendete Lipase Novozyme 435® in den beiden DES bestehend aus Cholinchlorid und Harnstoff, sowie Cholinchlorid und Glucose in der Lage war Glycolipide zu synthetisieren. Die so entstandenen Glycolipide konnten nach Extraktion aus den DES mittels ESI-Q-ToF, Tandem-Massenspektrometrie und NMR nachgewiesen werden. Zudem konnte gezeigt werden,

dass eine Glycolipidsynthese in der DES bestehend aus Cholinchlorid und Glucose ohne Zugabe von zusätzlicher Glucose möglich war, da diese DES als Lösemittel und Substrat zugleich fungierte.

Abstract

The efforts of the chemical industry to become more and more detached from petro-chemical processes and to synthesize certain products in a sustainable and environmentally friendly manner instead, have increased steadily since the turn of the millennium. Surfactants of biological origin, particularly glycolipids, already constitute an interesting alternative to chemically produced surfactants, since they can be produced biologically from renewable raw materials and under mild reaction conditions, e.g. by enzymatic processes.

The discovery of the stability of the synthetic activity of lipases in organic solvents in the 1980s led to numerous experiments targeting the synthesis of novel glycolipids via enzymatic acylation of sugars with fatty acids or fatty alcohols. This enzymatic synthesis of glycolipids can theoretically produce numerous different glycolipids. In contrast to chemical synthesis, distinct structures can be produced by using enzymes, which may differ in their surfactant properties and thus can be tailored to fit a wide range of applications.

The main goal of this work, which was embedded in the ERA-IB project “BioSurf – Novel Production Strategies for Biosurfactants”, was to selectively produce novel glycolipids using enzymatic methods. In addition, the basic building blocks for this synthesis should be obtained from renewable resources, which are not in competition with the food industry.

This was fulfilled by selecting the amino sugar N-acetyl glucosamine as sugar moiety of the glycolipid. N-acetyl glucosamine, the monomer of the world’s second most abundant polymer chitin, can be gained by hydrolysis from this renewable resource. As main component of e.g. crustacean shells, chitin is in no conflict to the food industry. For an enzymatic synthesis of glycolipids N-acetyl glucosamine is very interesting because of its additional amino group, which bears the possibility to both acylate sugars by forming an ester, as well as by an amide bond.

To simplify the selection of appropriate combinations of enzymes and organic solvents for an enzymatic synthesis of glycolipids a microtiter plate-based assay was developed, which is described in the first chapter of the result and discussion part. This assay is based on the lipase-catalyzed transesterification of 4-nitrophenol and a fatty acid ester, which may be measured directly by means of a color reaction. By using this assay, combinations of seven different lipases and six organic solvents were tested in a timesaving way, by applying only small volumes and in high throughput. To validate the results obtained, analog tests employing the same

enzymes and solvents were undertaken, aiming at a glycolipid synthesis using glucose. By comparing the results of the 4-nitrophenol assay and from the glycolipid synthesis, the feasibility to identify suitable combinations of enzymes and solvents for a successful glycolipid synthesis using the newly developed microtiter plate assay was demonstrated. It was shown that the two solvents, 2-methyl-2-butanol and tert-butanol and the two enzyme preparations Novozyme 435[®] and Lipozyme[®] were suitable best for the synthesis of glycolipids.

These results found applications in the work described in the second chapter of the results and discussion section. In the publication on which this chapter is based, the enzymatic acylation N-acetyl glucosamine using methyl octanoate was described for the first time, resulting in the synthesis of the novel glycolipid N-acetyl glucosamine-6-O-hexanoate. In addition, another novel glycolipid, N-butyryl glucosamine-6-O-hexanoate, was synthesized using the amino sugar N-butyryl glucosamine, so that a total of two novel glycolipids could be gained. For both syntheses it has been shown that one of the biggest obstacles of all enzymatic glycolipid syntheses, the low solubility of sugar in the organic solvents, can be circumvented by pre-dissolving the sugars in dimethyl sulfoxide (DMSO) before adding them to the reaction mixture. The better solubility of the sugars clearly improved the synthesis of glycolipids. Furthermore, it was possible to demonstrate the influence of the different hydrophobicities of each amino sugar on the synthesis, with the result that the increased hydrophobicity of N-butyryl glucosamine significantly promoted the synthesis by further enhancing the solubility of the sugars. In order to be able to compare these different experiments, analytical procedures including thin layer- liquid and gas chromatography had to be established. In addition, the surfactant properties of the two newly gained glycolipids were tested and their chemical structures were elucidated via nuclear magnetic resonance spectroscopy.

To characterize and to optimize the enzymatic synthesis of amino sugar fatty acid esters, further experiments were carried out to evaluate the impact of certain parameters on the synthesis, which are described in the third chapter of the results and discussion section. Among others, the substrate ratio between sugar and fatty acid ester, the water activity of the reaction medium representing the amount of unbound water present in the solvent and the reaction temperature are described as critical reaction parameters. Different substrate ratios might influence the synthesis, since high fatty acid concentrations can affect the solubility of the sugar negatively and might cause a substrate inhibition of the enzyme. The water activity of the reaction medium can be crucial for the reversal of the lipases hydrolytic activity, which is possible only at distinct low water activities. Too low water activities however might lead to a loss of enzyme activity. Higher reaction temperatures might promote the reaction up to a certain point; enzyme stability however might then be a concern. By performing experiments

with N-acetyl glucosamine and a total of three different fatty acid vinyl esters (vinyl hexanoate, vinyl laurate and vinyl palmitate), the influence on the corresponding glycolipid syntheses was shown for all named reaction parameters. Furthermore two additional glycolipids, N-acetyl glucosamine-6-O-laurate and N-acetyl glucosamine-6-O-palmitate, were synthesized. The best reaction conditions for these glycolipid syntheses could be found with a significant excess of fatty acid esters and the lowest possible water activity in the solvent present at the beginning of each reaction. Higher water activities led to significantly lower yields, since at these conditions increased hydrolytic activities were detected. The optimal reaction temperature was found to be 70°C. At this temperature, the synthetic activity of the lipase was highest while at the same time its hydrolytic activity was low. Further increase in temperature resulted in a shift in the enzymes activity, which in led to increased hydrolysis, resulting in distinct lower yields.

In the last chapter of the result and discussion part, the enzymatic synthesis of glycolipids in so-called deep eutectic solvents (DES) is described, mixtures consisting of two solids, which physically influence each other in a distinct way to form a liquid already at room temperature. By performing experiments in a total of six DES it could be shown that the lipase Novozyme 435[®] was able to synthesize glycolipids when employed in the DES consisting of cholin chloride and urea and cholin chloride and glucose. The resulting glycolipids were detected by ESI-Q-ToF, tandem mass spectrometry and NMR after extraction from the DES. Additionally it could be shown that in the DES consisting of choline chloride and glucose a glycolipid synthesis is possible without additional glucose, since the DES can function as solvent and substrate at the same time.

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I. THEORETICAL BACKGROUND

A. Surfactants

1. Introduction

Surfactants are amphiphilic molecules, consisting of a polar, hydrophilic and an unpolar, hydrophobic molecule moiety. The hydrophobic moiety is termed as “tail” of the molecule, whereas the hydrophilic moiety is called “head”. Figure 1 illustrates the schematic structure of a surfactant.

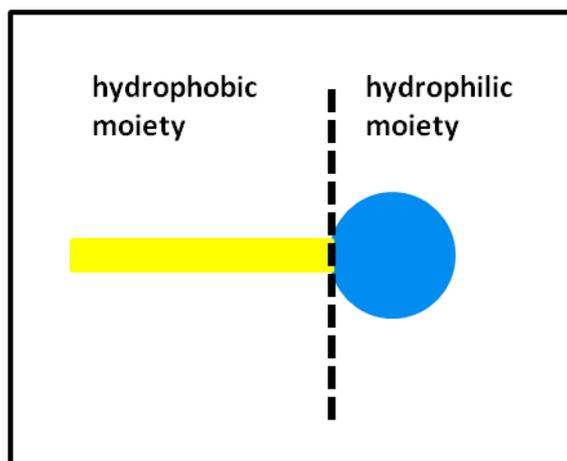


Figure 1: Schematic structure of a surfactant

Exemplary picture of an amphiphilic molecule consisting of a hydrophilic head and a hydrophobic tail.

The scheme depicted in figure 1 shows a simple surfactant molecule, consisting of one tail and one head group; likewise surfactants might consist of multiple head and tail moieties.

Whereas the unpolar moiety often consists of saturated or unsaturated alkyl side chains, the polar head is more versatile and can be non-ionic, anionic, cationic or amphoteric, dividing surfactants into different subclasses. Table 1 gives an overview of different surfactants subclasses, presenting examples of functional groups within the hydrophilic head of surfactants.

I. THEORETICAL BACKGROUND

Table 1: Different surfactant subclasses, distinguished by their hydrophilic head

Surfactant class	Schematic picture	Functional groups within the hydrophilic head
Non-ionic surfactants		alcohols (-OH) and ethers (-O-), carbohydrates
Anionic surfactants		carboxylates (-COO ⁻), sulfonates (-SO ₃ ⁻) and sulfates (-SO ₄ ²⁻)
Cationic surfactants		Quaternary ammonia groups (R ₄ N ⁺)
Amphoteric surfactants		e.g. carboxylates (-COO ⁻) and quaternary ammonia groups

As displayed, surfactants might appear in manifold combinations and shapes. In nature surfactants are versatile and ubiquitous. As main component of biological membranes phospholipids occur in every living creature, enabling the existence of cells, as well as the transport and exchange of materials [1].

In addition, surfactants find application in many products of our everyday life. Figure 2 shows some potential areas of application for surfactants.

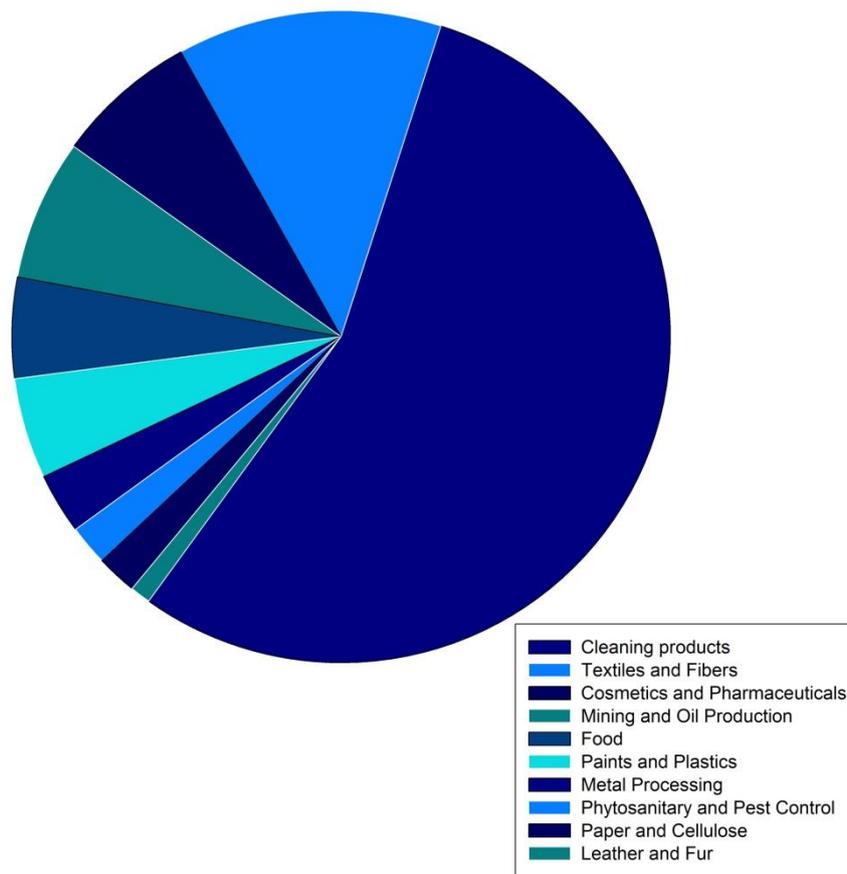


Figure 2: Areas of application for surfactants

Surfactants are mainly applied in cleaning products, but also find application in several other fields like cosmetics, pharmaceuticals and food. Modified from: http://www.basf.com/group/corporate/site-ludwigshafen/de_DE/about-basf/worldwide/europe/Ludwigshafen/Education/Lernen_mit_der_BASF/tenside/Anwendungsgebiete , 23.4.2014

Besides the use in cleaning formulations as main application, surfactants can be found in a wide range of products. While incorporated as emulsifiers in cosmetics, foods and pharmaceuticals, surfactants are as well employed during the production of textiles, paper and leather. Making surfactants valuable for these diverse applications are their unique physico-chemical properties.

2. Characterization of surfactants

Due to their structures, surfactants are (inter-)surface-active [2]. When dissolved in water, surfactants are located on the interface between water and air, as shown in figure 3.

I. THEORETICAL BACKGROUND

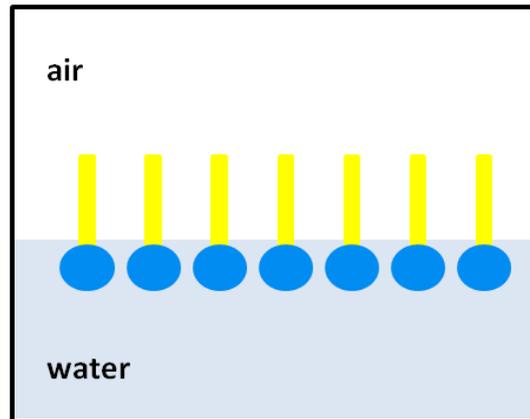


Figure 3: Schematic arrangement of surfactants on the surface between water and air

When covering a surface between water and air the hydrophilic head is located in the water; the hydrophobic tail is placed in contact with air.

Located at the liquid surface, amphiphiles reduce the surface tension of aqueous solutions. If, with increasing surfactant concentration, the surface area is covered with surfactant molecules, self-aggregation of the surfactant into micelles occurs, since it is thermodynamically favorable for the hydrophobic molecule moieties to avoid contact with the aqueous solution [3]. This aggregation takes place when the so called critical micelle concentration (CMC) of the surfactant is reached. At this concentration the surfactants take no further influence on the surface of the liquid; an increase in surfactant concentration now only leads to the formation of more micelles. Thus, the surface tension remains constant. Figure 4 schematically shows this relationship between surfactant concentration, surface tension and micelle formation.

I. THEORETICAL BACKGROUND

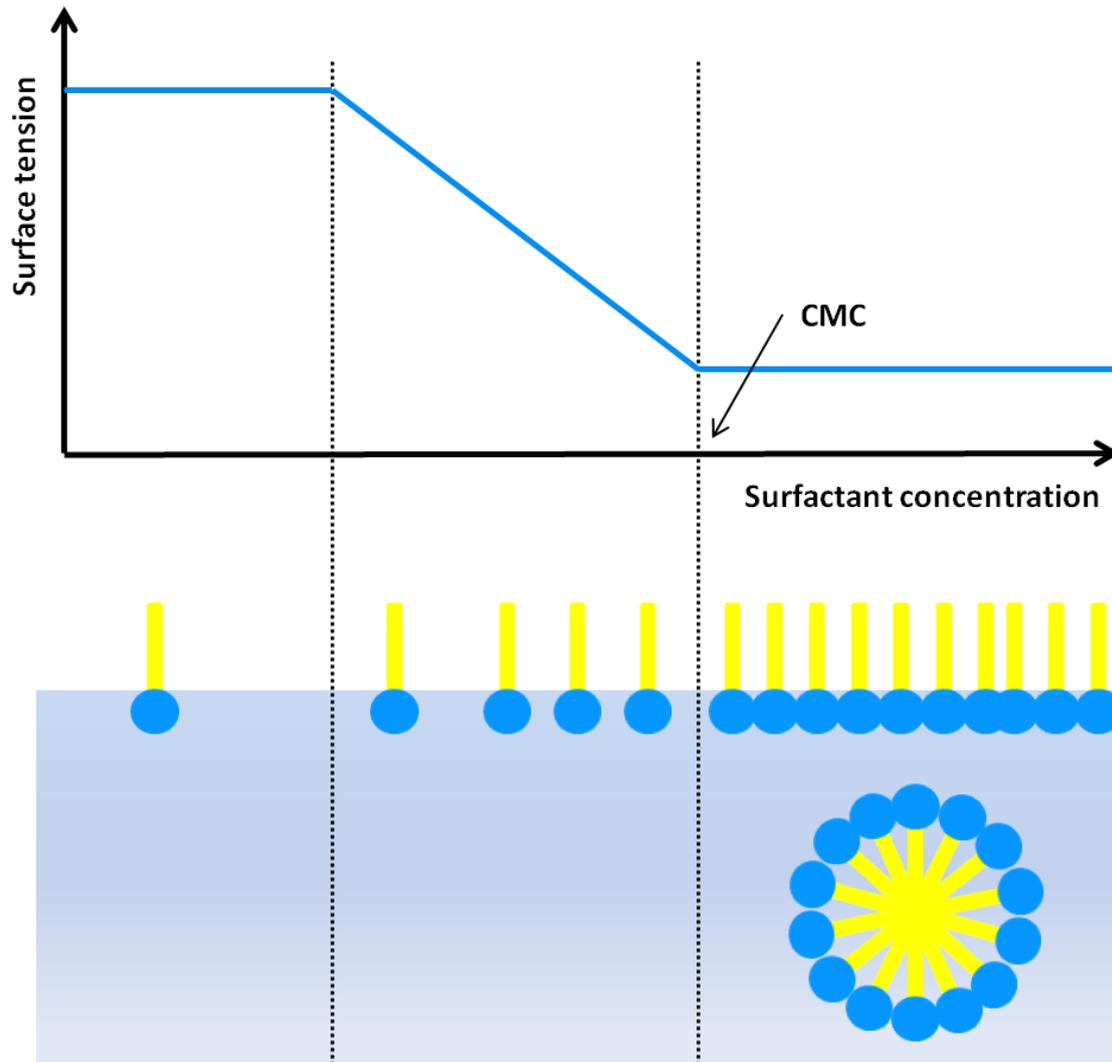


Figure 4: Correlation between the surface tension of water, surfactant concentration and micelle formation
With increasing surfactant concentrations the surface tension of water decreases until the critical micelle formation is reached. Beyond this concentration the surface tension remains constant and micelle formation is induced.

The critical micelle concentration exhibited by a surfactant depends not only on effects like temperature and electrolyte concentration of the liquid, but also heavily on the surfactant itself. Thus, surfactants can be characterized by their critical micelle concentration, which correlates with the shape of the surfactants head group and the chain length of its hydrophobic tail [4]. When forming micelles different surfactants might exhibit manifold micelle shapes, e.g. spherical or cylindrical. Forces that form and hold micelles together include hydrophobic interactions, Van-der-Waals forces, electrostatic interactions and hydrogen bridges [5].

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Their (inter-)surface active properties and the ability to form micelles enable surfactants to ease the mixing of non-miscible compounds. Thereby surfactants assist in cleaning processes (e.g. dissolving grease and fat stains in water) and the formation of emulsions (e.g. permanently dissolve oily phases in aqueous solutions).

Another way of characterizing surfactants can be accomplished by describing the molecules hydrophilic-lipophilic-balance (HLB). This value indicates the mass ratio between the molar mass of the non-polar part and the molar mass of the whole molecule. On a scale of 1 (lipophilic compound) to 20 (hydrophilic molecule), the surfactant is classified based on the molecular weights of the respective proportions using the formula:

$$HLB = 20 \times \left(1 - \frac{M_{lipo}}{M}\right)$$

with M_{lipo} being the molar mass of the lipophilic molecule moiety and M being the molar mass of the whole molecule.

Surfactants with a low HLB are described to provide good fat-dissolving properties, while amphiphiles with a high HLB exhibit good wetting of rather hydrophilic surfaces. A value between 3 and 8 is allocated to W/O emulsifiers, values between 8 and 18 to O/W emulsifiers. Typical detergents have HLB values of 13 to 15 and are rather hydrophilic and water-soluble [6]. A surfactants HLB value might as well determine which kind of micelle shape will be formed in aqueous solutions [4].

In addition, surfactants can be characterized by describing their potential to lower the interfacial tension between two liquids, their foaming behavior and their emulsification capacity, as well as through determination of their antibiotic properties [7]. Exhibiting manifold characteristics which make them interesting for numerous applications, surfactants naturally became a target for producing them via biotechnological routes.

3. Surfactants with biological origin

By today many surfactants are produced via chemical routes, which offer acceptable yields, thus allowing a cheap production in large scale. It however has to be considered that these chemical syntheses often rely on petroleum based substrates and result in the formation of a mixture of different products with vastly different, possible hazardous properties [8].

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When compared to the chemical production of surfactants, the synthesis of surfactants with biological origin, so called biosurfactants, bears several advantages and has gained more and more interest over the last two decades, as indicated by increasing numbers of studies aiming at their production. Biosurfactants are considered to show excellent physico-chemical and biological properties and hold several advantages over synthetic surfactants, like their low ecotoxicity [9]. Produced solely on biological routes, these surfactants are readily biodegradable, what makes them ecologically well accepted. Furthermore, biological synthesis routes can be based on the use of substrates gained from renewable resources, thus showing an alternative to petroleum-based surfactants.

The versatility of biosurfactants allows their application in many fields which employ synthetic surfactants thus far, as described in figure 2. Additional fields of application might include tertiary oil recovery, soil remediation and agricultural industry. Also the use as therapeutics can be considered, since biosurfactants often provide antibacterial, antifungal and antiviral or other beneficial properties [9] [10] .

Potential to further optimize the performance of biosurfactants lies in the fact that they can be tailored to the desired task, e.g. by producing them in enzyme-catalyzed reactions using chosen substrates, which results in the formation of specific products to meet the given requirements. Additionally, it is possible to modify already known biosurfactants enzymatically, in order to obtain novel structures with different properties, which might have the potential to alter and improve the physico-chemical characteristics of known biosurfactants to better fit the desired application [7] [11].

According to Lang and Trowitzsch-Kienast (2002) [12] surfactants with biological origin can be divided into different subclasses due to their structural differences. These subclasses are listed in table 2.

Table 2: Subclasses of surfactants with biological origin

Subclass	Example
Glycolipids	Rhamnolipids
	Sophorolipids
	Mannosylerythritol lipids
	Trehalose lipids
Lipopeptides, Lipoamino acids	Surfactin
	Ornithine lipids
Polymers	Proteins
	Lipoproteins (e.g., Liposan)
	Lipopolysachharides
Oil/membranes	Phospholipids
	Glycerolipids
	Fatty acids

Among these different subclasses, glycolipids are some of the best-studied group of surfactants.

B. Glycolipids

Glycolipids are surfactants characterized by having mono- di- or oligosaccharides as hydrophilic head moiety, while the hydrophobic tail can consist of one or more alkyl moieties with varying chain length. This variability leads to a great versatility of glycolipids, with different HLB values [13].

Many glycolipids are well-known and can be produced by various microorganisms. Named according to their saccharide moiety some of the best explored are rhamnolipids produced by *Pseudomonas aeruginosa*, sophorolipids by *Candida bombicola*, mannosylerythritol lipids by *Pseudozyma species* and trehalose lipids produced from *Rhodococcus species* [12].

Like other biosurfactants glycolipids exhibit several beneficial characteristics. Glycolipids are biodegradable under aerobic and anaerobic conditions and are non-toxic to the environment [14]. Being odor- and tasteless and non-irritant, they are very interesting for applications as emulsifier in food or cosmetic formulations [15] [16].

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Additionally, antimicrobial [17], antiviral [18], antifungal [19] and antiviral [20] properties of glycolipids, as well as their possible application as anti-tumor agent [21] are described in literature.

By providing selectivity and the possibility to work at mild reaction conditions, promising routes to synthesize glycolipids are enzyme-catalyzed coupling reactions, employing sugars and fatty acids or fatty alcohols. In order to give an introduction to this fundamental topic of this work, the next sections will give an introduction to enzymes and how they can be used to synthesize glycolipids.

C. Enzymes

1. Fundamentals

Enzymes are catalytic active proteins with the ability both to bind molecules and to effect reactions, thus being able to selectively and effectively catalyze a variety of biological reactions [22]. Functioning inside and outside of living cells, enzymes not only enable life itself, additionally their excellent properties are utilized in many of today's technologies [23].

Enzymes consist of a distinct sequential arrangement of amino acids ordered into special orientations. Due to this conformation enzymes only accept certain substrates, which are processed in a specified manner. Thus, when catalyzing reactions, enzymes can be characterized by their three dimensions of merit: activity, selectivity and stability. While activity (e.g. described by the enzymes specific activity or turnover frequency) and selectivity (e.g. described by the resulting enantiomeric excess of the reaction) evaluate the catalyzed reaction, stability (e.g. described by the enzymes melting point) assesses more the catalyst itself [22]. The enzymes provided chemo-, regio- and stereo selectivity without any need for high energy input clearly distinguish an enzymatic from a chemical process.

Classified regarding their different catalyzed reactions, enzymes are divided into six classes. For the synthesis of glycolipids experiments focused on employing enzymes belonging to the subgroup of hydrolases.

2. Hydrolases

Hydrolases (E.C. 3.x.x.x.) are enzymes that act on chemical bonds by using water as co-substrate. The most prominent reactions performed by hydrolases are the cleaving of a chemical bond by the addition of water, as shown in figure 5.

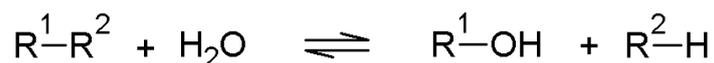


Figure 5: Reaction scheme of a hydrolysis

Hydrolysis occurs when a chemical bond is cleaved by the addition of water. During the reversed reaction (e.g. esterification) water is formed as side product.

Different hydrolases can act on many different types of bonds. Esterases (EC 3.1.x.x.) for example cleave esters, resulting in the formation of an alcohol and a carboxylate. Glycosidases (EC 3.2.x.x.) act on glycosidic linkages and peptidases (EC 3.4.x.x.) mainly hydrolyze peptide bonds.

The property making these enzymes valuable for the synthesis of glycolipids is their ability to reverse their natural hydrolytic activity under certain circumstances. When employed in almost water-free, so called anhydrous, organic solvents, few hydrolases are able to synthesize chemical bonds. The first enzymes with described synthetic activity in organic solvents were lipases [24].

3. Lipases

Lipases (EC 3.1.1.3.) are hydrolases which act on ester bonds, e.g. by hydrolyzing triglycerides, a common way of energy uptake, which is employed by almost every organism. Thus, lipases can be found as cellular or extracellular enzymes in all forms of life [25].

Lipases show a characteristic α/β hydrolase fold, consisting of six alpha helices and eight beta-sheets. Belonging to the group of serine hydrolases, the catalytic active region mainly consists of the so called *catalytic triad* of serine, histidine and aspartic acid.

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Many lipases exhibit surface or interfacial activation, a property which is provided by a structural feature called *lid*. When present in an enzyme, this *lid* covers the active center, shielding it from polar surroundings like aqueous solutions. Upon contact to unpolar surfaces, the enzymes conformation is changed, with the *lid* opening up and exposing the catalytic triad, thus enabling the conversion of the substrate.

The mechanism of each conversion is composed of four steps [23]:

- 1) The substrate reacts with the active site of the serine, forming a tetrahedral intermediate
- 2) Release of the alcohol, formation of an enzyme-acyl complex
- 3) Nucleophilic attack (from water in hydrolysis, from alcohol in (trans-)esterification) to form another tetrahedral intermediate
- 4) Collapse of the second intermediate, release of the product (acid or ester) and free enzyme

This reaction mechanism can be described as ping-pong-bi-bi-mechanism, following the Michaelis-Menten kinetics [26] [27].

Other structural characteristics of lipases are the so called *nucleophilic elbow*, a preserved loop region within the enzyme, containing the serine of the catalytic triad; as well as the so called *oxanion hole*, an element consisting of two amino acids, which stabilize the tetrahedral intermediate during the conversion.

Lipases are distinguished from other esterases by their surface activation and their broader substrate spectrum, which also includes water-insoluble, long chain fatty acids [23].

Lipases are widely used in many industrial processes today like the synthesis of biodiesel [28] and monoacylglycerols [29] or the racemic resolution of different molecules, e.g. chiral esters and amines [30]. Oftentimes lipases are employed in organic transformations, because they are stable even under harsh, water free conditions, while still providing excellent selectivity. These characteristics, together with the facts that lipases are not co-factor dependent, is what makes them attractive for many syntheses of pharmaceutical products [31].

Since the late 1980s it is known that lipases are able to perform hydrolysis in aqueous solutions, as well as reverse hydrolysis by catalyzing esterifications, alcohol- and acidolyses and transesterifications in water-free reaction media [24] [32]. Thus, lipases have been widely used to form ester bonds. Utilizing their broad substrate spectrum, various lipases have been used in organic solvents to synthesize different glycolipids [33] [34].

D. Enzymatic synthesis of glycolipids

Numerous experiments have been performed to synthesize different glycolipids via various enzymatic routes. Substrates were amongst others monosaccharides like glucose [33], mannose [35] or galactose [36], di- and trisaccharides like saccharose [37], fructose [38], lactose [39] or maltose [40] and oligo- and polysaccharides like starch, cellulose and pectin [41]. These carbohydrates have been acylated using different fatty acids or other fatty acid derivatives with varying chain length [37] [42]. Synthesizing glycolipids via enzymatic routes provides several advantages, like the fact that define structures can be obtained [39] and that the reaction can be carried out in less hazardous solvents than compared to chemical esterification with alkaline catalysts [43].

When enzymatically synthesizing glycolipids in organic solvents, several parameters influencing the reaction have to be considered. While most reaction conditions will not only affect the synthesis itself, but also interact with each other, optimal synthesis conditions might vary for each synthesis. The following section will list and discuss the most important reaction parameter influencing the enzymatic synthesis of glycolipids.

1. Substrates

The first crucial step for each glycolipid synthesis is the selection of the sugars and fatty acids or fatty alcohols, of which the final product will consist of. Theoretically every combination of carbohydrate and fatty acid should be possible, so that considerations regarding the use of cheap and renewable resources could be done. Additionally different characteristics of the product, like the HLB value, can be influenced by choosing different substrates.

a) Sugars

Several sugars have already been used to synthesize glycolipids, as listed above. A common problem of all sugars is their low solubility in organic solvents, which is a big obstacle for the synthesis of glycolipids. To handle this issue several attempts have been made, following different strategies, all with the common goal to

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improve the synthesis by enhancing the solubility of the sugars. Increasing the sugars hydrophobicity by methylation and acetylation led to promising results [39] [44], but also had a direct impact on the product properties or required additional purification steps. Employing additional solvents in which the sugars are soluble, e.g. dimethyl sulfoxide (DMSO), might increase the availability of the sugar for the enzyme, but might as well interfere with the enzymes activity and stability [45] [46].

b) Fatty acids

When selecting a fatty acid for an enzymatic synthesis mainly two characteristics have to be considered. Firstly the fatty acid chain length and secondly the use of fatty acid esters.

The chain lengths of the fatty acid will not only have a major impact on almost all product characteristics like the HLB; it will also influence several reaction parameters, like the reaction rate and the overall yield of the synthesis. Fatty acids with less than 8 C-atoms result in much higher starting reaction rates than those with more than 12 C-atoms [37]. On the other side it was shown that the overall yield in reaction with long chain fatty acids can be higher than compared to using short chain fatty acids [33]. Reasons for the different reaction kinetics appearing when using different the fatty acid chain length might be the differential behavior of these fatty acids upon entering and binding to the active center of the enzyme [47].

Fatty acid esters, like fatty acid methyl- ethyl- or vinyl esters provide several benefits to the reaction, e.g. leading to a transesterification reaction instead of an esterification. The benefit thereof is that water is no side product of the reaction, thus possibly preventing subsequent hydrolysis of the glycolipids. Vinyl esters of fatty acids additionally have the advantage that the generated vinyl alcohol will tautomerize into acetaldehyde, which evaporates at 20°C [48]. It has however to be considered that many side products from activated fatty acids, like methanol or acetaldehyde, can lower the reaction rate by inhibiting or denaturing the enzyme [49] [34].

2. Enzymes

Different hydrolases have been successfully used to synthesize glycolipids by reversing their hydrolytic activity. Esterases (EC 3.1.x.x.) and especially lipases (EC 3.1.1.x.) are frequently used for enzymatic syntheses of esters in organic solvents. Numerous lipases are commercially available, whether in solid, liquid or immobilized formulations. Often immobilized lipases like *Candida antarctica* lipase B immobilized on acrylic resins (Novozyme 435[®]) or the lipase from *Mucor miehei* immobilized on macroporous ion-exchange resin (Lipozyme[®]) are employed for reactions in organic solvents due to their enhanced stability. By performing esterifications or transesterification, the synthesis using lipases leads to glycolipids having an ester bond.

Proteases (EC 3.4.x.x.), which quite often also show esterase activity, have been used to synthesize carbohydrate fatty acid esters as well. Good results were achieved with the protease from *Bacillus licheniformis* (Subtilisin[®]) [50]. To synthesize glycolipids containing a glycosidic linkage instead of an ester bond glucosidases (E.C. 3.2.x.x.) can be used, employing a sugar together with a fatty alcohol [51]. Compared to carbohydrate fatty acid esters, these glycolipids have the advantage that they are very stable under alkaline conditions.

In contrast to lipases, proteases, glycosidases and esterases show a strong preference towards short chain fatty acids, making lipases the better choice when employing long chain fatty acids [46]. Thus, the choice of enzyme and enzyme formulation strongly depends on the desired reaction, reaction conditions and products.

3. Reaction media

a) Enzymatic reactions in organic solvents

The selection of the reaction media is a crucial step, since the chosen solvent has a major impact not only on the enzymes activity and stability, but also on the solubility of substrates. Additionally aspects like the water content of the solvent, its boiling point and possible downstream process have to be considered as well.

Organic solvents can be characterized by their polarity, oftentimes described in the solvents logP value. This value describes the solvents distribution coefficient between a polar (e.g. water) and an unpolar phase (e.g.

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octanol), thereby distinguishing unpolar solvents with positive logP values from polar solvent, having negative logP values.

By interfering with it, a solvent can basically influence every property of an enzyme. Unpolar solvents with high logP values do not interfere with the hydration state of the enzyme much, so that enzyme stability in these solvents is reported the highest and is even higher than in aqueous solutions [52]. For synthesizing glycolipids however, unpolar reaction media have the drawback that sugars are only poorly dissolvable in them. More polar solvents might be able to better dissolve sugars, but exhibit a much higher influence on the enzyme by detracting water from it [53], what leads to changes in the enzymes conformation and activity [54]. Additionally it was shown that not the solvents polarity alone, but also in combination with the solvents water content and w3ater activity, exhibits a major impact in the enzymes activity [55]. The presence of water or other polar solvents thereby might interfere with charged intermediates during the enzymatic conversion [56]. Similar, when using lipases, other enzyme features essential to the conversion, e.g. the *oxanion hole*, might be affected. In addition, the substrate recognition and binding of lipases, which oftentimes is due to hydrophobic interactions between the enzyme and the substrate, could be effected by the solvent [57].

Furthermore it is known that besides the solvents polarity, also its dielectric constant, describing the solvent relative permittivity, influences the enantioselectivity of enzymes, which decreases with increasing dielectric constants [58]. The solvents influences on the substrate specificity and the selectivity of the used enzymes are described in several works [26] [59] [60].

For the enzymatic synthesis of glycolipids several similar influences are known. When synthesizing e.g. xylitol oleic acid esters, using different solvents and solvent mixtures not only led to changes in enzyme activity and overall yield, but also to the formation of different products with varying degrees of acylation [61].

Many enzymatic syntheses of glycolipids were performed in organic solvents like pyridine, dimethyl formamide or dimethylpyrrolidone, because solubility of the carbohydrates was granted in these solvents [62] [50]. Also solvents with different logP values ranging from very hydrophobic solvents like hexane [15] to rather hydrophilic solvents like acetone or acetonitrile [63] have been tested as well. Numerous experiments have been carried out employing tertiary alcohols like 2-methyl-2-buthanol (2m2b) and tert-butanol or similar solvents like methyl tert-butyl-ether [64] [14]. These tertiary alcohols provide high enzyme stability and activity and are, due to their sterically hindered formation, no substrate to the lipase. Their occurrence in many natural formulations is also beneficial, allowing their use for the production of food additives as well.

b) Solvent-free reaction systems

Several attempts have been made to replace organic solvents or to avoid their use at all. In solvent-free reaction systems sugars are directly dissolved in the fatty acid and only few volumes of solvents, as so called adjuvants, are added [43]. This has the advantage that the use of hazardous solvents can be avoided and that the product often precipitates from the reaction media. As adjuvants acetone and tert-butanol showed good results [43].

c) Reactions in ionic liquids & deep eutectic solvents

Another alternative to the use of organic solvents might be the use of ionic liquids or deep eutectic solvents (DES). Recent experiments showed that lipases remain active when employed in ionic liquids like [Bmim][Tfo] [65] or [Bmim][PF₆] [66] and deep eutectic solvents like choline chloride and urea [67].

While the use of ionic liquids bears several disadvantages like high costs, uncertain toxicity and poor biodegradability of the solvent, DES are not limited by these factors [68]. DES typically consist of a mixture of an ammonium salt and a hydrogen-bond donor. Thereby the ammonium anion interacts with the hydrogen-bond donor, leading to a significant depression of the melting point of the mixture, resulting in the formation of a liquid [69] [70].

These solvents promise acceptable enzyme activity and stability and additionally can be tailored for the desired reaction, constituting a novel production route for enzymatic synthesis. A problem however might occur when extracting glycolipids from ionic liquids or DES, which often requires complex purification steps, what might complicate analytic procedures and downstream processing.

4. Various reaction parameters

a) *Reaction temperatures*

Although being stable in organic solvents already, enzymes and especially lipases may be employed in immobilized form when used in organic solvents to further enhance their thermo-stability. Commercially available immobilized lipases are very thermo stable and can be used at temperatures ranging from 20°C up to 60°C. Novozyme 435[®] might also be used at 80°C without a significant loss in activity [34]. Thus, a wide range of temperatures might be tested to find optimal conditions for the projected glycolipid synthesis. It however has to be considered that a change in temperature might have different effects on the enzymatic conversion. Higher temperatures might increase the reaction rate of the enzyme and the solubility of the carbohydrates, but at the same time there could be changes in the reaction equilibrium and in the stability and selectivity of the enzyme, leading to different glycolipids [71]. Additionally aspects like enzyme denaturation, the boiling point of the solvent employed and decomposition of the glycolipids have to be considered at temperatures higher than 80°C.

b) *Water activity*

Despite being called anhydrous solvents, many organic solvents still contain small amounts of bound and unbound water, which are essential to keep up the enzymes three-dimensional conformation and flexibility [72]. Thus the water content in an organic solvent, or more important its water activity representing the amount of unbound water present in the solvent is a very crucial parameter influencing the enzymes activity. In order to reverse the hydrolytic activity of hydrolases the solvent should exhibit low water activities to prevent hydrolysis, whereas at the same time the water layer bound to the enzyme, which is essential to keep the enzyme in an active state, should not be affected by the solvent [73] [74]. Hence only suitable combinations of enzymes and organic solvents with distinct water activities enable enzymatic syntheses in organic solvents.

When synthesizing fructose monopalmitate using Novozyme 435[®] in 2m2b it was shown that water activities lower 0.2 resulted in the highest reaction rates [75]. Furthermore the water activity also influenced the yield of these syntheses, as well as the stability and the selectivity of the lipase employed in these experiments.

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Additionally the water activity is known to have an impact on the degree of acylation, e.g. when synthesizing fructose oleic acid esters in acetone [71].

Several approaches have been tested to reduce the water content and water activity of the reaction mixture before and during the reaction. Molecular sieves (pore size: 4Å) remove water from the reaction media by adsorbing it, thus keeping the water activity low throughout the reaction. In addition, the water activity of solvents can be pre-adjusted to a desired value by incubating the solvent together with different saturated salt solutions. Another strategy could be to work *in vacuo* or to remove the unwanted side product water by azeotropic distillation.

To avoid the formation of water during the reaction a transesterification could be pursued instead of a reversed hydrolysis, using fatty acid esters instead of free fatty acids. If however the water activity in the solvent is too high, the synthesized glycolipids will likely be hydrolyzed by the employed hydrolase, no matter what fatty acid formulation is used.

c) Ratio of substrates

The molar ration between sugar and fatty acid is another parameter influencing the enzymatic synthesis of glycolipids. Using fatty acids in excess might influence the reaction rate and the yield of the experiment, since free fatty acids can have an inhibiting effect on the enzyme [76]. Additionally the substrate ratio might have an impact on the product regarding its degree of acylation, possibly leading to polyacylated glycolipids at high fatty acid : sugar ratios [42].

Predictions on what ratio might lead to the highest yield are ambiguous, since the outcome is described differently in literature. Tarahomjoo et al. (2003) [15] claimed the reaction could be optimized by setting the glucose : palmitic acid ratio 1 : 1, whereas Yan et al. (1999) [77] found that the glucose : stearic acid ration of 1 : 3 worked best for them. Sakaki et al. (2006) [78] state in their work that a glucose : palmitic acid ration of 1 : 5 gave best results. While Coulon et al. (1996) described the most suitable ratio for the synthesis of fructose oleate as 1 : 5, successful syntheses at even higher excesses are described within this work as well [34]. In addition, it has to be considered that the best substrate ratio is also depended on the fatty acid chain [79]. So when synthesizing novel glycolipids several variations of all parameters described before have to be considered, which at the same time offers potential to optimize every synthesis.

II. RESEARCH PROPOSAL

The enzymatic synthesis of sugar fatty acid esters is a well-studied way to obtain glycolipids, known for almost exactly 30 years by now. With theoretical any given combination of sugar and fatty acid possible, countless novel glycolipids are still to be synthesized. Thus, besides relying on a broad fundament of experience considerations regarding the ecology of the synthesis and the use of renewable resources can be done when synthesizing novel glycolipids.

The first and most important objective of this thesis was to selectively produce novel glycolipids, using substrates originating from renewable resources. Therefore unusual sugars have to be selected, which lead to the formation of new surfactants. To establish such new syntheses, suitable reaction setups have to be tested and validated.

In order to characterize and optimize the synthesis of these new glycolipids, the influence of critical reaction parameters like fatty acid chain length, initial water activity of the organic solvent, substrate ratio and reaction temperature should be investigated, thereby promoting the synthesis, regarding aspects like enzyme activity and overall yield.

To perform such optimizations adequate analytical methods have to be established, ideally being able to not only measure the glycolipid formation, but also elucidating their chemical structure. Being able to measure the contents of sugars and fatty acids, classical chromatographic procedures like high performance liquid – and gas chromatography would be desired. Additionally, a small-scale assay facilitating the choice of enzyme and solvent employed for the synthesis should be developed.

To characterize the novel gained glycolipids, suitable purification steps should be selected, thus, being able to obtain pure products. Besides describing the synthesis and chemical structures of the novel amino sugar fatty acid esters, their specific properties as surfactants should be exploited, e.g. by determination of their critical micelle concentration, emulsification capacity or possible antibiotic properties.

III. PUBLICATIONS & MANUSCRIPTS

A. Development of a microtiter plate-based assay for the detection of synthetic lipase activity in organic solvents

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III. PUBLICATIONS & MANUSCRIPTS

This manuscript is prepared for publication and will be submitted for publication to “Biotechnology Letters”.

Author’s contribution to this manuscript:

Martin Pöhnlein designed this study, developed the methodology, collected and evaluated the data and wrote the manuscript.

Tim Finkbeiner assisted in performing experiments as student worker supervised by Martin Pöhnlein.

Professor Christoph Syldatk supervised the project and proofread the manuscript.

Professor Rudolf Hausmann supervised the project and proofread the manuscript.

1. Abstract

A microtiter plate-based assay was developed to evaluate the ability of lipases to synthesize ester bonds when employed in organic solvents. Therefore a 4-nitrophenol assay was carried out employing seven different lipase formulations and two fatty acid methyl esters with different chain lengths in a total of six organic solvents. This assay delivered results within comparatively short times measured by a color reaction.

By monitoring the synthetic activity of lipases this assay might facilitate the choice of an enzyme – solvent combination for the synthesis of glycolipids. Thus, in order to validate the findings gained from the assay, glycolipid syntheses have been performed using the same lipase formulation in the same solvents. When comparing the results obtained with the assay to the results of the glycolipid syntheses using the same lipases and solvents, matching results could be obtained. These findings demonstrated the ability of the new assay to detect solvent-enzyme combinations suitable for glycolipid syntheses. It was shown that the immobilized lipases Novozyme 435[®] and Lipozyme[®] employed in 2-methyl-2-butanol or tert-butanol delivered the best results of all combinations tested.

2. Introduction

Lipases (E.C. 3.1.1.3, triacylglycerol acyl hydrolases) are hydrolases that act on ester bonds between a fatty acid and an alcohol. They are widely used in many processes today like the synthesis of biodiesel [28] and monoacylglycerols [29] or the racemic resolution of different molecules [30]. Since the late 1980s lipases have gained interest due to their stability and reverse hydrolytic activity in water-free reaction media [80]. Thus, lipases have been widely used to synthesize ester bonds, for example for the enzymatic synthesis of glycolipids, where different lipases in varying formulations have been used in several organic solvents [34] [64]. When performing enzymatic glycolipid syntheses, the choice of solvent and catalyst is a crucial parameter.

To evaluate the activity of lipases, several screening assays have been proposed [81] [82] and have been carried out in aqueous solutions mainly focusing on the enzymes hydrolytic activity, which however is not always correlated to its synthetic activity [83]. Thus, to test lipase activity in organic solvents, several assays have been developed or existing assays have been refined and modified to determine the lipases synthetic activities as well [84] [85]. Many assays however are carried out in unpolar solvents like hexane or heptane [86] [87]. Although providing enzyme stability these solvents are not suitable to monitor reactions like glycolipid syntheses, since sugars do not dissolve in them. To circumvent this problem a 4-nitrophenol (para-nitrophenol, pNP) assay has been developed to determine the synthetic activity of different lipases in organic solvents with a lower logP value ranging from approximately 1 to -1, which is described within this work.

3. Materials and Methods

a) Materials

4-nitrophenol, lipase B from *Candida antarctica*, immobilized on acrylic resin (Novozyme 435[®]), lipase from *Rhizomucor miehei* immobilized on macroporous ion-exchange resin (Lipozyme[®]), lipase from *Candida rugosa*, lipase from *Rhizomucor miehei* and lipase from *Thermomyces lanuginosus* were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Glucose monohydrate, Triton X 100 and Tris were purchased from Carl Roth (Carl Roth KG, Karlsruhe, Germany). Lipase A (CalA) and lipase B (CalB) from *Candida antarctica* (both lyophilized) were gifts from cLEcta (cLEcta GmbH, Leipzig, Germany). Methyl n-octanoate and methyl n-palmitate were purchased from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium). The solvents acetone, 2-methyl-2-butanol (2m2b), tert-butanol (t-butanol), 2-propanol, methyl tert-butyl ether (MTBE) and acetonitrile (ACN) were purchased from Sigma Aldrich in highest purity available and used without further treatment.

b) Methods

(1) Synthesis of 4-nitrophenol esters in organic solvents

10mM 4-nitrophenol (pNP) solutions were prepared in all used solvents listed in table 3.

Table 3: Solvents employed for the synthesis of 4-nitrophenol esters

Name	logP value	Chemical formula
MTBE	1.18	$\text{H}_3\text{C}-\text{C}(\text{CH}_3)(\text{CH}_3)-\text{O}-\text{CH}_3$
2m2b	1.06	$\text{H}_3\text{C}-\text{CH}_2-\text{C}(\text{CH}_3)_2-\text{OH}$
tert-butanol	0.54	$\text{H}_3\text{C}-\text{C}(\text{CH}_3)_2-\text{OH}$
2-propanol	0.25	$\text{H}_3\text{C}-\text{CHOH}-\text{CH}_3$
acetone	0.11	$\text{H}_3\text{C}-\text{CO}-\text{CH}_3$
acetonitrile	-0.17	$\text{H}_3\text{C}-\text{CN}$

Since tert-butanol tends to appear in solid state at room temperature all pNP solutions were preheated to 40°C prior to the reaction. After addition of 10 mg or 10 µL of the enzyme formulation to be tested the reaction was started by adding 60 mM methyl octanoate or methyl palmitate respectively. The reaction took place in 2 mL reaction vessels under constant shaking at 1200 rpm and 40°C with a total reaction volume of 1 mL. Samples were withdrawn after 0, 10, 20 and 30 minutes to follow the exemplary projected transesterification in figure 6.

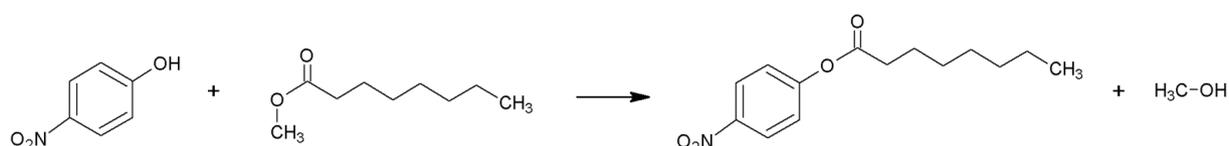


Figure 6: Schematic transesterification between 4-nitrophenol and methyl octanoate

During the synthesis of 4-nitrophenol esters using 4-nitrophenol and methyl octanoate a transesterification occurs, resulting in the formation of the 4-nitrophenol octanoate and methanol. When employing methyl palmitate the transesterification would result in 4-nitrophenol palmitate and methanol.

By following the depletion of pNP it was thus possible to determine and to compare the initial transesterification activity of the employed enzyme. This reaction was carried out with a total of seven different lipases, which are listed in table 4.

Table 4: Lipases employed for the synthesis of 4-nitrophenol esters

Name	Formulation	Description
Novozyme 435®	Immobilized	Lipase B from <i>Candida antarctica</i> , immobilized on acrylic resin
Lipozyme®	Immobilized	Lipase from <i>Rhizomucor miehei</i> , immobilized on macroporous ion-exchange resin
CalA	lyophilized	Lipase A from <i>Candida antarctica</i>
CalB	lyophilized	Lipase B from <i>Candida antarctica</i>
Lipase <i>T. lanuginosus</i>	liquid	Lipase from <i>Thermomyces lanuginosus</i>
Lipase <i>R. miehei</i>	liquid	Lipase from <i>Rhizomucor miehei</i>
Lipase <i>C. rugosa</i>	lyophilized	Lipase from <i>Candida rugosa</i>

When using the immobilized lipases Novozyme 435® and Lipozyme® the enzyme was allowed to settle before taking a 5 µL sample, which was directly mixed with 250 µL tris buffer solution (50 mM tris buffer (pH 7.0) + 0,1% Triton X, by vol.) in a microtiter plate. To extract the pNP, the microtiter plate was shaken for ten seconds on a thermo mixer at 1200 rpm, followed by the measurement of absorption at 412 nm in a microtiter plate reader. At 412 nm only the substrate pNP exhibits an absorption maximum, while the resulting pNP esters show

no absorption at this wave length. Thus it is possible to monitor this reaction by following the decrease of absorption and subsequently calculating the enzymes synthetic activity.

Experiments using lipases in liquid or powdered formulations were treated as follows to avoid the transfer of enzymes to the aqueous phase. After 0, 10, 20 and 30 minutes 100 μ L sample were withdrawn to a 1.5 mL reaction vessel which was then centrifuged at 13000 rpm for 1 minute to precipitate free enzymes. 5 μ L of the clear supernatant was then mixed with the Tris buffer solution and treated as described before. All experiments, including blanks without fatty acids or without enzymes, were carried out as triplets.

(2) *Enzymatic synthesis of glucose esters*

18 mg (10 mM) of glucose were mixed with the corresponding solvent together with 100 mg or 100 μ L of lipase formulation. To start the reaction 60 mM methyl octanoate (94 μ L) or methyl palmitate (162 μ L) were added, to a total volume of 10 mL. The reaction was incubated for 48 hours at 40 °C and 300 rpm in a shaking water bath. All experiments were carried out as triplicates. Samples from each reaction were drawn at timed intervals to follow the proposed glycolipid synthesis shown in figure 7.



Figure 7: Schematic reaction for the synthesis of glucose ester

When using glucose and methyl octanoate for the lipase-catalyzed synthesis of glucose esters, a transesterification occurs, resulting in glucose octanoate, here exemplarily depicted as glucose-6-O-hexanoate and methanol. When using methyl palmitate, glucose palmitate and methanol are formed.

(3) *Detection of glycolipids via thin layer chromatography*

Thin layer chromatography (TLC) was performed for qualitative analysis of the projected glycolipid synthesis. Therefore 10 μ l samples were applied onto an analytical silica gel 60 TLC plate (10 x 20 cm, film thickness 0.25 mm). The plates were developed in a mobile phase consisting of chloroform : methanol : acetic acid (65 : 15 : 2, by vol.). Visualization was accomplished by dipping the plate in an anis aldehyde solution (anis aldehyde : sulfuric acid : acetic acid, 0,5 : 1 : 100, by vol.) followed by heating at 200°C under constant air flow for about 5 min. Glucose octanoate typically has a retention factor (Rf) of approximately 0.43, while glucose palmitate yields Rf values of approximately 0.56.

4. Results and Discussion

a) *Synthesis of 4-nitrophenol esters in organic solvents*

The synthetic activity of each lipase towards the formation of an ester bond was determined by following the occurring color reaction. All experiments were carried out with methyl octanoate and methyl palmitate, which results will be discussed separately in this chapter.

(1) *4-nitrophenol ester synthesis using methyl octanoate*

To compare the synthetic activities of the different lipases in all solvents, the results obtained using pNP and methyl octanoate are shown in table 5.

Table 5: Results of the lipase-catalyzed synthesis of 4-nitrophenol esters using methyl octanoate

Lipase activity [$\mu\text{mol pNP /min}$]	MTBE	2m2b	t-butanol	2-propanol	acetone	ACN
Novozyme 435[®]	-	51.2 \pm 33.3	93.6 \pm 8.1	9.9 \pm 5.3	27.6 \pm 12.1	112.5 \pm 21.2
Lipozyme[®]	-	141.4 \pm 31.6	51.1 \pm 23.3	-	52.9 \pm 22.1	31.8 \pm 31.1
Cal A	-	64.2 \pm 10.3	-	11.5 \pm 0	-	-
Cal B	-	27.0 \pm 8.4	-	-	-	-
Lipase <i>T. lanuginosus</i>	-	58.4 \pm 18.1	5.8 \pm 0.4	-	-	-
Lipase <i>R. miehei</i>	-	-	54.2 \pm 46.5	-	-	16.7 \pm 6.2
Lipase <i>C. rugosa</i>	-	25.9 \pm 22.7	-	-	-	-

In 40% (17 out of 42) of all possible combinations of lipase and solvent a synthetic activity of the lipase could be deduced. Especially the two immobilized lipases Novozyme 435[®] and Lipozyme[®] showed activities in most of the solvents tested. Novozyme 435[®] for example exhibited synthetic activity in all tested solvents except for MTBE, Lipozyme[®] in all solvents, except MTBE and 2-propanol. For all other lipases synthetic activities could only be measured in a maximum of two solvents. Possible explanations for the lack of activity of the lipases in certain solvents might be the polarity of the given solvent, what might lead to the disruption of the water layer

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around the enzyme, essential for its flexibility and activity [53]. Thus, lipases like Novozyme[®] and Lipozyme[®] might show higher activities, since they are more stable in polar solvent due to their immobilization. But also other factors like the initial water content might influence the enzymes synthetic activity.

The most unpolar solvent tested was MTBE, in which no activity could be observed for any lipases. 2m2b and t-butanol are less unpolar and seem to provide a good combination of substrate solubility, enzyme stability and activity, thus showing synthetic activities for most of the lipases employed. In the more polar solvents 2-propanol, acetone and acetonitrile the immobilized lipases Novozyme 435[®] and Lipozyme[®] could synthesize ester bonds between pNP and the fatty acid, but mostly with moderate activity. Only the combination of Novozyme 435[®] in acetonitrile presented a distinct exception, showing a very high synthetic activity. These observations confirmed the assumption that more polar solvents reduce the stability of enzymes, which can be prevented by immobilization.

Summed up, the highest activities were measured when using Novozyme 435[®] in acetonitrile or t-butanol and Lipozyme[®] in 2m2b, making these combinations interesting for an enzymatic glycolipid synthesis using fatty acid esters with medium chain length.

(2) *4-nitrophenol ester synthesis using methyl palmitate*

In order to investigate the influence of the fatty acid chain length and to find suitable enzyme-solvent combinations for transesterification reactions using long chain fatty acid esters, experiments, analog to the ones with methyl octanoate, were carried out employing methyl palmitate. The results of these experiments are shown in table 6.

Table 6: Results of the lipase-catalyzed synthesis of 4-nitrophenol esters using methyl palmitate

Lipase activity [$\mu\text{mol pNP /min}$]						
	MTBE	2m2b	t-butanol	2-propanol	acetone	ACN
Novozyme 435[®]	84.9 \pm 24.4	76.2 \pm 28.5	48.5 \pm 6.5	-	-	-
Lipozyme[®]	41.9 \pm 7.2	75.8 \pm 27	32.1 \pm 2.3	-	-	-
CalA	-	72.1 \pm 20.1	8.8 \pm 0	5.1 \pm 0	-	49.1 \pm 4
CalB	-	-	-	-	-	-
Lipase <i>T. lanuginosus</i>	-	8.6 \pm 2.5	-	-	-	35.9 \pm 0.4
Lipase <i>R. miehei</i>	59.8 \pm 13.7	33.6 \pm 17.9	7 \pm 6.3	3.7 \pm 0	-	64.3 \pm 10.5
Lipase <i>C. rugosa</i>	-	11 \pm 12.3	-	-	-	-

The results from the experiments with methyl palmitate clearly differed from these using methyl octanoate. In contrast to the former described experiments, synthetic activities of these lipases were observed in MTBE when employing the longer chained methyl palmitate. A possible explanation might be the better solubility of the more hydrophobic fatty acid in this rather unpolar solvent, which favors its transesterification. Similar to the experiments with methyl octanoate, synthetic activities could be proven for various lipases in 2m2b and t-butanol. These two solvents thus seem capable of dissolving fatty acids with short to medium chain length as well as long chained fatty acids.

In the rather polar solvents 2-propanol and acetone almost no activity could be detected. The most polar of all solvents tested was acetonitrile, in which the lipases from *T. lanuginosus* and *C. rugosa*, as well as Cal A were synthetically active. This however contradicts the before stated negative influence of the solvents polarity.

Comparison to the results presented in table 5 allows the description of some lipases preference towards fatty acids with distinct chain length. The lipase from *R. miehei* e.g. was able to synthesize ester bonds in only two solvents when methyl octanoate was employed, but in five solvents when methyl palmitate was used.

The highest activities were measured when using Novozyme 435[®] in MTBE and 2m2b. Additionally the combination of Lipozyme[®] or Cal A in 2m2b resulted in quite high synthetic activities.

b) Glucose ester synthesis in organic solvents

(1) Glucose ester synthesis using methyl octanoate

All possible combinations of lipases and solvents were used in experiments for the synthesis of glycolipids employing glucose and methyl octanoate or methyl palmitate. To qualitatively monitor possible successful syntheses all experiments were analyzed via TLC. The results of the experiments using methyl octanoate are shown in table 7. Since in all experiments the obtained results just varied between whether a positive glycolipid synthesis (additional bands appearing after one hour reaction time with a Rf value of approximately 0.43) or no glycolipid synthesis at all (no additional bands detected) just positive results are highlighted.

Table 7: Results of the lipase –catalyzed synthesis of glucose esters using methyl octanoate

Bands detected by TLC (Rf = 0.43)						
	MTBE	2m2b	t-butanol	2-propanol	acetone	ACN
Novozyme 435[®]		+	+		+	+
Lipozyme[®]		+	+			+
CalA						+
CalB		+	+		+	+
Lipase <i>T. lanuginosus</i>						
Lipase <i>R. miehei</i>						
Lipase <i>C. rugosa</i>						

The results of the glycolipid syntheses using methyl octanoate showed that the use of most enzyme-solvent combinations exhibiting positive results using the pNP assay also led to the formation of glycolipids. It however was as well observed that some lipases, like the lipase from *Thermomyces lanuginosus* or *Rhizomucor miehei*, which both were used in liquid formulations, didn't exhibit any synthetic activity in any solvent tested, although synthetic activity in the pNP assay was detected. Also the lipases from *Candida rugosa*, which was lyophilized,

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didn't successfully synthesize glycolipids. CalA only showed synthetic activity in acetonitrile, what also depicts a clear difference to the results obtained from the pNP ester synthesis, where a synthetic activity of CalA was observed in 2m2b and 2-propanol. Thus, it might indicate, that these lipases can utilize pNP as a substrate, but not glucose.

In addition it was observed that all experiments using MTBE or 2-propanol resulted in no glycolipid synthesis. A possible explication might be the relatively high logP value of MTBE. It was observed during the experiments that the employed sugars precipitated and cannot dissolve properly in unpolar MTBE. In the only slightly less unpolar solvent 2m2b however, glycolipid synthesis could be observed. In the case of 2-propanol its relative polar nature might decrease the stability of enzymes by disrupting the essential water layer around the enzyme. On the other hand glycolipid synthesis in the even more polar solvent acetone was detected. This shows that the possible outcome of a synthesis with a distinct solvent – enzyme combination can only hardly be predicted and is likely to be influenced by more parameters than just the solvent polarity. Other factors having an impact on enzyme activity and stability might be the water content of the solvent or the presence of functional groups and their conformation in the solvent itself, which affects the solvent enzyme interaction, especially in the active center of the lipase. Thereby also conformational changes of the enzyme might be caused, leading to a shift or loss of activity or even denaturation of the enzyme.

The solvents providing the best combination of substrate solubility and enzyme stability and activity are 2m2b, tert-butanol, acetone and acetonitrile. Especially the lipase B from *Candida antarctica* exhibited positive results, with Novozyme 435[®] (immobilized Lipase B) and CalB (free, lyophilized Lipase B) being able to synthesize glycolipids in four different solvents. Further the other immobilized lipase tested Lipozyme[®] was able to synthesize glycolipids in 2m2b, t-butanol and acetonitrile.

(2) *Glucose ester synthesis using methyl palmitate*

Similar to the experiments for the pNP ester synthesis, all different glycolipid syntheses were also performed using methyl palmitate. The results of the TLC analyses of these experiments are shown in table 8.

Table 8: Results of the lipase –catalyzed synthesis of glucose esters using methyl palmitate

Bands detected by TLC (Rf = 0.56)	MTBE	2m2b	t-butanol	2-propanol	acetone	ACN
Novozyme 435[®]		+	+		+	+
Lipozyme[®]		+	+			+
CalA						+
CalB		+	+		+	+
Lipase <i>T. lanuginosus</i>						
Lipase <i>R. miehei</i>						
Lipase <i>C. rugosa</i>						

When comparing the results from the glycolipid syntheses with methyl palmitate to the results from the experiments using methyl octanoate (see tables 7), it was shown that exactly the same combinations of enzymes and solvents led to the formation of glycolipids. This proves that the lipases from *Candida antarctica* (Novozyme 435[®] and CalB) and Lipozyme[®] are able to accept fatty acids with medium chain length as well as fatty acids with longer chain length as substrates for a transesterification to yield glycolipids.

Thus, for the glycolipid synthesis using methyl palmitate, the same enzyme - solvent combinations might be suitable, mainly showing that the lipases Novozyme 435[®], Lipozyme[®] and CalB employed in the solvents 2m2b, t-butanol and acetonitrile might be a good choice.

5. Conclusions

A microtiter plate based 4-nitrophenol assay was developed which was suitable to measure the synthetic activity of lipases in organic solvents in a timesaving and high throughput manner. In addition to already known assay this assay allows the use of less unpolar solvents also suitable for glycolipid syntheses, allowing the effective screening of enzyme-solvent combinations to fit this cause. Furthermore this assay might be used to characterize other enzymes with unknown synthetic activities or facilitate the screening for new synthetic active enzymes.

Out of the seven lipases and six solvents tested the highest synthetic activities were observed when employing the immobilized lipases Novozyme 435[®] and Lipozyme[®] with methyl octanoate or methyl palmitate in 2m2b. In addition the pNP esters syntheses in t-butanol were feasible using both fatty acids esters, while for methyl octanoate activities in acetonitrile and for methyl palmitate activities in MTBE were detected.

When comparing these results to the results of the glycolipid syntheses performed with the same enzyme – solvent combination, the majority of the measured activities were confirmed. Only in a few combinations, which led to positive results in the pNP ester synthesis, no synthetic activity towards glycolipid synthesis could be detected.

It hence was possible to pre-select four enzyme-solvent combinations, which enabled the synthesis of glycolipids using fatty acids with varying chain length. The immobilized lipases Novozyme 435[®] and Lipozyme[®] employed in the solvents 2m2b or t-butanol provide the best combination of enzyme activity and stability for the aimed glycolipid synthesis, proving the benefit of an enzyme immobilization. The use of other possible solvents that gave positive results, like MTBE or acetonitrile has to be considered carefully, since these solvents have lower boiling points (MTBE = 55°C, ACN = 82°C), which could complicate testing a possible glycolipid synthesis at higher temperatures. In addition it has to be considered that t-butanol is solid at room temperature, what might lead to complication during analytical methods.

B. Enzymatic synthesis of amino sugar fatty acid esters

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This manuscript was accepted for publication in the “Journal of Lipid Science and Technology” in January 2014 and is currently available under: Pöhnlein et al., Eur. J. Lipid. Sci. Technol. 2014, 116, 423 – 428; doi:101002/ejilt.201300380.

Author's contribution to this manuscript:

Martin Pöhnlein designed this study, developed the methodology, collected and evaluated the data and wrote the manuscript.

Christin Slomka performed experiments employing DMSO to pre-dissolve N-acetyl glucosamine during her master thesis supervised by Martin Pöhnlein.

Olga Kukharensko performed experiments to reproduce the results regarding the acylation of N-butyryl glucosamine during her internship supervised by Martin Pöhnlein.

Dr. Tobias Gärtner performed the structure elucidation via NMR spectroscopy and proofread the manuscript.

Dr. Lars Wiemann proofread the manuscript.

Professor Volker Sieber proofread the manuscript.

Professor Christoph Syldatk supervised the project and proofread the manuscript.

Professor Rudolf Hausmann supervised the project and proofread the manuscript.

1. Abstract

N-acetyl glucosamine fatty acid esters were synthesized by a lipase-catalyzed transesterification with methyl hexanoate and N-acetyl glucosamine (GlcNAc), which resulted in the formation of 2-(acetylamino)-2-deoxy-6-O-hexanoate-D-glucose (GlcNAc-6-O-Hex), a novel glycolipid. Additionally N-butyryl glucosamine (GlcNBu) was used for a similar synthesis, leading to the formation of 2-(butyrylamino)-2-deoxy-6-O-hexanoate-D-glucose (GlcNBu-6-O-Hex). The higher hydrophobicity of GlcNBu led to an increase in the overall yield and the initial reaction rate when compared to the reaction with GlcNAc. By pre-dissolving GlcNAc and GlcNBu in dimethyl sulfoxide (DMSO), it was possible to completely dissolve both sugars in the organic solvent, thus further enhancing the initial reaction rate and yield respectively. To further characterize the newly gained amino sugar fatty acid esters their surfactant properties were evaluated.

2. Introduction

Fatty acid sugar esters, belonging to glycolipids, are amphiphilic, non-ionic molecules that consist of a hydrophilic carbohydrate moiety and one or more fatty acids as lipophilic moiety. As surfactants showing high emulsifying, stabilizing and detergent properties, they find applications in different food, cosmetic, pharmaceutical and cleaning products [88]. Glycolipids typically are non-toxic, odor- and tasteless, completely biodegradable and may be produced using cheap substances from renewable sources [89].

Among other routes, glycolipids can be synthesized by enzyme-catalyzed reactions in organic solvents. Enzymatic syntheses hold several advantages, like mild reaction conditions and the chemo- and regioselectivity of the enzyme [64], which should be exploited in this work. By using suitable hydrolases, e.g. lipases (E.C. 3.1.1.3), it is possible to aim at gaining only the desired product without formation of byproducts via reversed hydrolysis or transesterification reactions [90] [91].

Most of the sugars used are monomers from different polymers regarded as renewable resources e.g. glucose from cellulose or maltose from starch. Further sugars for synthesizing glycolipids could be the amino sugars N-acetyl-glucosamine (GlcNAc) and glucosamine. Both sugars originate from the world's second most abundant biopolymer: chitin [92]. Chitin stands in no conflict to food industry, since it is the main component in crustacean shells [93]. Having an amino- instead of a hydroxyl group at its C₂-atom these sugars possess differing binding possibilities and might show different properties than comparable sugar esters [94].

A limiting factor for the enzymatic synthesis of sugar esters is the low solubility of sugars in non-polar organic solvents, in which lipases exhibit their esterification activity, whereas *vice versa* most lipases lose their esterification activity in more polar solvents, in which sugars are soluble [13]. To handle this problem different strategies have been proposed. A promising approach is to increase the hydrophobicity of the sugar, thus making it more soluble in organic solvents [95] [96]. Further the use of solvent mixtures with DMSO was described [97] [98] in which both, sugar solubility and remaining enzyme activity, is given [61].

In this report we describe a method to synthesize glycolipids from two amino sugar derivatives via transesterification with fatty acid methyl esters. It could be demonstrated that the different hydrophobicity of the sugars has a major impact on the reaction. Moreover was shown that prior dissolving of the sugars in DMSO enhances the initial reaction rate and the yield of the synthesis.

3. Methods & Materials

a) Substrates, Lipases and solvents

Lipase B from *Candida antarctica*, immobilized on acrylic resin (Novozyme 435[®]), N-acetyl glucosamine, glucosamine HCl, butyric acid anhydride and 2-methyl-2-butanol were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Methyl n-hexanoate was purchased from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium). DMSO was purchased from Carl Roth (Carl Roth KG, Karlsruhe, Germany). All solvents were purchased in highest purity available and used without further treatment.

b) Glycolipid synthesis

(1) Chemical acylation of glucosamine

N-butyryl glucosamine (GlcNBu) was chemically synthesized using glucosamine HCl and butyric acid anhydride according to the method described by Brockhausen et al. (2005) [99] and purified using Soxhlet extraction.

(2) Enzymatic glycolipid synthesis

To achieve complete solubilization of the carbohydrates 100 mg of N-acetyl glucosamine (0.45 mmol) or N-butyryl glucosamine (0.40 mmol) respectively were dissolved in 400 μ L DMSO at 40°C prior to the reaction. 400 μ L methyl hexanoate and the DMSO/amino sugar mixture were then dissolved in 9.2 mL 2-methyl-2-butanol. Thus, a total volume of 10 mL was reached. In experiments without DMSO, 9.6 mL 2m2b was added. The reaction was started by the addition of 50 mg of lipase (Novozyme 435[®]) and incubated for 48 hours at

40 °C and 300 rpm in a shaking water bath. All experiments were carried out as triplicates. Samples from each reaction were drawn at timed intervals to follow the reactions as shown in Figure 8.

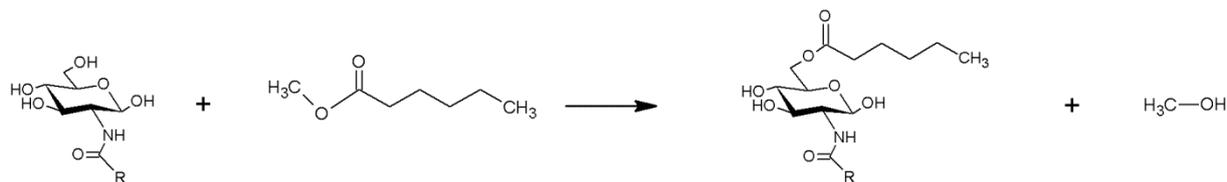


Figure 8: Transesterification between GlcNAc (R = CH₃) or GlcNBu (R = CH₂-CH₂-CH₃) and methyl hexanoate
The projected lipase-catalyzed reactions result in the formation of GlcNAc-6-O-Hex or GlcNBu-6-O-Hex and methanol

c) Purification of the glycolipids

After completion of the reaction, undissolved sugars and immobilized enzymes were removed via filtration with a cellulose filter. After evaporation of the organic solvent in a scanvac BA-VC 300 H vacuum centrifuge (Saur, Reutlingen, Germany), the remaining reaction mixture was dissolved in chloroform. By using a flash chromatography system the glycolipids could be separated from substrates by employing a hydrophilic silica gel column and a gradient of chloroform and methanol as eluent. Fractions containing glycolipids were unified and chloroform/methanol was evaporated. So gained products were used for structure elucidation and as standards for HPLC.

d) Analytical procedures

(1) Concentration determination by high performance liquid chromatography

The time course of the glycolipid concentration during the reaction was followed via high performance liquid chromatography (HPLC). Therefore, the supernatants of centrifuged samples from the reaction mixture were analyzed in an Agilent series 1100 HPLC (Agilent, Santa Clara, USA) consisting of a Hyperclone C-18 column

and a diode array detector. Injection volume was 5 μL and the oven temperature was held at 25°C. The separation of the different components was reached using a gradient of acetonitrile and water at a flow rate of 0.5 mL/min, by increasing the acetonitrile content from 10 % to 60 % in 25 minutes. Concentrations of all reactants were calculated using internal standards. Based on these data overall yield after 48 h and initial reaction rate during the first three hours were calculated.

(2) *Structure elucidation via nuclear magnetic resonance spectroscopy*

Nuclear magnetic resonance spectroscopy (NMR) structure elucidation via 1D ^1H , ^{13}C and 2D ^1H , ^1H COSY (correlation spectroscopy), ^1H , ^{13}C HSQC (heteronuclear singlequantum correlation) and ^1H , ^{13}C HMBC (heteronuclear multibond correlation) spectra was performed on an JEOL ECS 400 (Jeol Ltd, Akishima, Japan). For sample preparation 35 mg of GlcNBu-6-O-Hex and 32 mg of GlcNAc-6-O-Hex were dissolved each with 0.6 mL DMSO- d_6 . Chemical shifts were referenced via the residual solvent signal.

e) *Determination of the critical micelle concentration in water*

The surface tension of aqueous solutions containing different glycolipid concentration was measured versus air with a Lauda TD1 Tensiometer (Lauda, Lauda-Königshofen, Germany) using the du Noüy ring method.

4. Results and Discussion

a) *Glycolipid synthesis using N-acetyl glucosamine and methyl hexanoate as substrates*

In a first set of experiments untreated GlcNAc and GlcNAc dissolved in DMSO were used as substrates in 2m2b, in order to investigate the influence of DMSO on the synthesis. It was expected that the synthetic activity of the lipase led to a transesterification between the primary hydroxyl group of the amino sugar and methyl hexanoate, which would form methanol and 2-(acetylamino)-2-deoxy-6-O-hexanoate-D-glucose (GlcNAc-6-O-Hex), a yet unexplored glycolipid. The time course of the synthesis was followed by HPLC and is shown in figure 9:

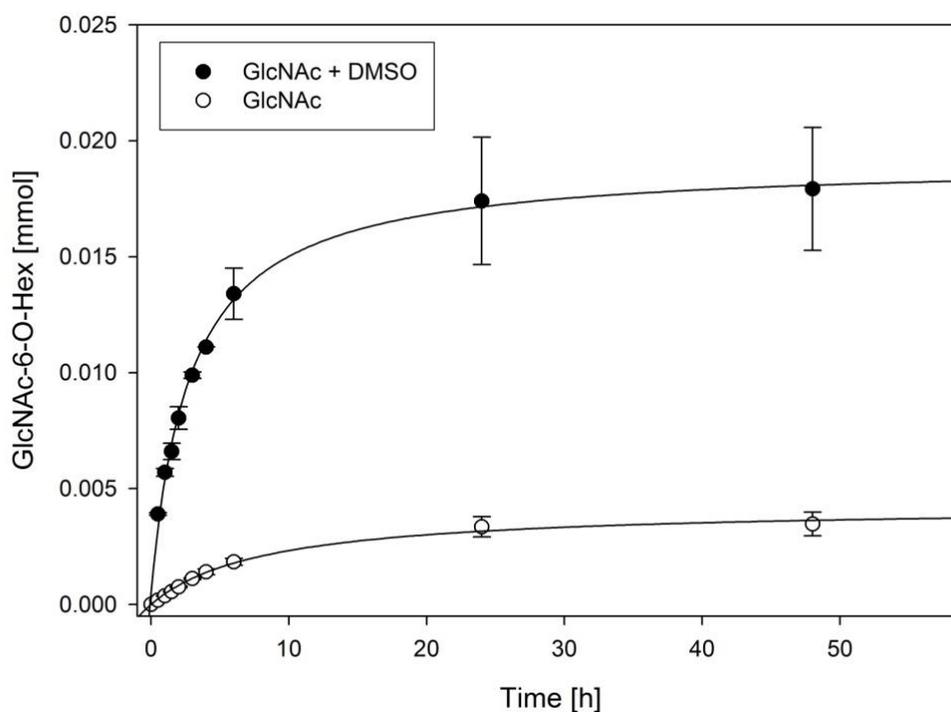


Figure 9: Time course of the enzymatic synthesis of GlcNAc-6-O-Hex using untreated GlcNAc (empty circles) or GlcNAc dissolved in DMSO (filled circles) and methyl hexanoate in 2-methyl-2-butanol with Novozyme 435® at 40°C and 300 rpm.

The experiments using untreated GlcNAc yielded ca. 0.003 mmol GlcNAc-6-O-Hex, while the experiments using pre-dissolved GlcNAc yielded in 0.018 mmol GlcNAc-6-O-Hex and exhibited a faster increase in GlcNAc-6-O-Hex concentration during the first six hours.

An increase of the glycolipid concentration was observed in all experiments, showing that the transesterification was successful. Values for both curves stayed constant after 24 hours with the reaction reaching its equilibrium. While the experiments with untreated GlcNAc resulted in the formation of approximately 0.003 mmol GlcNAc-6-O-Hex, in the analog synthesis with pre-dissolved GlcNAc, 0.018 mmol GlcNAc-6-O-Hex were obtained, showing a significantly higher overall yield (six fold increase). Additionally, the initial reaction rate is higher during the first three hours in the experiment with GlcNAc dissolved in DMSO.

b) Glycolipid synthesis using N-butyryl glucosamine and methyl hexanoate as substrates

An increased hydrophobicity of carbohydrates typically facilitates their use in organic solvents, but even with more unpolar sugars, a complete dissolution is hard to achieve. Since GlcNBu has a longer hydrophobic side chain attached to its C₂-atom it is more hydrophobic, thus expected to be more soluble in 2m2b than GlcNAc. To compare the two substrates GlcNAc and GlcNBu, hence examining the influence of the hydrophobicity of the carbohydrate on the synthesis, experiments analogous to the ones described before with untreated GlcNBu and pre-dissolved GlcNBu were performed. Corresponding results are shown in Figure 10.

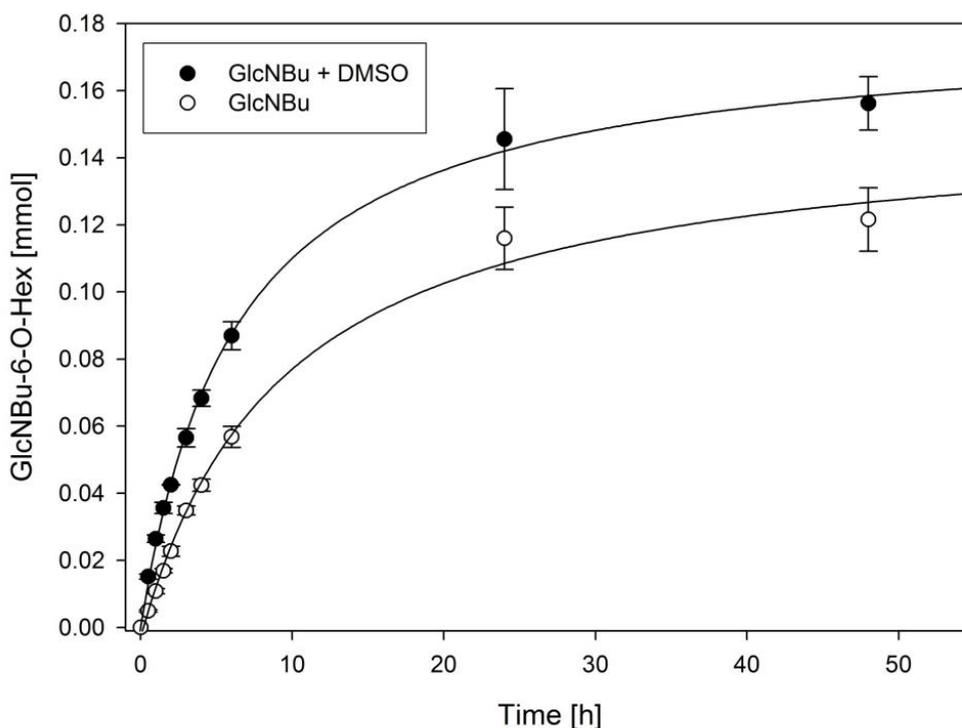


Figure 10: Time course of the transesterification between untreated GlcNBu (empty circles) and GlcNBu pre-dissolved in DMSO (filled circles) and methyl hexanoate in 2-methyl-2-butanol with Novozyme 435[®] at 40°C and 300 rpm.

The experiments using untreated GlcNBu yielded ca. 0.12 mmol GlcNBu-6-O-Hex, while the experiments using pre-dissolved GlcNBu yielded in 0.156 mmol GlcNBu-6-O-Hex and exhibited a faster increase in GlcNBu-6-O-Hex concentration during the first six hours.

Increasing glycolipid concentrations indicate that, with GlcNBu as substrate, the ester synthesis was successful as well. Both curves show a fast increase which even might proceed after the last sample was taken, indicating that the synthesis may have not been terminated at this point. After 48 hours the experiment with untreated GlcNBu yielded 0.122 mmol GlcNBu-6-O-Hex, while with prior dissolving of the amino sugar 0.156 mmol GlcNBu-6-O-Hex could be obtained.

c) Effect of pre-dissolving the amino sugar in DMSO

To compare both reactions including all results, the prior dissolving of the amino sugars in DMSO and the different hydrophobicity of the amino sugars, reaction parameters of all experiments are summarized in table 9.

Table 9: Characteristics of the enzymatic synthesis of GlcNAc-6-O-Hex and GlcNBu-6-O-Hex using untreated GlcNAc and GlcNBu or GlcNAc and GlcNBu dissolved in DMSO and methyl hexanoate in 2-methyl-2-butanol with Novozyme 435® at 40°C and 300 rpm. Yield after 48 h calculated by $\frac{n \text{ sugar } (t=0)}{n \text{ glycolipid } (t=48h)}$

	initial reaction rate [mU]	specific activity [mU/mg]	yield [mg/mL]	yield [mmol]	yield [%]
GlcNAc	0.0004 ± 0	0.067 ± 0	0.13 ± 0.02	0.003 ± 0.001	0.8 ± 0.1
GlcNAc + DMSO	0.0038 ± 0.0001	0.633 ± 0.021	0.66 ± 0.08	0.018 ± 0.003	4.0 ± 0.6
GlcNBu	0.0114 ± 0.0005	1.9 ± 0.077	4.88 ± 0.33	0.122 ± 0.009	30.3 ± 2.3
GlcNBu + DMSO	0.0207 ± 0.0006	3.45 ± 0.095	6.27 ± 0.28	0.156 ± 0.008	38.9 ± 2.0

In both figures 9 and 10, as well as in table 9, it is observable that the prior dissolving of the corresponding amino sugar improves the synthesis and led to higher overall yields and higher initial reaction rates. When employing dissolved GlcNAc the initial reaction rate and specific activity were 9.5 times higher than compared to untreated GlcNAc. Additionally a five times higher yield proved that DMSO favored both crucial reaction parameters. Likewise the synthesis with GlcNBu was enhanced when the carbohydrate was dissolved beforehand, resulting in a 1.8 times as high initial reaction rate and specific activity of the enzyme and an increased overall yield. Thus, it could be shown that the use of the DMSO to pre-dissolve the amino sugars improved the synthesis, which is most likely due to its better solubility in the organic solvent, so that the sugar might be more easily available for the lipase. Despite its positive effects, the use of DMSO has to be considered critically, since it complicates the purification dramatically.

d) Influence of the hydrophobicity of the amino sugar

When comparing the results of all GlcNAc and GlcNBu experiments it is obvious that all experiments with the more hydrophobic sugar GlcNBu exhibited significantly higher reaction rates and yields. The specific activity of the lipase in the experiment using GlcNBu dissolved in DMSO was 5.4 times higher than the specific activity in the corresponding experiment with pre-dissolved GlcNAc, while the overall yield was 8.7 times higher. The quite high reaction rate and the yield in the experiment using untreated GlcNBu showed that the hydrophobicity of the substrate had a much stronger impact than the use of DMSO, since this yield was eminently higher than

the yield in any reaction with GlcNAc. This proved that it is very important for the synthesis of glycolipids that the carbohydrate is properly dissolved, which can be achieved best if the sugar is more hydrophobic.

e) Further parameters influencing the synthesis

Another way to optimize the solubility of the amino sugars might be the use of more hydrophilic organic solvents. But here it has to be considered that with the use of more polar solvents the enzyme activity and the solubility of the fatty acid might be negatively influenced. 2m2b has a logP value of 1.06 and miscible with DMSO [61]. And, since it's a tertiary, sterically hindered alcohol it is a substrate only converted by few lipases [100].

A clear drawback of the discussed synthesis is the use of fatty acid methyl esters. Although it is an advantage that no water is produced during the transesterification, the formation of methanol is a limiting factor to the lipase action, since it's known that high methanol concentrations might lead to the inactivation of lipases [28].

f) Structure elucidation

Proton and carbon NMR measurements proved that both GlcNBu-6-O-Hex and GlcNAc-6-O-Hex were suitable for the detailed structure analysis. $^1\text{H}, ^1\text{H}$ COSY spectra showed the protons connected via 3J coupling and $^1\text{H}, ^{13}\text{C}$ HSQC spectra of both samples showed the direct connection of proton and carbon signals whereby the diastereotope splitting of the protons attached to C_6 could be assigned. Due to the missing 1J coupling the carbonyl carbon atoms of the ester and the amide and the N-H-proton could be clearly identified. The most important evidence that clearly characterized the proposed structures are the detected couplings in the $^1\text{H}, ^{13}\text{C}$ HMBC spectra of both samples. Here crosspeaks between the protons at C_6 and the carbonyl-carbon atom of the synthesized ester were observed. Furthermore, crosspeaks between the N-H-proton and the carbonyl-carbon atom of either the butyryl-group in GlcNBu-6-O-Hex or the acetyl-group in GlcNAc-6-O-Hex could be detected. Due to these directly observed couplings the molecular structure could be confirmed as predicted. Detailed chemical shift assignment and the corresponding spectra are given in the appendix.

g) Hydrophilic-lipophilic balance

With the chemical structure of both glycolipids confirmed, it was possible to calculate their HLB value, with was 13.85 for GlcNAc-6-O-Hex and 12.73 for GlcNBu-6-O-hex. Thus, both novel glycolipids might be classified as detergents, which classically have HLBs of 13 to 15.

h) Critical micelle concentration

To examine their surfactant characteristics the ability to lower the surface tension of aqueous solutions was tested for both glycolipids. The surface tension of aqueous solutions containing different concentrations of GlcNAc-6-O-Hex and GlcNBu-6-O-Hex is shown in figure 11.

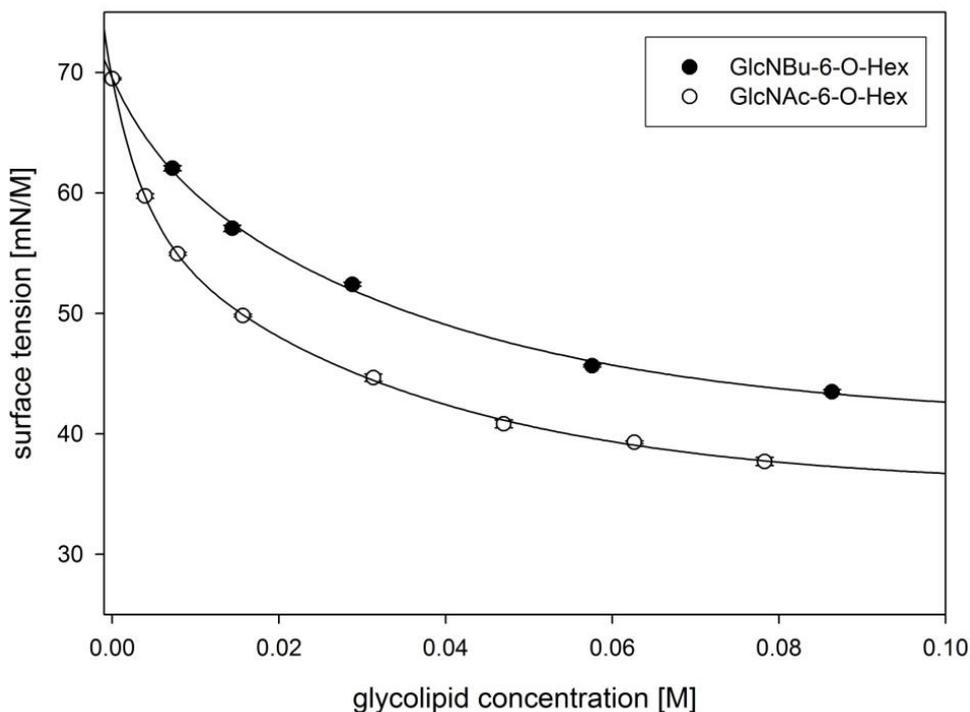


Figure 11: Surface tension of aqueous solutions containing different concentrations of GlcNBu-6-O-Hex (filled circles) and GlcNAc-6-O-Hex (empty circles) versus air.

GlcNAc-6-O-Hex lowered the surface tension of water from approx. 70 mN/m to 40 mN/m, while GlcNBu-6-O-Hex lowered the surface tension of water to ca. 45 mN/m.

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Both glycolipids were able to lower the surface tension of water in already low concentrations, showing their good surfactant properties. GlcNAc-6-O-Hex lowered the surface tension from 70 mN/m to 39 mN/m, while GlcNBu-6-O-Hex lowered the surface tension to 45 mN/m. When reaching a concentration of approximately 0.08 M (approx 20 mg/mL) it seemed that the surface tension stays constant with even higher concentration, meaning that the critical micelle concentration might have been reached. Higher concentrations could have not been tested, due to the maximal solubility of the glycolipids in water was reached.

5. Conclusions

Two novel glycolipids, namely amino sugar fatty acid esters were synthesized in an enzymatic reaction using commercially available lipases, thus further broadening the range of known glycolipids synthesized via biological routes. The structure of both glycolipids was clearly elucidated as 2-(acetylamino)-2-deoxy-6-O-hexanoate-D-glucose and 2-(butyrylamino)-2-deoxy-6-O-hexanoate-D-glucose by NMR. By calculation their HLB value and examining their surfactant characteristics, both GlcNAc-6-O-Hex and GlcNBu-6-O-Hex could be characterized as detergents, although the critical micelle concentration was comparable high.

The synthesis was characterized by following the course of each reaction via HPLC, showing that a) the use of the more hydrophobic amino sugar N-butyryl glucosamine leads to a significantly higher reaction rate and yield than the synthesis with less hydrophobic N-acetyl glucosamine and b) prior dissolving of both sugars in DMSO increased the initial reaction rate and the overall yield of the synthesis even further. By proving the importance of the solubility of substrates, these finding might help to further characterize and optimize enzymatic syntheses in organic solvents.

C. The effects of substrate ratio, water activity and reaction temperature on the enzymatic synthesis of amino sugar fatty acid esters

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This manuscript is currently under preparation for publication.

Author's contribution to this manuscript:

Martin Pöhnlein designed this study, developed the methodology, collected and evaluated the data and wrote the manuscript.

Tim Finkbeiner performed experiments evaluating the influence of the water activity on the synthesis of GlcNAc-6-O-Hex during his bachelor thesis supervised Martin Pöhnlein.

Robin Wagner performed experiments to test the influence of the reaction temperature on the synthesis of amino sugar fatty acid esters during his bachelor thesis supervised by Martin Pöhnlein.

Dr. Tobias Gärtner performed the structure elucidation via NMR spectroscopy and proofread the manuscript.

Dr. Lars Wiemann proofread the manuscript.

Professor Volker Sieber proofread the manuscript.

Professor Christoph Syldatk supervised the project and proofread the manuscript.

Professor Rudolf Hausmann supervised the project and proofread the manuscript.

1. Abstract

N-acetyl glucosamine was acylated in lipase-catalyzed transesterifications using three different fatty acid vinyl esters with chain lengths ranging from six to sixteen C-atoms, resulting in the formation of novel glycolipids, namely amino sugar fatty acid esters. By comparing these reactions, the influence of the fatty acid chain length on the synthesis could be demonstrated. In order to characterize the synthesis crucial reaction parameters for the transesterification like the molar substrate ratio between amino sugar and fatty acid ester, the initial water activity of the organic solvent and the reaction temperature were varied, all showing a significant influence on the synthesis. For every particular reaction the specific synthetic and specific hydrolytic activity of the lipase was determined, allowing the characterization and optimization of the synthesis. It hence was demonstrated that besides the initial water activity of the organic solvent also the reaction temperature exhibits an influence on the lipases synthetic and hydrolytic activity. Highest yields of amino sugar fatty acid esters were obtained at the lowest water activity tested, a distinct fatty acid ester excess and a temperature of 70°C.

2. Introduction

Previous experiments demonstrated that the enzymatic synthesis of glycolipids is influenced by various factors. Besides the selection of substrates, which determines the composition of the final product and the choice of enzyme and reaction media, crucial parameters influencing the synthesis might be the molar substrate ratio, the water activity of the reaction media and the reaction temperature.

An excess of fatty acids might affect the synthesis, since high fatty acid concentrations can lower the solubility of sugars by making the reaction media more hydrophobic. Additionally the chance of obtaining multiple acylation of the sugar molecules increases with higher fatty acid concentration.

When employing lipases as catalysts, it has to be considered that their natural activity is hydrolysis. Only at distinct low water activities in the solvent, hydrolysis can be suppressed, resulting in the reversal of this activity, which leads to syntheses. Small amounts of water on the other hand are essential to provide the enzymes flexibility and activity. Thus the water activity, representing the amounts of free water present in the solvent is a crucial reaction parameter for enzymatic activity in organic solvents. Increasing reaction temperatures might favor the enzymatic activity up to a certain point, but every enzyme will denature at a certain temperature. Another benefit of high reaction temperatures might be the better solubility of sugars in organic solvents. Within this work the influence of all named parameters will be investigated.

3. Methods & Materials

a) Substrates, lipases and solvents

Lipase B from *Candida antarctica*, immobilized on acrylic resin (Novozyme 435[®]), N-acetyl glucosamine and 2-methyl-2-butanol were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Vinyl n-hexanoate, vinyl n-laurate and vinyl n-palmitate were ordered from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium). Dimethyl sulfoxide, 4Å molecular sieves, lithium chloride, sodium chloride and magnesium chloride were purchased from Carl Roth (Carl Roth KG, Karlsruhe, Germany). All solvents were purchased in highest purity available and if not stated otherwise used without further treatment.

b) Adjustment of the water activity in 2-methyl-2-butanol and determination of the water content

In order to determine the influence of the initial water activity on the synthesis, experiments were carried out in a total of five different 2m2b formulations, all representing different water activities. In addition to experiments employing 2m2b without further treatment, the water activity of 2m2b was adjusted before experiments. Therefore 2m2b was incubated with three different saturated salt solutions (LiCl, MgCl₂ and NaCl) for 7 days in an airtight vessel, according to Chamouleau et al. (2011) [101].

In additional experiments, the water content of 2m2b was reduced by adding 2 g molecular sieves (pore size 4Å) to each reaction, which were beforehand activated at 200°C for two hours, if not stated otherwise. It was assumed that by reducing the water content of the solvent also its water activity might be lowered.

To monitor the difference between these solvent preparations, the total water content of all prepared solvent formulations was determined via coulometric Karl-Fischer-titration using a Titroline 7500 KF trace titrator (SI Analytics GmbH, Mainz, Germany). Measuring the water content of the three 2m2b variants with known, pre-adjusted water activities then allowed the correlation between water content and water activity. This linear

correlation was applied to calculate the water activities of 2m2b without further treatment and 2m2b with added molecular sieves (see appendix).

c) *Enzymatic synthesis of N-acetyl glucosamine esters*

To achieve complete solubilization of the amino sugars, the necessary amount of N-acetyl glucosamine was dissolved in 400 μ l DMSO at 40°C prior to the reaction. The DMSO/amino sugar mixture then was dissolved in 2m2b, together with 100 mg Novozyme 435[®]. The volume of 2m2b depended on the fatty acid employed, which was added to start the reaction, reaching a total volume of 20 mL. The amounts of amino sugar and fatty acid vinyl ester matching the specific ratios are listed in table 10.

Table 10: Experimental details of the enzymatic synthesis of N-acetyl glucosamine esters

Fatty acid ester	Substrate Ratio	GlcNAc [mg] - dissolved in 400 μL DMSO	Fatty acid [μL]	2m2b [mL]
Vinyl hexanoate	1 : 1.4	200 (0.90 mmol)	200 (1.26 mmol)	19.400
	1 : 3	100 (0.45 mmol)	217 (1.35 mmol)	19.383
	1 : 5.5	100 (0.45 mmol)	400 (2.47 mmol)	19.200
Vinyl laurate	1 : 1.4	200 (0.90 mmol)	332 (1.26 mmol)	19.278
	1 : 3	100 (0.45 mmol)	349 (1.35 mmol)	19.251
	1 : 5.5	100 (0.45 mmol)	643 (2.47 mmol)	18.957
Vinyl palmitate	1 : 1.4	200 (0.90 mmol)	407 (1.26 mmol)	19.193
	1 : 3	100 (0.45 mmol)	437 (1.35 mmol)	19.163
	1 : 5.5	100 (0.45 mmol)	807 (2.47 mmol)	18.793

The reaction was incubated for 48 hours at 40 °C and 300 rpm in a shaking water bath. All experiments were carried out as triplicates. Samples from each reaction were withdrawn at timed intervals to follow the formation of the glycolipids as projected in figure 12.

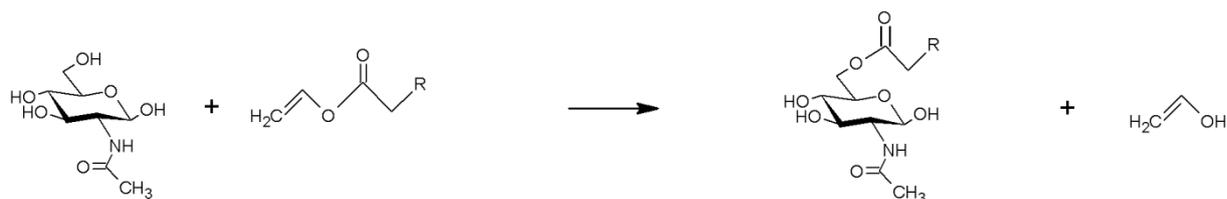


Figure 12: Schematic reaction of a transesterification between N-acetyl glucosamine and different fatty acid vinyl esters

When employing vinyl hexanoate [R = $-(\text{CH}_2)_3-\text{CH}_3$] the transesterification results in the formation of 2-(Acetylamino)-2-deoxy-6-O-hexanoate-D-glucose and ethanol; when using vinyl laurate [R = $-(\text{CH}_2)_9-\text{CH}_3$] 2-(Acetylamino)-2-deoxy-6-O-laurate-D-glucose and ethanol is formed; the use of vinyl palmitate [R = $-(\text{CH}_2)_{13}-\text{CH}_3$] leads to the formation of 2-(Acetylamino)-2-deoxy-6-O-palmitate-D-glucose and ethanol.

d) Purification of the glycolipids

After completion of the reaction, undissolved sugars and immobilized enzymes were removed via filtration with a cellulose filter. By using flash chromatography the glycolipids could be separated from unreacted substrates by employing a hydrophilic silica gel column and a gradient of chloroform and methanol as eluent. Fractions containing glycolipids were unified and chloroform/methanol was evaporated. So gained pure products were used for structure elucidation and as standards for concentration determination.

e) Analytical procedures

(1) Concentration determination of amino sugar fatty acid esters using high performance liquid chromatography

The time course of each reaction was followed via high performance liquid chromatography (HPLC). Therefore centrifuged samples from the reaction mixture were analyzed using an Agilent Series 1200 system (Agilent Technologies, Santa Clara, USA) consisting of a reversed phase column (Hyperclone C-18) and a diode array detector. Injection volume was 5 μl and the oven temperature was held at 25°C. The separation of the different components was reached using different gradients of acetonitrile and water at a flow rate of 0.5 ml/min.

Concentrations of all reactants were calculated using internal standards. Based on these data, initial and maximal reaction rate during the first three hours and overall yield after 48 hours were calculated.

(2) *Concentration determination of fatty acid vinyl esters using gas chromatography*

In order to monitor the fatty acid vinyl ester concentration during each reaction, samples were analyzed using a 6890N Network GC system from Agilent (Agilent Technologies, Santa Clara, USA), employing an methyl siloxane column (HP-1) and a flame ionization detector. Sample volume was 1 μ L. Separation was achieved by increasing the temperature from 50°C to 250°C in 20 minutes, with front inlet and detector temperatures of 220°C and 250°C respectively. Gas flows were 40 mL/min for hydrogen and helium and 450 mL/min for pressured air. Concentrations of all fatty acid esters were calculated using internal standards. Based on these data the specific hydrolytic activity of the enzyme against fatty acid vinyl esters within the first hour of the reaction was calculated.

(3) *Structure elucidation via nuclear magnetic resonance spectroscopy*

Nuclear magnetic resonance spectroscopy (NMR) structure elucidation via 1D ^1H , ^{13}C and 2D ^1H , ^1H COSY (correlation spectroscopy), ^1H , ^{13}C HSQC (heteronuclear singlequantum correlation) and ^1H , ^{13}C HMBC (heteronuclear multibond correlation) spectra was performed on an JEOL ECS 400 (Jeol Ltd, Akishima, Japan). For sample preparation 50 mg of 2-(acetylamino)-2-deoxy-6-O-laurate-D-glucose (GlcNAc-6-O-Lau) and 50 mg of 2-(acetylamino)-2-deoxy-6-O-palmitate-D-glucose (GlcNAc-6-O-Palm) were dissolved each with 0.6 mL DMSO-*d*₆. Chemical shifts were referenced via the residual solvent signal.

f) Calculation of process parameters

To compare and optimize the various experiments, the following process parameters were calculated as follows:

Specific synthetic activity [$\text{U}\cdot\text{mg}^{-1}$]: glycolipid formed [μmol] \cdot time⁻¹ [min] \cdot enzyme used⁻¹ [mg]

Specific hydrolytic activity [$\text{U}\cdot\text{mg}^{-1}$]: fatty acid ester hydrolyzed [μmol] \cdot time⁻¹ [min] \cdot enzyme used⁻¹ [mg]

Overall yield [%]: glycolipid formed after 48h [mmol] \cdot amino sugar used⁻¹[mmol] \cdot 100

4. Results and Discussion

a) *Determination of the water content in 2m2b and calculation of the water activity*

After the variation of the initial water activity due to the use of saturated salt solutions or molecular sieves, the water content of 2m2b after each treatment was measured via Karl-Fischer-titration. The results thereof are shown in table 11.

Table 11: Measured water content of 2m2b treated in different ways using saturated salt solutions or molecular sieves

Treatment of the solvent	Assumed Water activity	Measured water content [ppm]	Measured water content [%]
2m2b without further treatment	0,1	6823	0,68
Addition of 2 g molecular sieves (4 Å) to the reaction	0,01	999	0,1
LiCl	0,13	9622	0,96
MgCl ₂	0,33	26728	2,67
NaCl	0,75	50075	5,0

The results of the titrations proved that it was possible to generate five 2m2b variants with water contents ranging from approximately 0.1 % to 5 %. Thus it was shown that the incubation with saturated salt solution not only exhibited the known impact on the water activity of solvents [101], it also could be shown that this procedure also has an influence on the water content of the solvent.

The linear correlation between water content and water activity observed in these three 2m2b variants allowed the designation of approximate water activities to the 2m2b variants without further treatment and with added molecular sieves. The water activities of untreated 2m2b and 2m2b with added molecular sieves were the lowest tested, showing that only the addition of molecular sieves led to a reduced water activity in the solvent.

b) Influence of the molar substrate ratio on the synthesis

To monitor the possible impact upon variation of the substrate ratio between amino sugar and fatty acid vinyl ester on the synthesis, the time courses of the synthesis using N-acetyl glucosamine and vinyl laurate in untreated 2m2b with substrate ratios ranging from almost equimolar (1 : 1.4) to a distinct fatty acid ester excess (1 : 5.5) are shown in figure 13.

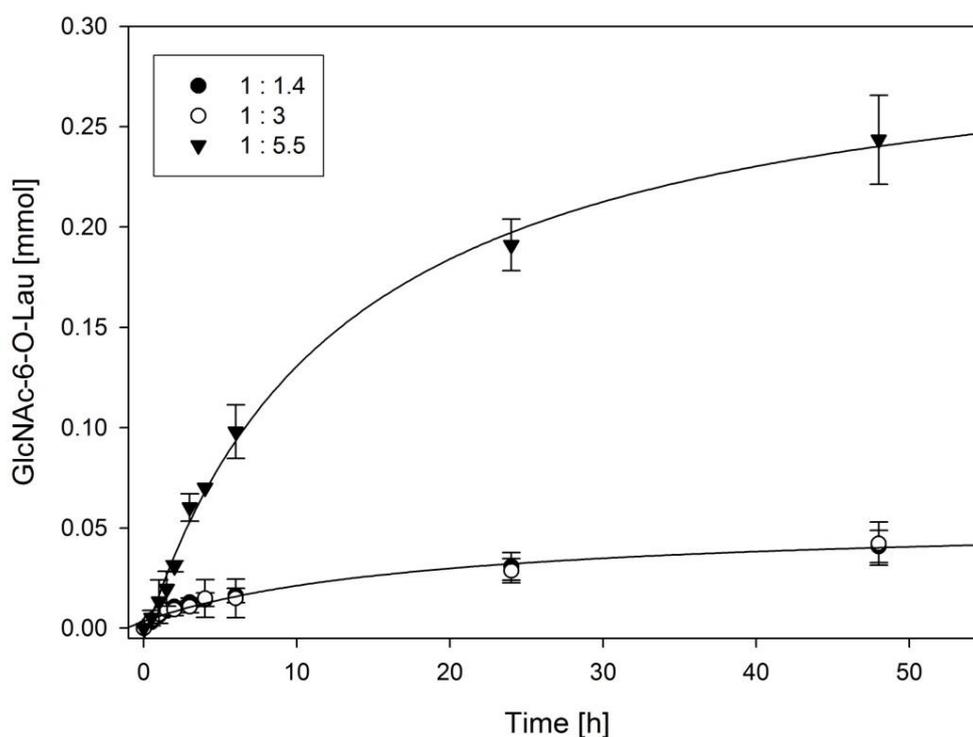


Figure 13: Time course of the transesterification between N-acetyl glucosamine and vinyl laurate at substrate ratios of 1 : 1.4 (filled circles), 1 : 3 (empty circles) and 1 : 5.5 (filled triangles) in 2-methyl-2-butanol with Novozyme 435® at 40°C and 300 rpm.

The experiments using a substrate ratios of 1 : 1.4 and 1 : 3 yielded ca. 0.04 mmol GlcNAc-6-O-Lau, while the experiments using a substrate ratio of 1 : 5.5 yielded in 0.25 mmol GlcNAc-6-O-Lau and exhibited a faster increase in GlcNAc-6-O-Lau concentration during the first six hours.

The time course of all experiments showed increasing GlcNAc-6-O-Lau concentrations, thus proving successful glycolipid syntheses in all cases. The experiments with substrate ratios of 1 : 1.4 and 1 : 3 gained almost matching results, yielding in a GlcNAc-6-O-Lau concentration of approximately 0.04 mmol after 48 hours.

When compared to the other two variants, the GlcNAc-6-O-Lau concentration during the first six hours in the experiments employing an amino sugar : fatty acid ester ratio of 1 : 5.5 increased faster, indicating a higher initial reaction rate. After 48 hours these experiments resulted in a six-fold higher yield, reaching a GlcNAc-6-O-

Lau concentration of ca. 0.24 mmol. Increasing concentration between 24 and 48 hours indicate that the synthesis, didn't reach its equilibrium and might have proceeded further.

Based on these reaction courses, the maximal reaction rate of the lipases maximal specific synthetic activity was calculated for each experiment, which is presented in figure 14.

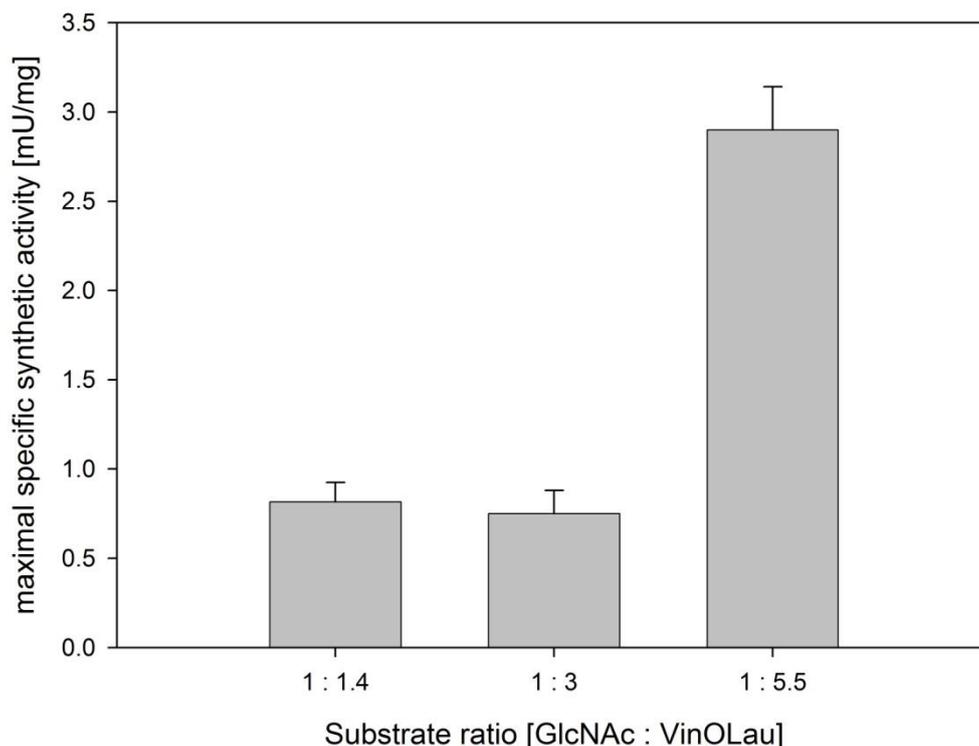


Figure 14: Maximal specific synthetic activities of Novozyme 435[®] for the transesterification between N-acetyl glucosamine and vinyl laurate at different substrate ratios in 2-methyl-2-butanol at 40°C and 300 rpm. While the maximal specific synthetic activities didn't exceed 1 mU/mg when employing substrate ratio of 1 : 1.4 and 1 : 3, the experiments with a substrate ratio of 1 : 5.5 yielded a maximal, specific, synthetic activity of approximately 3 mU/mg.

The results shown in figure 14 confirm the findings presented in figure 13 by illustrating that, when compared to the experiments with lower substrate ratios, the maximal specific synthetic activity of the enzyme was approximately three times higher in the experiments with an amino sugar : fatty acid ester ratio of 1 : 5.5.

These results demonstrated that an excess of fatty acid esters favors this synthesis of amino sugar fatty acid esters by improving important reaction parameter like maximal reaction rate and overall yield, likely by increasing the chance of a formation of an enzyme-acyl complex due to the higher fatty acid ester concentrations. Negative influences of a high fatty acid ester concentration, like a possible enzyme inhibition or a change in the polarity of the solvent, were not observed.

These findings were observed in all further experiments employing all three different fatty acid esters, indicating that out of the tested three substrate ratios 1 : 5.5 favors the synthesis the most. Thus, for all results presented in chapter III.C.3.c) and III.C.3.d), only the results from the experiments with an amino sugar : fatty acid ester ratio of 1 : 5.5 are discussed.

c) *Influence of the water activity on the synthesis*

To investigate the influence of the initial water activity of 2m2b on the synthesis of amino sugar fatty acid esters, experiments have been carried out using 2m2b treated in five different ways. As chosen examples thereof the time courses of the experiments using GlcNAc and VinOLau are shown in figure 15.

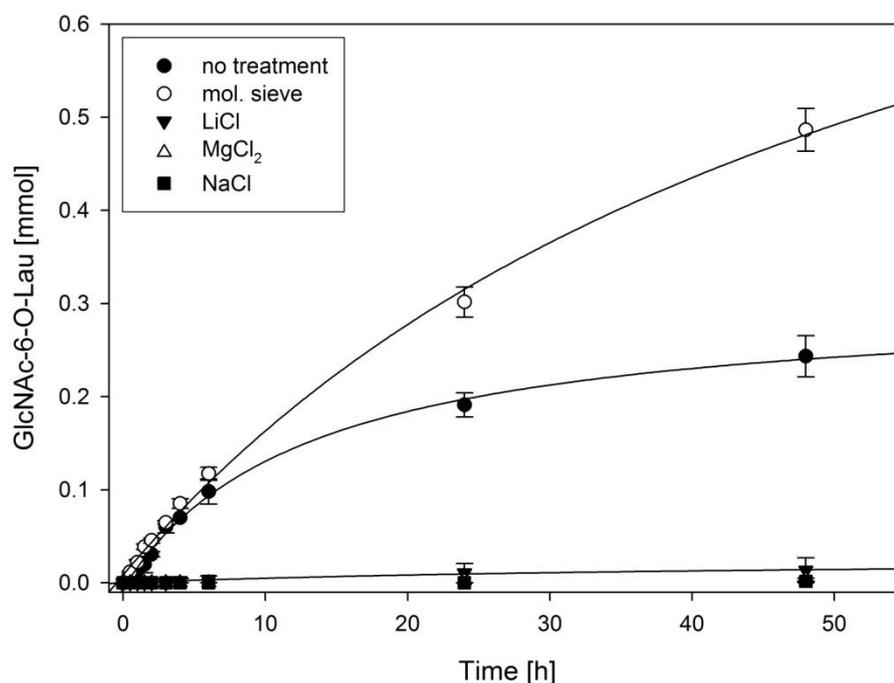


Figure 15: Time course of the transesterifications between N-acetyl glucosamine and vinyl laurate using 2m2b without further treatment (filled circles), 2m2b with additional molecular sieves (empty circles), 2m2b pre-treated with LiCl (filled triangles), 2m2b pre-treated with MgCl₂ (empty triangles) and 2m2b pre-treated with NaCl (filled squares) using Novozyme 435® at 40°C and 300 rpm.

The experiments employing 2m2b without further treatment yielded ca. 0.22 mmol GlcNAc-6-O-Lau, while the experiments using 2m2b pre-treated with various saturated salt solutions all yielded ca. 0.01 mmol GlcNAc-6-O-Lau. The experiments with additional molecular sieves resulted in the formation of 0.48 mmol GlcNAc-6-O-Lau and exhibited a faster increase in GlcNAc-6-O-Lau concentration.

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The results of the experiments using 2m2b variants with different water activities clearly demonstrated the influence of the initial water activity on the synthesis of GlcNAc-6-O-Lau. Although in all five experiments glycolipids were synthesized, the differences in the time courses between the experiments with lower water activity were evident. While the GlcNAc-6-O-Lau concentration increased steadily during the first six hours in the experiments with added molecular sieves and 2m2b without further treatment, no glycolipids were gained in the other three experiments with water activities greater than 0.1 during this time. Only after 24 hours GlcNAc-6-O-Lau could be detected, resulting in concentration of approximately 0.01 mmol GlcNAc-6-O-Lau after 48 hours for the experiments with LiCl treated 2m2b and even lower concentrations for the two experiments with MgCl₂ and NaCl treated 2m2b.

The synthesis carried out in 2m2b without further treatment resulted in the formation of ca. 0.22 mmol GlcNAc-6-O-Lau after 48 hours, while the synthesis with added molecular sieves yielded 0.48 mmol GlcNAc-6-O-Lau. Thus, it was shown, that the reduction of the initial water activity to 0.01 by adding molecular sieves had a positive effect on the synthesis. For both reactions the synthesis did not reach its equilibrium and might have proceeded after 48 hours. This however could not be possible in the case of the experiment employing molecular sieves, since all the applied amino sugar was acylated already. The fact that the whole amount of amino sugar employed could be detected as GlcNAc-6-O-Lau proved, that no side products like diacylated glycolipids were formed during the reaction.

These results demonstrated the major impact of the water activity on the synthesis, by showing that the initial reaction rate and overall yield were favored when the water activity was comparably low. To validate these findings, the maximal specific synthetic activity of Novozyme 435[®] for the transesterifications of GlcNAc and all three fatty acid vinyl esters respectively are shown in figure 16.

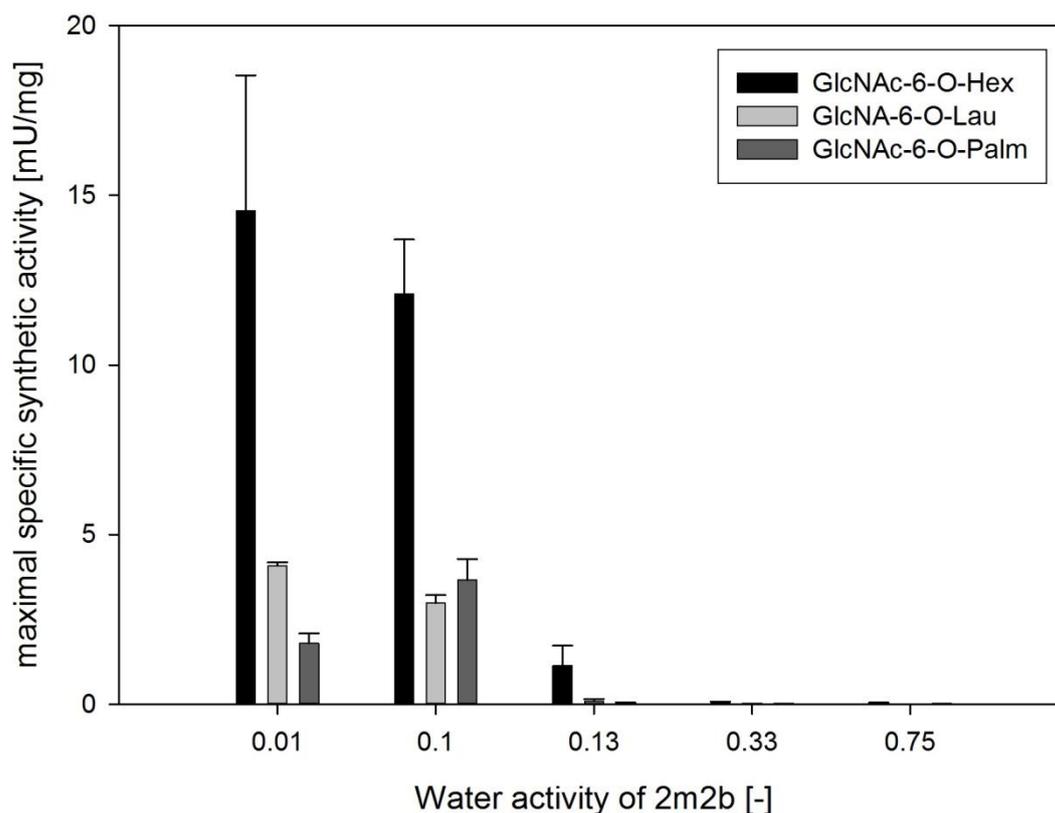


Figure 16: Maximal specific synthetic activities of Novozyme 435[®] during the transesterifications between N-acetyl glucosamine and vinyl hexanoate resulting in the formation of GlcNAc-6-O-Hex (black bars), vinyl laurate resulting in the formation of GlcNAc-6-O-Lau (light grey bars) and vinyl palmitate resulting in the formation of GlcNAc-6-O-Palm (dark grey bars) using 2m2b preparations with varying water activity at 40°C and 300 rpm.

Experiments with different fatty acid vinyl esters all presented the same trend of increasing maximal specific synthetic activities with decreasing water activities. Maximal specific synthetic activities of experiments with 2m2b pre-treated with saturated salt solutions were just above minimal detection limits. Highest activities of ca. 15 mU/mg were detected when employing VinOHex in 2m2b with additional molecular sieves.

Analog to the findings described in figure 15, the results presented in figure 16 showed that in experiments employing 2m2b pre-treated with saturated salt solutions, much lower maximal specific synthetic activities were observed. Thus, only small differences were found when comparing the different results from experiments with different fatty acid vinyl esters. The experiment using VinOHex in LiCl treated 2m2b showed a maximal specific synthetic activity of ca. 1 mU/mg.

The experiments without further treatment of the solvent resulted in maximal specific synthetic activities ranging from approximately 4 mU/mg when using VinOLau and VinOPalm to ca. 12 mU/mg when using VinOHex. The experiments with addition of molecular sieves gained similar values, resulting in maximal specific synthetic activities of ca. 2 mU/mg for the synthesis of GlcNAc-6-O-Palm, ca. 5 mU/mg for GlcNAc-6-O-Lau and ca. 15 mU/mg for GlcNAc-6-O-Hex. These findings confirm, that the highest synthetic activities were observed when adding molecular sieves to the reaction.

In addition, the fact that the highest activities were measured in the experiments using VinOHex indicated, that for the synthesis of glycolipids fatty acid esters with smaller chain length were converted faster by the enzyme. Short chained fatty acids like VinOHex are likely to be able to enter and find optimal positioning in the active center of the lipase faster than long chained fatty acids.

To further investigate the influence of the initial water activity on the enzymatic reaction, the specific hydrolytic activity of Novozyme 435[®] at different water activities is shown in figure 17.

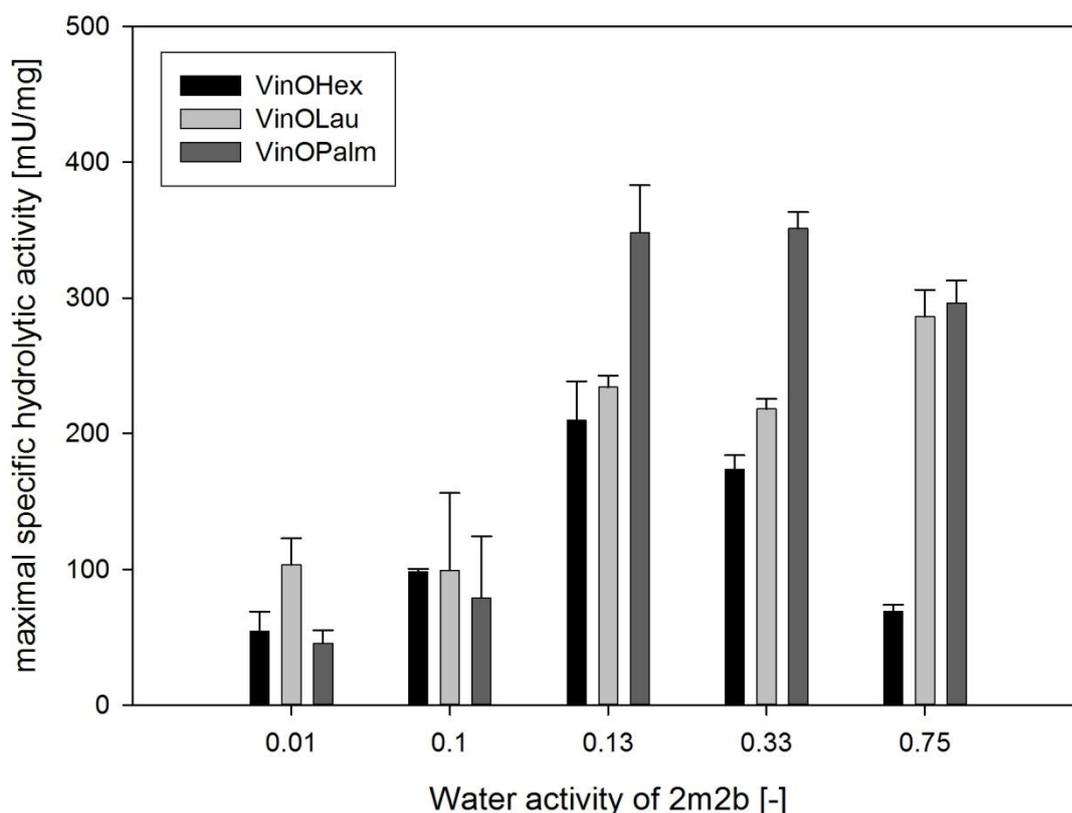


Figure 17: Maximal specific hydrolytic activities of Novozyme 435[®] using vinyl hexanoate (black bars) , vinyl laurate (light grey bars) and vinyl palmitate (dark grey bars) for transesterification with GlcNAc in 2m2b preparations with varying water activity at 40°C and 300rpm.

The experiments with different fatty acid vinyl esters all presented the same trend of increasing specific hydrolytic activities with increasing water activities. Highest hydrolytic activities of more than 300 mU/mg were detected when employing VinOPalm in 2m2b pre-treated with LiCl or MgCl₂.

When comparing the specific hydrolytic activities with the specific synthetic activities shown before, two facts became obvious. Firstly, all measured hydrolytic activities were higher than the corresponding synthetic, for example, in the experiments using LiCl treated 2m2b an almost 200-fold higher hydrolytic activity was detected. Secondly, the trend of the activities detected was exactly contrary to the synthetic activities regarding the water activity. For most experiments the hydrolytic activity increased with increasing water activity. Only the

experiments employing VinOHex seem to contradict this thesis, with lower maximal specific hydrolytic activities detected in the experiments using MgCl_2 and NaCl treated 2m2b. This, however was due to the fact that in these experiments the hydrolysis of the fatty acid esters proceeded so fast, that the initial fatty acid ester concentration measured was too low (2.47 mmol applied, only 1.81 detected), thus leading to smaller values when calculating the initial hydrolytic activity.

The determination of the hydrolytic activities of the lipase proved that no loss of activity was observed at higher water activities. Moreover the increased hydrolytic activities at higher water activities might explain the lower synthetic activities, which occurred because of a shift in the enzyme activity, probably due to more unbound water present in the solvent. This assumption can be validated by comparing the overall yields of the different reactions. Thus, in figure 18 the overall yield of all reactions, as well as the synthetic and hydrolytic activities of one chosen example using GlcNAc and VinOLau, are compared.

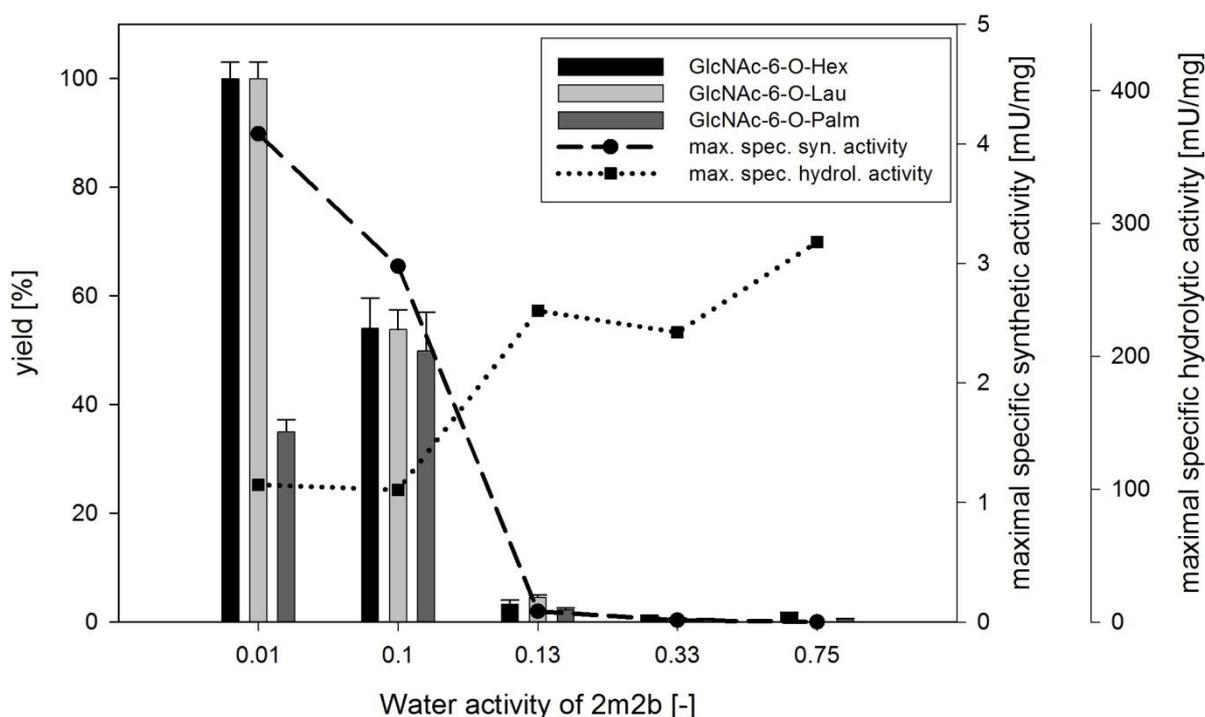


Figure 18: Yields of the transesterifications between N-acetyl glucosamine and vinyl hexanoate (black bars), vinyl laurate (light grey bars) and vinyl palmitate (dark grey bars) using Novozyme 435[®] in 2m2b preparations with varying water activity at 40°C and 300rpm.

The experiments employing 2m2b pre-treated with saturated salt solutions only resulted in yields lower than 5%. The experiments using 2m2b without further treatment yielded conversions of approximately 50 – 55% for all different fatty acid esters used. Highest yields were obtained when using VinOHex or VinOLau in 2m2b with additional molecular sieves, resulting in the acylation of all employed amino sugars.

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The overall yields showed results matching the synthetic activity during experiments with different water activities. Highest yields were achieved when molecular sieves were added with the water activity being comparably the lowest. The experiments using VinOHex and VinOLau in 2m2b with additional molecular sieves even resulted in a yield of 100 %, corresponding to every amino sugar being acylated with one fatty acid. While in the experiments using untreated 2m2b approximately 50 – 55% yield were reached, the three experiments with 2m2b pre-treated with saturated salt solutions didn't exceed yields of more than 5 %.

In order to summarize and to illustrate all findings, the maximal specific synthetic and the maximal specific hydrolytic activities measured in the experiments using VinOLau are projected in figure 18 as well. As described before, contradicting trends are shown, with the synthetic activity being the highest at low water activities, while the hydrolytic activity increases with increasing water activity.

The measured yields and activities can be explained by summing up all discussed results. The higher synthetic and the reduced hydrolytic activity at low water activity led to high glycolipid yields, since the reaction equilibrium between synthesis and hydrolysis was shifted towards synthesis. The low yields at high water activities were due to a dominant hydrolytic activity of the lipase, what likely led low synthesis rates and the hydrolysis of eventually synthesized glycolipids. Thus, the effect of the water activity on the lipase activity clearly was demonstrated. In order to optimize the synthesis of amino sugar fatty acid esters, the water activity of 2m2b should be as low as 0.01 or even lower.

In addition, these results can partially be compared to the syntheses of GlcNAc-6-O-Hex described in chapter III.B. Carried out under the same conditions the experiments only differed in the fatty acid esters employed. The positive effects of using vinyl fatty acid esters could clearly be demonstrated resulting in approximately 25-fold higher specific activities and yields, when compared to experiments using methyl hexanoate. This distinct enhancement of the synthesis can be explained by formation of acetaldehyde as side product of the transesterification instead of methanol. While methanol is known to have a negative effect in the enzymatic synthesis, acetaldehyde already evaporates at 20°C, thus being permanently removed from the reaction mixture.

d) Influence of the temperature on the synthesis

In order to examine the influence of the reaction temperature on crucial reaction parameters like specific enzyme activity and overall yield, experiments have been performed at different temperatures ranging from 30°C to 80°C. To be able to detect possible influences of the temperature, these experiments were modified from the ones described before, since the experiments using VinOHex and VinOLau already reached yields of 100 % in previous experiments at 40°C. Thus, in the experiments investigating the influence of the reaction temperature, the added molecular sieves were only activated one hour at 100°C to diminish their positive effect on the synthesis.

Figure 19 shows exemplarily the time courses of the transesterification between GlcNAc and VinOLau in 2m2b at six different temperatures.

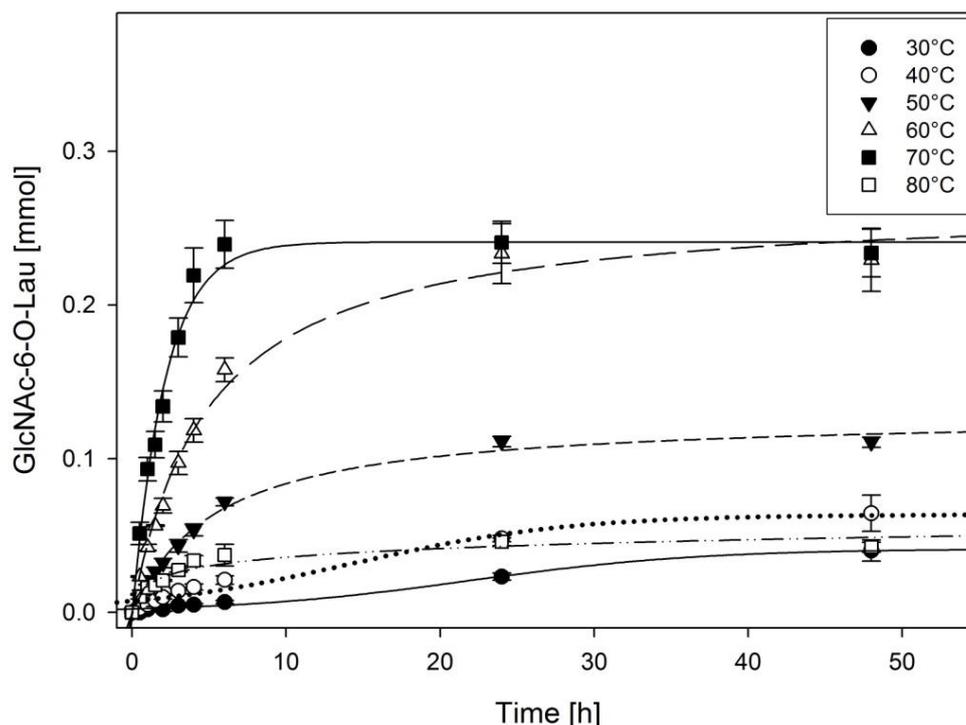


Figure 19: Time course of the transesterifications between N-acetyl glucosamine and vinyl laurate in 2m2b using Novozyme 435® at 300 rpm and 30°C (filled circles), 40°C (empty circles), 50°C (filled triangles), 60°C (empty triangles), 70°C (filled squares) and 80°C (empty squares).

Increasing GlcNAc-6-O-Lau concentrations confirmed transesterifications taking place at every temperature tested. The experiments carried out at 30°C and 80°C yielded approximately 0.04 mmol GlcNAc-6-O-Lau, while experiments at 40°C resulted in 0.06 mmol GlcNAc-6-O-Lau. When performing the transesterification at 50°C 0.1 mmol GlcNAc-6-O-Lau were obtained. The experiment carried out at 60°C and 70°C both yielded ca. 0.22 mmol GlcNAc-6-O-Lau. The GlcNAc-6-O-Lau concentration increased the fastest during the first 6 hours when the transesterification was carried out at 70°C.

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The variation of the reaction temperature showed a major impact on the reaction, resulting in very diverse time courses of the different experiments. During the first six hours in the experiments carried out at 30°C and 40°C the GlcNAc-6-O-Lau concentration only increased to values of approximately 0.02 mmol. After 48 hours the experiments performed at 30°C yielded in a concentration of ca. 0.04 mmol, while in the experiment carried out at 40°C ca. 0.06 mmol GlcNAc-6-O-Lau were synthesized. This proved two things. Firstly, that at these temperatures the yield increased when applying the higher temperature and secondly, when compared to the experiments with molecular sieves described before (see figure 15) lower GlcNAc-6-O-Lau concentrations were obtained. Thus, also the yield was lower, likely due to the different treatment of the molecular sieves, which made it possible to detect influences of the reaction temperature after all.

When comparing the time courses of the reactions carried out at 50°C, 60°C and 70°C, further increasing yields were observed. While at 50°C 0.1 mmol GlcNAc-6-O-Lau were gained after 48 hours, the experiments at 60°C and 70°C resulted both in approximately 0.22 mmol after 48 hours. Additionally, increasing initial reaction rates were observed with increasing temperature. The reaction at 70°C showed the fastest incline in GlcNAc-6-O-Lau concentration during the first six hours, after which the concentration seemed to decrease slightly.

During the first three hours of the experiments carried out at 80°C values similar to the ones obtained from the experiment carried out at 50°C were measured. After that the time course flattened, resulting in a yield of approximately 0.04 mmol GlcNAc-6-O-Lau after 48 hours, a concentration that was already reached after six hours. This decline in initial reaction rate and overall yield compared to the experiment carried out at 70°C, demonstrated a distinct influence of the temperature, whether on the enzymes activity or its stability.

For further examination, the maximal specific synthetic activity of the enzyme at different temperatures is shown in figure 20.

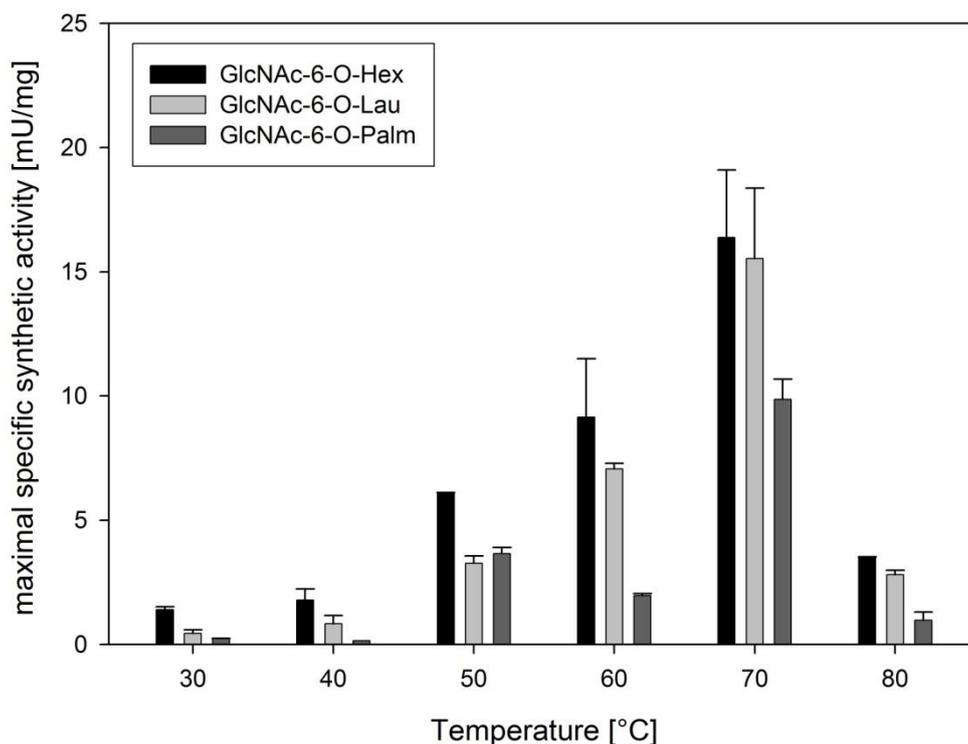


Figure 20: Maximal specific synthetic activities of Novozyme 435[®] during the transesterifications between N-acetyl glucosamine and vinyl hexanoate (black bars), vinyl laurate (light grey bars) and vinyl palmitate (dark grey bars) in 2m2b at 300 rpm and different reaction temperatures.

Experiments with different fatty acid vinyl esters all presented the same trend of increasing maximal specific synthetic activities with temperatures increasing up to 70°C. Highest activities of ca. 15 mU/mg and 16 mU/mg were detected when employing VinOHex and VinOLau in experiments carried out at 70°C. Increasing the temperature to 80°C led to maximal specific synthetic activities not exceeding 5 mU/mg.

The specific synthetic activities presented in figure 20 concur with the results shown in figure 19, by exhibiting increasing activities with increasing temperatures. While the calculated activities at 30°C and 40°C were 1 – 2 mU/mg, values of approximately 6 mU/mg were reached in the experiments with VinOHex carried out at 50°C. The synthetic activities of the experiments carried out at 60°C varied when comparing the results obtained using different fatty acid esters. While the maximal specific synthetic activity was approximately 10 mU/mg when using VinOHex, only 2 mU/mg were detected when using VinOPalm. The highest maximal specific synthetic activities for all variations tested were found in experiments carried out at 70°C, with maximal specific synthetic activities of approximately 15 mU/mg for the experiments using VinOHex or VinOLau.

When compared to the experiments described in section III.C.3.c) it was observed that although the maximal specific synthetic activities were lower in these experiments, the overall yield was higher. This could be explained with the fact that in these experiments almost every synthesis reached its equilibrium after 48 hours, while the modified experiments performed to examine the influence of the temperature already reached their

equilibrium after 6 – 24 hours. This might be caused by the different treatment of the applied molecular sieves. Congruently to the results described in III.C.3.c) it was found that short chained fatty acid esters are converted faster by the lipase than medium or long chain fatty acids.

The activities obtained at 80°C were distinct lower, not exceeding 5 mU/mg for any fatty acid ester used. Since these finding might as well have indicated a loss of activity at 80°C, the hydrolytic activity was determined for every experiment as well. The maximal specific hydrolytic activities of every synthesis at different temperatures are shown in figure 21.

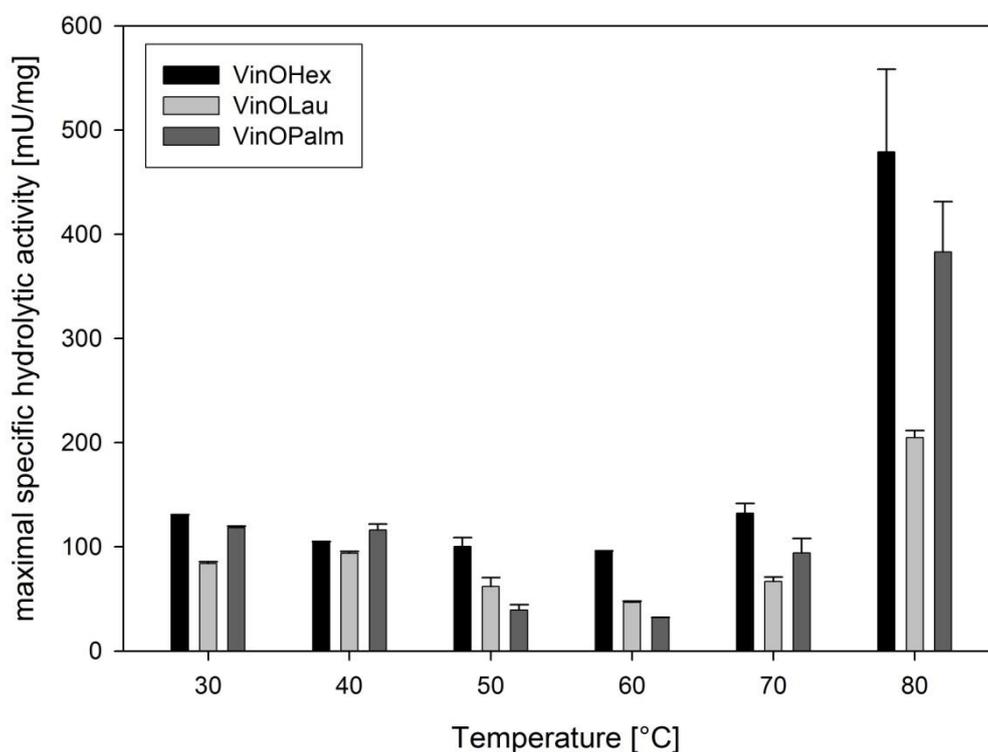


Figure 21: Maximal specific hydrolytic activities of Novozyme 435[®] using vinyl hexanoate (black bars) , vinyl laurate (light grey bars) and vinyl palmitate (dark grey bars) for transesterification with GlcNAc in 2m2b 300rpm and different reaction temperatures.

Experiments with different fatty acid vinyl esters all presented initial specific hydrolytic activities of ca. 100 mU/mg up to 70°C. Experiments using VinOLau and VinOPalm resulted in decreasing hydrolytic activities in experiments carried out at 50°C and 60°C. Highest hydrolytic activities of more than 400 mU/mg were measured when employing VinOHex and VinOPalm in experiments carried out at 80°C.

Similar to the findings described in III.C.3.c), all calculated maximal specific hydrolytic activities were higher than all maximal specific synthetic activities described in figure 20. But while the synthetic activities increased with increasing temperature, the hydrolytic activities decreased. The experiments using VinOHex exhibited relative stable hydrolytic activities of approximately 100 mU/mg at temperatures between 30°C and 70°C. The measured activity regarding the hydrolysis of VinOPalm decreased from ca. 120 mU/mg detected in the

experiment carried out at 30°C to ca. 50 mU/mg measured in the experiment carried out at 60°C. In all experiments performed at 70°C higher hydrolytic activities were observed than compared to experiments at lower temperatures, a trend which got even more explicit in the experiments carried out at 80°C. These experiments resulted in the highest activities measured, ranging between 200 mU/mg when employing VinOLau to almost 500mU/mg when using VinOHex. Thus, the lipases possible loss of activity at 80°C was clearly confuted.

The obtained overall yields of all experiments, together with the exemplary synthetic and hydrolytic activity from the experiments using VinOLau is shown in figure 22.

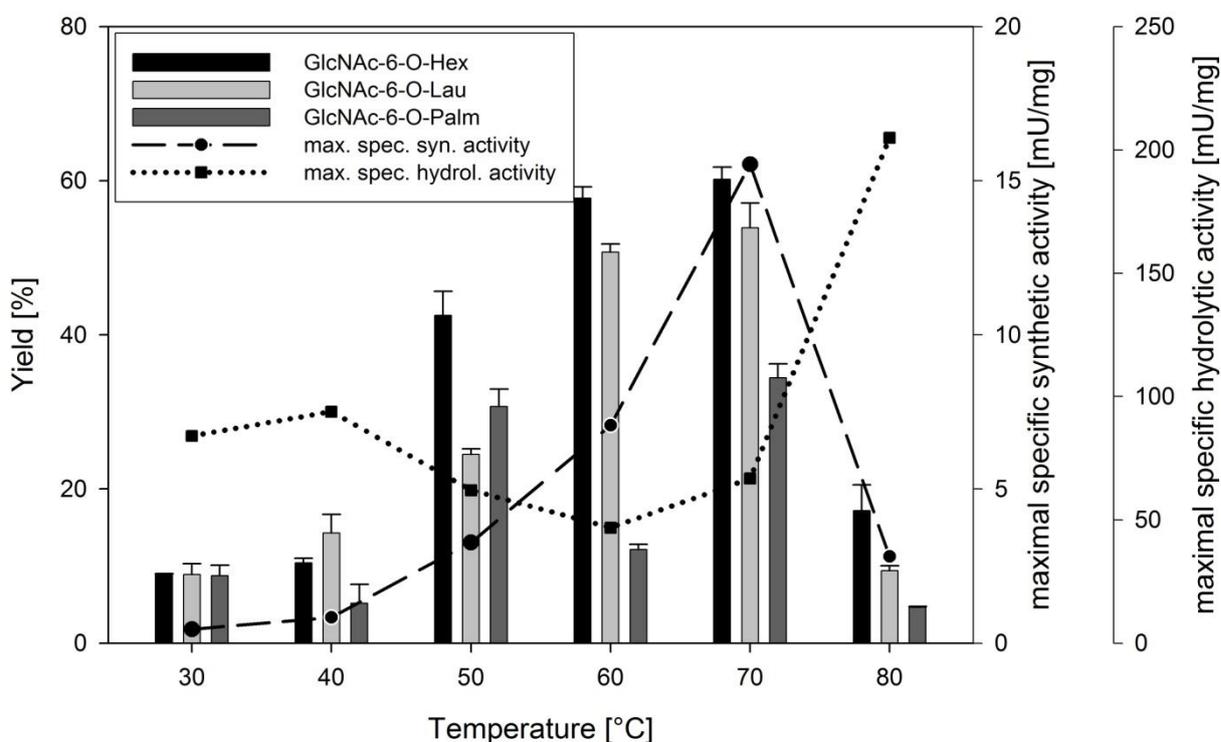


Figure 22: Yields of the transesterifications between N-acetyl glucosamine and vinyl hexanoate (black bars), vinyl laurate (light grey bars) and vinyl palmitate (dark grey bars) using Novozyme 435[®] in 2m2b at 300 rpm and different temperatures.

The experiments carried out at 30°C and 40°C resulted in yields lower than 20%. The experiments performed at 50°C yielded conversions of approximately 40% when employing VinOHex and 25 -30 % when using VinOLau and VinOPalm. Highest yields were obtained when using VinOHex in transesterification carried out at 60°C and 70°C, resulting in the acylation of approximately 60% of all employed amino sugars. The transesterification using VinOLau led to slightly lower yields, while with the use of VinOPalm the yield decreased to approximately 15% (60°C) and 40% (70°C). The experiments carried out at 80°C didn't exceed yields of more than 20%.

The overall yields shown in figure 22 clearly demonstrated the influence of the reaction temperature on the enzymatic synthesis. In almost all experiments performed at temperatures between 30°C and 60°C the higher

yields were obtained with increasing temperature. Only the yields calculated from the experiments at 40°C and 60°C did not follow this trend when applying VinOPalm.

The highest yields of all experiments were reached in the experiments carried out at 70°C, with approximately 60% of the amino sugars acylated. The experiments carried out at 80°C yielded conversions of 20% when using VinOHex and approximately 12% and 5% when applying VinOLau and VinOPalm respectively. This confirmed the results found by comparing the maximal specific synthetic activities in figure 20.

A possible explanation therefore is illustrated by the additionally presented synthetic and hydrolytic activities from the corresponding experiments with VinOLau. It is shown that at lower temperatures, the maximal specific synthetic activity is rather low, while the maximal specific hydrolytic was approximately 100 mU/mg. This might have led to low yields due to a shift in the enzymes activity towards hydrolysis, possibly causing hydrolysis of the eventually formed glycolipids. When increasing the temperature, the hydrolytic activity decreased, while at the same time the synthetic activity increased, having its maximum at 70°C. This resulted in the highest yields at 70°C, where the synthetic activity was at its highest, while the hydrolytic activity measured was still comparable low.

The experiments performed at 80°C then showed, that the lipase did not lose any activity at this temperature. Instead the hydrolytic activity was the highest being approximately 200 mU/mg, while the corresponding synthetic activity was ca. 3 mU/mg. This led to lower yields, due to the fact that the equilibrium of this reaction was shifted towards hydrolysis.

The results obtained from the experiments carried out at 50°C support this finding. The measured maximal specific synthetic activity in these experiments was ca. 3mU/mg when using VinOLau, a value close to the one measured at 80°C. But in contrary to the experiments at 80°C the maximal specific hydrolytic activity was comparable low, being approximately 60 mU/mg, meaning that in the experiments at 50°C the equilibrium of the synthesis was shifted more towards synthesis. This resulted in higher yield of 25% for the experiments carried out at 50°C. Thus, it was shown that the reaction temperature has a major impact on the enzymatic synthesis, by determining whether hydrolysis or synthesis is favored by the lipase.

e) Structure elucidation

In order to confirm the proposed structures of N-acetyl glucosamine-6-O-laurate and N-acetyl glucosamine-6-O-palmitate 1D- ^1H , 1D- ^{13}C , 2D $^1\text{H}^1\text{H}$ COSY, 2D- $^1\text{H}^{13}\text{C}$ HSQC and 2D- $^1\text{H}^{13}\text{C}$ HMBC measurements were performed. By comparison between these different spectra assignment of the gained signals from the ^1H - and ^{13}C spectra to specific atoms or atom groups was possible. The presence of the postulated ester linkage at C6 was proven by $^1\text{H}^{13}\text{C}$ HMBC where couplings between C7 and H6/6' were detected for both glycolipids. Also the missing of couplings between C7 and H4, H3 or H1 confirmed the proposed structures. In addition couplings between H 19 and C20 and between H2 and H20 confirm the presence of an amid bond at C2 for N-acetyl glucosamine-6-O-laurat. For N-acetyl glucosamine-6-O-palmitate couplings between H23 and C24 as well as between H2 and H23 confirm the presence of an amid bond at C2. Thus, the proposed structures were validated. Detailed chemical shift assignment and the corresponding spectra are given in the appendix.

f) Hydrophilic-lipophilic balance

With the chemical structure confirmed for both glycolipids, it was possible to calculate their corresponding HLB value, which was 10.96 for GlcNAc-6-O-Lau and 9.62 for GlcNAc-6-O-Palm. Thus, these glycolipids are more hydrophobic than the GlcNAc-6-O-Hex and GlcNBu-6-O-Hex, which were described before. With HLB values of around 10 GlcNAc-6-O-Lau and GlcNAc-6-O-Palm might not exhibit classical detergent properties, but might more serve as water in oil emulsifiers.

g) Critical micelle concentration

The critical micelle concentration of GlcNAc-6-O-Hex was described before in chapter III.B. Since both, GlcNAc-6-O-Lau and GlcNAc-6-O-Palm are only poorly soluble in water, determination of the critical micelle concentrations was not feasible.

5. Conclusions

Lipase-catalyzed transesterifications using GlcNAc and three fatty acid vinyl esters with varying chain length led to the formation of novel amino sugar fatty acid esters, namely 2-(acetylamino)-2-deoxy-6-O-laurate-D-glucose and 2-(acetylamino)-2-deoxy-6-O-palmiate-D-glucose. The structure of both new amino sugar fatty acid esters could be elucidated via NMR, showing that it was possible to monoacylate the amino sugar GlcNAc with fatty acids having different chain lengths by employing Novozyme 435[®] in 2m2b.

Crucial reaction parameters like the substrate ratio, the initial water activity of the reaction media and the reaction temperature were varied to determine their influence on the synthesis.

By examining three different ratios between amino sugar and fatty acid ester it could be shown that highest yields were obtained by employing an excess of fatty acid vinyl ester. Thus, from the tested variants, the ratio between amino sugar and fatty acid ester of 1 : 5.5 gave the best results. By employing more fatty acid esters per reaction the synthesis might be favored even more, but already the ratio of 1 : 5.5 resulted in yields of 100% for certain experiments. Applying even higher fatty acid ester concentrations might further improve the syntheses not achieving complete conversion, but high ratios might lead to possible substrate inhibition of the enzyme and higher costs.

The well-known impact of water on the enzymatic synthesis in organic solvents was investigated as well. The results confirmed that the water activity of the solvent has a distinct influence on whether the enzymes hydrolyzes the fatty acid vinyl esters employed or performs a transesterification. Thus, the highest synthetic activity of the enzyme was observed when carrying out the experiments at the comparably lowest water activity which was achieved by adding molecular sieves to the reaction. In all experiments it could be shown that the hydrolytic and the synthetic of the enzyme are connected, with synthetic activities being the highest when the hydrolytic activity was the lowest. The experiments with 2m2b pre-treated with saturated salt solutions only gained low yields of amino sugar fatty acid esters. Although described differently in literature for different lipases, the findings of the lowest hydrolytic activities measured at the lowest water activity only seem reasonable. Additionally the fact that the used Novozyme 435[®] not only remains its stability and flexibility at a low water activity, but also exhibits its highest synthetic activity, approves the choice of this enzyme in the first place.

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The influence of the reaction temperature was examined by performing experiments at temperatures between 30°C and 80°C. These experiments showed that the highest yields were measured when performing the synthesis at 70°C. At 70°C the synthetic activity was the highest, while the corresponding hydrolytic activity was comparable low, what led to high yields. Further it was shown that the experiments carried out at 80°C resulted in much lower yields. This was not due to the enzyme losing its activity, but due to fact that the equilibrium of these experiments was shifted towards hydrolysis, with the highest hydrolytic activities measured of all experiments performed. Thus, it could be shown that not only the initial water activity of the solvent determines whether hydrolysis or transesterification is performed by the enzyme, also the temperature has an influence on this important reaction parameter, which is a new gained insight into the lipases activity when synthesizing glycolipids.

All described finding allow the characterization and optimization of the synthesis of amino sugar fatty acid esters and might furthermore be beneficial to establish and optimize other lipase-catalyzed syntheses in organic solvents.

D. Lipase-catalyzed synthesis of glucose-6-*O*-hexanoate in deep eutectic solvents

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This manuscript was submitted for publication to “European Journal of Lipid Science and Technology”.

Author’s contribution to this manuscript:

Martin Pöhnlein designed this study, developed the methodology, collected and evaluated the data and wrote the manuscript.

Jonas Ulrich assisted in performing experiments as student worker supervised by Martin Pöhnlein.

Frank Kirschhöfer performed the ESI-Q-ToF measurements and proofread the manuscript.

Michael Nusser performed the tandem mass spectrometry measurements and proofread the manuscript.

Dr. Claudia Muhle-Goll performed the NMR analyses and proofread the manuscript.

Bastian Kannengiesser proofread the manuscript.

Dr. Gerald Brenner-Weiß proofread the manuscript.

Professor Burkard Luy proofread the manuscript.

Professor Andreas Liese proofread the manuscript.

Professor Christoph Syldatk supervised the project and proofread the manuscript.

Professor Rudolf Hausmann supervised the project and proofread the manuscript.

1. Abstract

Enzymatic synthesis of sugar fatty acid esters in organic solvents is a well-described procedure to synthesize glycolipids. This study aims at replacing these solvents with deep eutectic solvents (DES), a group of solvents that gained more and more interest during the last years, since they can be easily produced from non-toxic resources. The enzymatic synthesis of glycolipids using the substrates glucose and vinyl hexanoate in deep eutectic solvents was investigated, employing *Candida antarctica* lipase B (Novozyme 435[®]) in a total of six different deep eutectic solvents. A lipase-catalyzed synthesis of glucose fatty acid esters was observed in two different deep eutectic solvents consisting of choline chloride and urea (CC : U) and choline chloride and glucose (CC : Glc). Additionally the DES consisting of choline chloride and glucose was observed to act as solvent and substrate for the synthesis at the same time.

2. Introduction

Lipases (E.C. 3.1.1.3, triacylglycerol acyl hydrolases) are hydrolases that act on ester bonds between a fatty acid and an alcohol. They are widely used in many industrial biotechnological processes today. Since the 1980s it is known, that amongst other enzymes, lipases are able to reverse their hydrolytic activity in anhydrous organic solvent, thus being able to synthesize ester bonds [32]. The enzymatic synthesis of sugar fatty acid esters has been investigated in numerous experiments since, employing different substrates and lipases in various organic solvents [64] [76]. A common problem of most approaches is the low solubility of sugars in organic solvents. This and the fact that organic solvents are not necessarily regarded as environmentally friendly, led to efforts which focused on solvent-free syntheses or experiments in ionic liquids [102] [44].

Seldom applied types of solvents are the so called deep eutectic solvents (DES), which gained more and more attention since their definition in 2003. DES typically consist of a mixture of an ammonium salt and a hydrogen-bond donor. Thereby the ammonium anion interacts with the hydrogen-bond donor, leading to a significant depression of the melting point of the mixture, resulting in the formation of a liquid [69]. Compared to similar solvents like ionic liquids DES are not limited by poor biodegradability, toxicity or high cost [68].

Recent publications demonstrated the use of DES for enzyme-catalyzed reactions, proving that lipase B from *Candida antarctica* (Novozyme 435[®]) was able to perform transesterification reactions in these media [103]. Thus, it might be possible to synthesize glycolipids in DES by using lipases. Furthermore the ability of several DES to dissolve hydrophilic sugars and hydrophobic fatty acid derivatives at the same time might be beneficial to this reaction. In addition it is possible to use tailored DES to enhance the enzymatic reaction even further, by improving the substrate solubility or enzyme stability.

Within this work the enzymatic synthesis of glucose fatty acid esters in six different DES was investigated.

3. Methods & Materials

a) Materials

Lipase B from *Candida antarctica*, immobilized on acrylic resin (Novozyme 435[®]), betaine and choline chloride were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Glucose (anhydrous), glycerol, ethylene glycol, urea, methanol, acetic acid and ethyl acetate were purchased from Carl Roth (Carl Roth KG, Karlsruhe, Germany). Vinyl n-hexanoate was purchased from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium).

b) Methods

(1) Preparation of deep eutectic solvents

All DES listed in table 12 were prepared by mixing the named ammonium salts and hydrogen-bond donors in the given molar ratio under constant stirring using a stirring rod and heating to approximately 100°C until a clear liquid was formed.

Table 12: Deep eutectic solvents used for glycolipid synthesis experiments

DES	Ammonium salt (AS)	Hydrogen-bond donor (HBD)	Molar ratio (AS : HBD)
CC : U	Choline chloride	Urea	1 : 2
CC : Gly	Choline chloride	Glycerol	1 : 2
CC : Glc	Choline chloride	Glucose (anhydrous)	1 : 1
CC : EtG	Choline chloride	Ethylene glycol	1 : 2
CC : EtG	Choline chloride	Ethylene glycol	1 : 1
B : EtG	Betaine	Ethylene glycol	1 : 2

(2) *Glycolipid synthesis in deep eutectic solvents*

250 mg glucose (1.38 mmol) and 400 μ L vinyl hexanoate (2.72 mmol) were dissolved in 3.5 mL of the corresponding DES in a glass tube. The reaction was started by the addition of 100 mg of Lipase (Novozyme 435®) and incubated for three days at 70 °C and 1500 rpm in an orbital shaker. All experiments including negative controls without enzyme or carbohydrate were carried out as triplicates in all DES. Samples from each reaction were drawn at timed intervals to follow a possible reaction as shown in Figure 23.

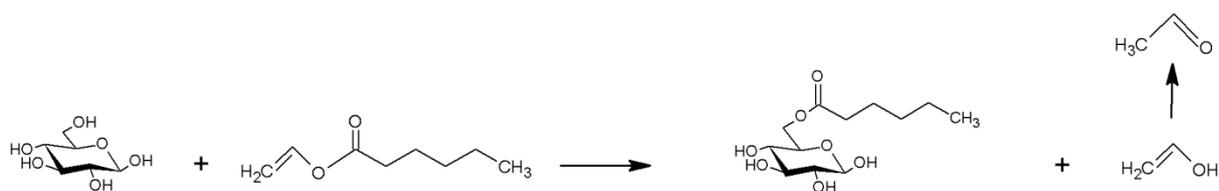


Figure 23: Possible transesterification between glucose and vinyl hexanoate which leads to the formation of glucose hexanoate and a vinyl alcohol.

Exemplary shown is the transesterification leading to glucose-6-O-hexanoate. The acylation might also take place at any other hydroxyl groups or at multiple hydroxyl groups in the same glucose molecule. The formed vinyl alcohol will tautomerize into acetaldehyde.

(3) *Glycolipid extraction*

In order to follow the progress of the glycolipid synthesis by thin layer chromatography, all reaction samples were extracted by the following scheme. 150 μ L DES sample was withdrawn from the reaction and dissolved in 150 μ L of ddH₂O by shaking each sample at 1500 rpm and 70 °C until full dissolution of the DES was achieved. After the addition of 350 μ L ethyl acetate extraction was reached by vigorous mixing for one minute. Afterwards the ethyl acetate phase was separated and analyzed.

(4) *Detection of glycolipids using thin layer chromatography*

Thin layer chromatography was performed for qualitative analysis. 10 μ l of extracted samples were applied onto an analytical silica gel 60 TLC plate (10 x 20 cm, film thickness 0.25 mm). The plates were developed in a mobile phase consisting of chloroform : methanol : acetic acid (65 : 15 : 2, by vol.). Visualization was

accomplished by dipping the plate in an anis aldehyde solution (anis aldehyde : sulfuric acid : acetic acid 0,5 : 1 : 100, by vol.) followed by heating at 220°C under constant air flow for about 5 min.

(5) *Determination of the accurate masses via electro spray ionization quadrupole time of flight mass spectrometry (ESI-Q-ToF)*

Mass determination of extracted samples was performed using ESI-Q-ToF (Q-Star Pulsar, AB SCIEX) in positive measuring mode. Samples were diluted 1:100 in a solvent mixture consisting of methanol : 0,1 % acetic acid (50 : 50, by vol.). After mixing, samples were continuously infused via a syringe pump at a flow of 10 µL/min. Heater temperature was 350°C; spray tip voltage 5000V, declustering potential 30 V and focusing potential was 60 V. Nebulizer gas was nitrogen 5.0, curtain gas was nitrogen 5.0. Spectra were recorded in ToF - MS mode in a mass range from m/z 100 to m/z 1000 in the activated “enhance all” mode and an accumulation time of one second.

(6) *Structural characterization via tandem mass spectrometry*

Tandem mass spectrometry MS/MS was performed by using a tandem mass spectrometer (API 4000, Applied Biosystems, MDS Sciex, Toronto Canada) equipped with an electro spray ionization source (ESI) in positive ionization mode. Samples were diluted depending on their concentration in a solvent mixture consisting of methanol : 10mM NH₄OAc (50 : 50, by vol.). After mixing, samples were used for infusion experiments and measured in full scan modus (Q1 scan) as well as in specific MS/MS experiments (product ion scan, PIC). Samples were infused into the ESI source using a syringe pump (Havard Apparatus Inc., South Natick, USA) at a flow rate of 10 µL/min. Adjustment of the instrument was as follows: Ion spray voltage: 4800V, declustering potential: 60V, entrance potential: 10V. MS/MS experiments were optimized using the compound optimization mode of the Analyst 1.6. software (Applied Biosystems, MDS Sciex, Toronto, Canada).

(7) *Structure elucidation via nuclear magnetic resonance spectroscopy*

22 mg of purified glycolipids were dissolved in 0.8 ml CDCl₃ / CD₃OD (70:30 v/v) One dimensional ¹H NMR spectroscopy and two dimensional ¹H-¹H double quantum filtered correlation spectroscopy (DQF-COSY), nuclear Overhauser effect spectroscopy (NOESY) with 800 ms mixing time, ¹H-¹³C heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded on a Bruker AVANCE II+ 600 MHz spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a BBI probe head. Spectra were analyzed with Topspin 3.2 (Bruker AG). Intensities were measured from a 1D ¹H spectrum acquired with a single scan. Chemical shifts are referenced to the ¹H and ¹³C resonances of tetramethylsilan.

4. Results and Discussion

a) *Detection of glycolipids using thin layer chromatography*

In order to monitor the possible formation of glycolipids all samples were analyzed via TLC after extraction. After separation glycolipids can be visualized with comparable glycolipids consisting of one hexanoate moiety bound to one sugar molecule having a retention factor (Rf) of approximately 0.4. The results of TLC analyses of all experiments are summarized in table 13.

Table 13: Retention factor of bands detected in extracted reaction samples via TLC

Experiment	Rf of bands detected
CC : U	0.25; 0.40; 0.66
CC : U – Blank without lipase	-
CC : U – Blank without add. glucose	-
CC : Gly	0.37
CC : Gly – Blank without lipase	-
CC : Gly – Blank without add. glucose	-
CC : Glc	0.40
CC : Glc – Blank without lipase	-
CC : Glc – Blank without add. glucose	0.40
CC : EtG (1:2)	-
CC : EtG (1:2) – Blank without lipase	-
CC : EtG (1:2) – Blank without add. glucose	-
CC : EtG (1:1)	-
CC : EtG (1:1) – Blank without lipase	-
CC : EtG (1:1) – Blank without add. glucose	-
B : EtG	-
B : EtG – Blank without lipase	-
B : EtG – Blank without add. glucose	-

Out of all tested combinations of DES, lipase and substrate only the thin layer chromatography of the experiments carried out in CC : U, CC : Gly and CC : Glc, including the blank without additional glucose,

showed bands with a similar retention factor than glycolipids. Thus a possible glycolipids synthesis might be assumed for these four experiments. For the experiment in CC: U a total of three bands was detected, indicating possible side products like diacylated glycolipids or impurities. Since TLC only provides qualitatively results, those samples possibly containing glycolipids have been characterized further by mass determination using electro spray ionization quadrupole time of flight mass spectrometry.

b) Determination of the accurate masses via ESI-Q-ToF

To confirm the formation of glycolipids, the extracts of the four experiments, which exhibited positive results by TLC, were analyzed performing electro spray ionization quadrupole time of flight mass spectrometry, which allows the determination of the accurate molecular masses of the compounds contained. These detected m/z values are listed in table 14.

Table 14: m/z values detected via ESI-Q-ToF MS. Masses compared to glucose-6-*O*-hexanoate with a theoretical, neutral, monoisotopic molar mass of 278.13 Da (=M).

Experiment	m/z values detected via ESI-Q- ToF	Possible correspondent
CC : U	202.08	Choline - hexanoate
	279.13	M + H ⁺
CC : Gly	173.01	-
	202.08	Choline - hexanoate
	271.13	M + Na ⁺ - CHO
CC : Glc	202.09	Choline - hexanoate
	271.12	M + Na ⁺ - CHO
	301.08	M + Na ⁺
	399.27	diacylated glucose + Na ⁺
CC : Glc – Blank without add. glucose	202.09	Choline - hexanoate
	243.04	M – 2 x H ₂ O
	271.12	M + Na ⁺ - CHO
	281.11	-
	301.08	M + Na ⁺
	399.26	diacylated glucose + Na ⁺

The proposed transesterification shown in figure 23 might lead to glucose-6-*O*-hexanoate with a molecular mass of 278.13 Da, thus when measuring in positive mode m/z values of 279.13 (M + H⁺) and 301.13 (M + Na⁺) would confirm the presence of these glycolipids. As shown in table 3, matching masses could be observed in the three experiments using CC : U and CC : Glc, including the blank without additional glucose in CC : Glc. Noticeable thereby is the fact, that in the experiments carried out in CC : U ions of m/z 279.13 (M + H⁺) were detected, whereas ions of m/z 301.08 representing the possible sodium adduct (M + Na⁺) were observed in the experiments carried out in CC : Glc. Both values would represent glycolipids, but the absence of m/z 301.08 in the samples from CC : U raised the question if these detected signals truly proved the presence of glycolipids, since m/z signals of approximately 279 can also be explained by known impurities like phthalate derivatives.

Additionally several other signals were detected in most experiments, which might also explain the additional bands detected by TLC. These signals are very likely due to the poor purification of the extracts. A possible explanation of the m/z signal of 202.09, which was measured in all experiments, is the formation of choline hexanoate. Since choline also contains a free hydroxyl group it might act as a substrate for a lipase-catalyzed transesterification as well, indicating lipase activity in all four tested DES. The signal of m/z 399.27 detected in the two experiments carried out in CC : Glc indicates the formation of a diacylated glycolipid, which would have a molecular mass of 376.44 Da. Thus the m/z signals of 399.27 might represent the sodium adduct of diacylated glycolipids. The m/z signal of 271.12 was detected in the extracts of three experiments, including those in CC : Gly. A possible explanation for this observation might be the sodium adduct of glucose-*O*-hexanoate lacking a CHO fragment. This finding would also indicate lipase activity in these DES, albeit not proving it.

c) Structural characterization via tandem mass spectrometry

In order to verify the presence of monoacylated glycolipids the three samples from the experiments in CC . U and CC : Glc, which possibly contained glycolipids, were analyzed via tandem mass spectrometry. MS/MS experiments of masses corresponding to possible glycolipids should lead to specific fragments indicating the proposed molecules. In addition to the protonated molecule ions ($M + H^+$) charged ammonium as well as sodium adducts of molecules should be considered due to the different solvent composition. The results of all MS/MS experiments are listed in table 15.

Table 15: m/z signals and fragments detected using tandem mass spectrometry MS/MS. Masses compared to glucose-*O*-hexanoate with a theoretical, neutral, monoisotopic molar mass of 278.13 Da (=M).

Experiment	m/z signals fragmented [possible correspondent]	m/z values detected after fragmentation	Possible explanation of the fragment
CC : U	296.2 [M + NH ₄ ⁺]	296.2.	M + NH ₄ ⁺
		279.0	M + H ⁺
		261.2	M – H ₂ O
		243.2	M – 2 x H ₂ O
		99.0	Hexanoate residue
CC : Glc	296.2 [M + NH ₄ ⁺]	296.0	M + NH ₄ ⁺
		279.2	M + H ⁺
		261.0	M – H ₂ O
		243.0	M – 2 x H ₂ O
		99.0	Hexanoate residue
CC : Glc – Blank without add. glucose	296.3 [M + NH ₄ ⁺]	296.0	M + NH ₄ ⁺
		279.0	M + H ⁺
		261.2	M – H ₂ O
		243.0	M – 2 x H ₂ O
		99.0	Hexanoate residue

In all three examined samples signals corresponding to glucose-6-*O*-hexanoate were detected. Exemplary depicted in table 4 are the m/z values of 296.2 detected in each Q1 scan prior to fragmentation. Furthermore in all three experiments other m/z values possibly representing glucose-6-*O*-hexanoate, like 279.3 (M + H⁺) and 301.2 (M + Na⁺) were observed as well. Since upon fragmentation all mentioned signals revealed the same fragmentation patterns the fragmentation of the most intensive adduct mass m/z 296.2 will be discussed exemplarily.

In all MS/MS experiments the same fragments could be detected by analyzing m/z 296.2. Besides the precursor ion at m/z 296.2, several other characteristic fragments for glucose-*O*-hexanoate were detected. Additionally as also described in table 4, it was possible to assign reasonable explanations to the five main fragments of the assumed glycolipid for every sample, like glycolipids minus one or two water molecules (m/z 261.2 and m/z 243.0) or residues of the hexanoate (m/z 99.0). Thus, the presence of glucose-hexanoate was confirmed, proving

that glucose-6-*O*-hexanoate was synthesized in the DES CC : U and CC : Glc, including the blank without additional glucose in CC : Glc. In order to completely elucidate the chemical structure of the obtained glycolipids samples were analyzed using NMR.

d) Confirmation of glucose-6-O-hexanoate by nuclear magnetic resonance spectroscopy (NMR)

One and two-dimensional ^1H and ^{13}C NMR spectra confirmed the presence of a glucose moiety attached to a hexanoate chain. Full chemical shift assignment of the sugar moiety was achieved in ^1H DQF-COSY and $^1\text{H}^{13}\text{C}$ -HSQC spectra. The acyl chain is attached to C6 of the glucose moiety clearly indicated by a cross peak between the carbonyl atom at 174.5 ppm and the two protons attached to C6 at 4.27 and 4.37 ppm in the $^1\text{H}^{13}\text{C}$ HMBC spectrum. Thus the presence of glucose-6-*O*-hexanoate was confirmed.

Besides glucose-6-*O*-hexanoate NMR also revealed the presence of a second molecule in the examined sample, namely 4,6-methyliden glucose, which was not detected using mass spectrometry. This acetal is likely formed during the synthesis reaction between glucose and acetaldehyde, the tautomer of vinylalcohol. For detailed results of the NMR analysis, all mass spectrometry experiments, as well as examples of TLC results; please consider the data shown in the appendix

5. Conclusions

The lipase-catalyzed acylation of glucose in DES is presented, leading to the formation of glucose-6-*O*-hexanoate, which was detected using ESI-Q-ToF and tandem mass spectrometry. Thereby *m/z* signals and fragments corresponding to the proposed glycolipid glucose-6-*O*-hexanoate were observed. Furthermore the chemical structure of glucose-6-*O*-hexanoate was elucidated using NMR. Thus, the known transesterification activity of Novozyme 435 in DES was validated and expanded with carbohydrates as newly described substrates, proving that the enzymatic synthesis of glycolipids is feasible in the deep eutectic solvents CC : U and CC : Glc. Further it was shown that when employing the DES CC : Glc as solvent no additional glucose needs to be added to the reaction, since this DES can function as solvent and substrate at the same time. While it is known for lipase-catalyzed reactions, that the lipophilic substrate can function as substrate and solvent at the same time, this case presents an exception with the polar carbohydrate acting as such.

With DES like CC : U are characterized in detail and known to provide enzyme stability and activity, there are less reports mentioning CC : Glc. The fact that the glucose in the DES CC : Glc was used as substrate might present an option to facilitate the downstream processing, since when glucose is used in the transesterification reaction, it is removed from the eutectic solution which finally will solidify if the molar ratio is changed. During the experiments in CC: Glc without additional glucose beginning solidification was observed, but only led to a higher viscosity of the DES.

IV. CONCLUDING REMARKS AND OUTLOOK

Within this work, aiming at a selectively production of novel glycolipids using amino sugars, several methods and production routes for the enzymatic synthesis of sugar fatty acid esters have been described. By developing and employing a newly described microtiter plate-based 4-nitrophenol assay to detect the synthetic activity of lipases in different organic solvents, it was possible to pre-select those enzyme-solvent combinations showing the highest activity in small scale and time saving manner. Additionally this assay might be beneficial to any kind of screening for synthetic enzyme activity performed in less unpolar organic solvents.

The selected combination of the lipase Novozyme435[®] and 2-methyl-2-butanol was employed to synthesize amino sugar fatty acids esters, using the two amino sugars N-acetyl glucosamine and N-butyryl glucosamine. Both newly gained glycolipids N-acetyl glucosamine-6-O-hexanoate and N-butyryl glucosamine-6-O-hexanoate were described for the first time. By comparing the results of the syntheses using two sugars with different hydrophobicities, the influence of this characteristic could be demonstrated. Thus it could be shown, that the higher hydrophobicity of N-butyryl glucosamine clearly favors the enzymatic synthesis. Additionally was shown, that pre-dissolving any sugar in dimethyl sulfoxide further enhances overall yield and initial reaction rate of the syntheses. These finding showing the importance of the solubility of the sugar might be helpful to further improve and understand enzymatic syntheses in organic solvents. Additionally both novel glycolipids exhibited good surfactant properties and might be suitable for further performance testing.

In order to characterize and to optimize the synthesis of amino sugar fatty acid esters, the influences of critical reaction parameters like the substrate ratio between sugar and fatty acid ester, the initial water activity of the organic solvent and the reaction temperature have been investigated. All named parameters showed a major impact on the synthesis, favoring the reaction most at an excess of fatty acid esters employed, possible low water activity and a reaction temperature of 70°C. Comparison to other related works shows that the influence of these reaction parameter is described differently, e.g. proposing the optimal water activity of 2m2b for the synthesis of fructose palmitate using Novozyme 435[®] being 0.2 [101]. This illustrates that each parameter and its influence on the synthesis has to be tested separately for each combination of substrates, solvents and enzymes.

By employing fatty acid esters with different chain length not only the influence of this reaction parameter could be demonstrated, also the two novel amino sugar fatty acid esters N-acetyl glucosamine-6-O-laurate and N-

IV. CONCLUDING REMARKS AND OUTLOOK

acetyl glucosamine-6-O-palmitate could be synthesized for the first time. Thus a total of four newly described glycolipids, namely amino sugar fatty acid esters have been synthesized within this work.

Glycolipids are used in a wide range of applications, ranging from food, cosmetic and pharmaceutical formulations to classic cleaning products. Further applications may include fields like membrane protein extraction, bioremediation or tertiary oil recovery. Novel glycolipids with tailor-made properties might be useful to improve any of the named applications and widen the diversity of available environmentally friendly surfactants.

Future works regarding the enzymatic synthesis of glycolipids might focus on the use of different sugars and fatty acids or fatty alcohols, ideally gained from renewable resources as well. Several methods described within this work might be employed to establish newly described syntheses, e.g. by facilitating the selection of enzyme and solvent.

To further optimize the synthesis of amino sugar fatty acid esters, all named reaction parameters might be varied, e.g. by employing an even higher excess of fatty acid esters or working at temperature exceeding 80°C. The variation of these reaction parameters might gain even more insights into possible enzyme inhibition or further shifts of activity. Also the use of other techniques to reduce the water activity of the organic solvent, like working *in vacuo*, might be applied, to validate and possibly optimize the synthetic activity of Novozyme 435[®] at even lower water activities. Additionally syntheses in solvent-free reaction systems or liquid two phase systems might be considered.

In order to replace the organic solvent used, experiments have been performed in deep eutectic solvents, a solvents class which has not been described for enzymatic glycolipid syntheses before. Within the six deep eutectic solvents tested, it could be shown that it was possible to synthesize glycolipids in the DES consisting of choline chloride : urea and choline chloride : glucose. In addition to the described glycolipid synthesis, it could be shown that when using choline chloride : glucose no additional glucose has to be added to the reaction, since this DES can act as solvent and as substrate at the same time.

These findings propose a novel synthesis route for the enzymatic synthesis of glycolipids, employing deep eutectic solvents which can be gained by using only biological resources like glucose or urea. Thus deep eutectic might be an ecological alternative to organic solvents.

IV. CONCLUDING REMARKS AND OUTLOOK

For future works in deep eutectic solvents, there are still numerous deep eutectic solvents to be tested for the enzymatic synthesis of glycolipids. In order to quantify the reaction methods facilitating the extraction of glycolipids from the deep eutectic solvents should be developed, enabling the use of e.g. high performance liquid chromatography or gas chromatography.

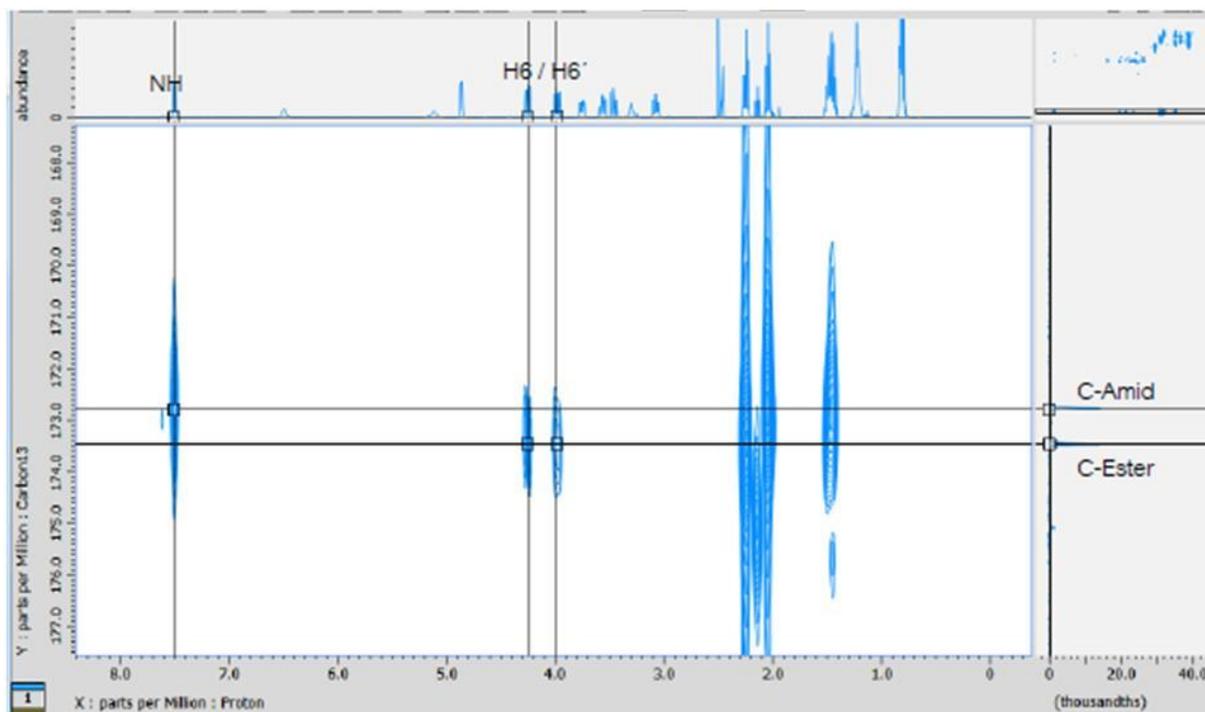
V. APPENDIX

B. Enzymatic synthesis of amino sugar fatty acid esters

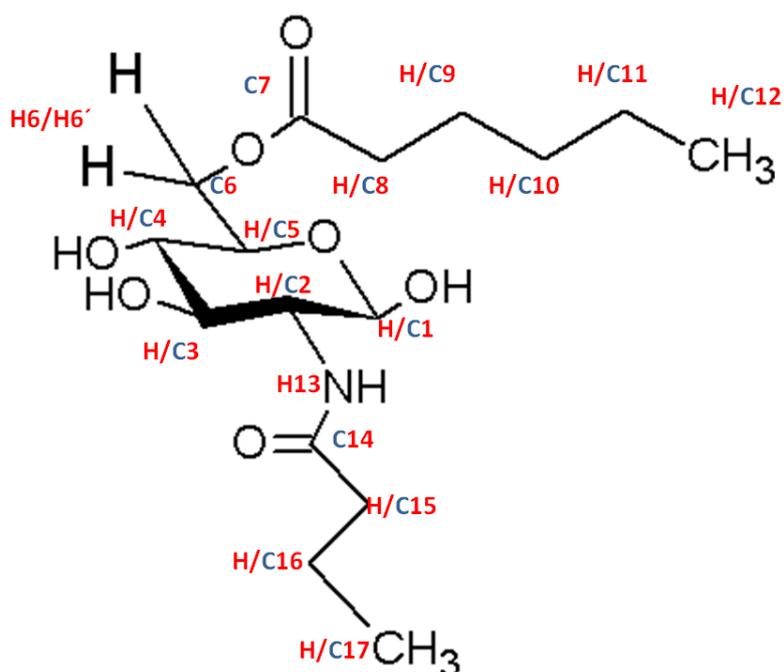
Structure elucidation

NMR Spectra: GlcNBu-6-O-Hex

CH-Number	¹ H [ppm]	¹³ C [ppm]	Integral	J(H,H) [Hz]	Comment
1	4,902 (d)	91,66	1	J(H1,H2)=3,18	
1OH	6,523 (s)	--	0,7	--	Assigned via comparison with GlcNAc-6-O-Hex
2	3,605 (ddd)	55,01	1	J(H1,H2)=3,18 J(H2,H13)=8,10 J(H2,H3)=10,70	--
3	3,499 (dd)	71,18	1	J(H3,H2)=10,70 J(H3,H4)=8,68	--
3OH	--	--	--	--	Not visible, too wide
4	3,115 (dd)	72,08	1,12	J(H4,H3)=8,68 J(H4,H5)=9,83	Slight overlay
4OH	5,146 (s)	--	0,7	--	Assigned via comparison with GlcNAc-6-O-Hex
5	3,790 (ddd)	70,20	1	J(H5,H4)=9,83 J(H5,H6)=6,02 J(H5,H6')=1,74/2,02	--
6/6'	H6: 4,019 (dd) H6': 4,292 (dd)	64,69	H6: 1,14 H6': 1,12	J(H6,H5)=6,02 J(H6',H5)=1,74/2,02 J(H6/H6')=11,57	Slight overlay, diastereotop
7	--	173,89	--	--	
8	2,284 (t)	34,37	2,23	--	Slight overlay
9	1,2 - 1,35	31,59	5,85	--	Overlay with H11
10	1,4 - 1,6	25,11	5,0	--	Left multiplett, overlay with H16
11	1,2 - 1,35	22,74	5,85	--	Overlay with H9
12	0,8 - 0,9	14,76	7,57	--	Overlay with H17
13	7,530 (d)	--	1	J(H13,H2)=8,10	--
14	--	173,18	--	--	--
15	2,082 (t)	38,09	2,10	--	Slight overlay
16	1,4 - 1,6	19,64	5,0	--	Right multiplett, overlay with H10
17	0,8 - 0,9	14,60	7,57	--	Overlay with H12



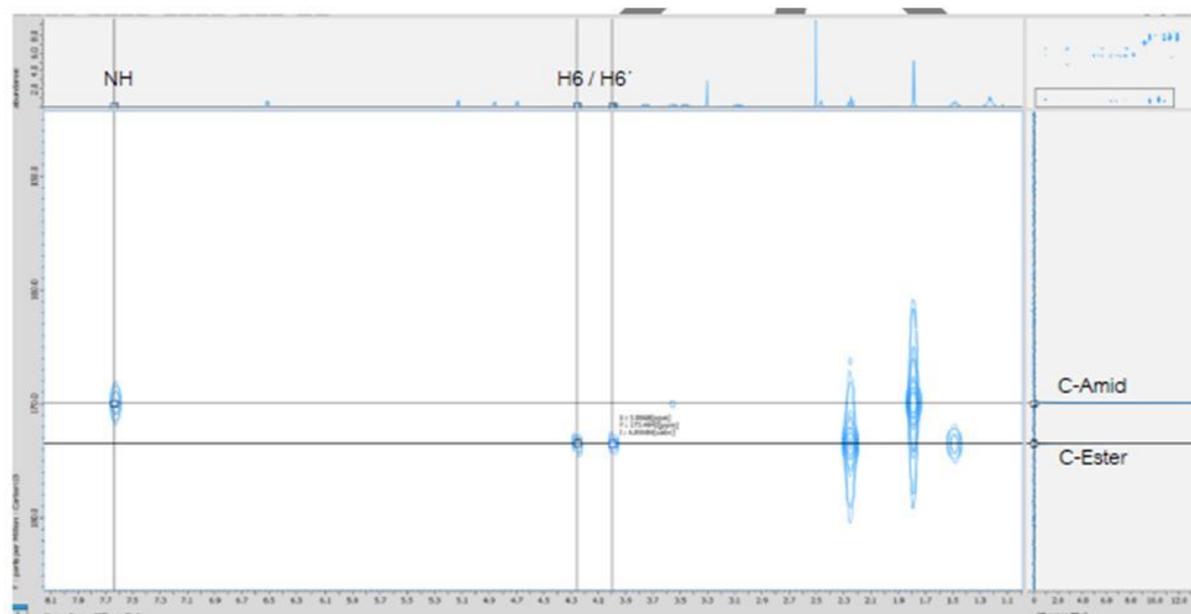
To elucidate the postulated structure 1D-1H, 1D-13C, 2D1H1H COSY, 2D-1H13C HSQC und 2D-1H13C HMBC measurements were performed. Comparison between the single spectra allowed assignment of the single atoms of the molecule. Proof of the postulated regioisomer gave the 1H13C HMBC. Here a coupling between C7 and H6/6' was detected. The absence of a coupling between C7 and H4, H3 or H1 also proofs the position of the ester bond at C6. Further the coupling between H13 and C14 and between H2 and H13 verify the amid binding at C2.



NMR Spectra GlcNAc-6-O-Hex

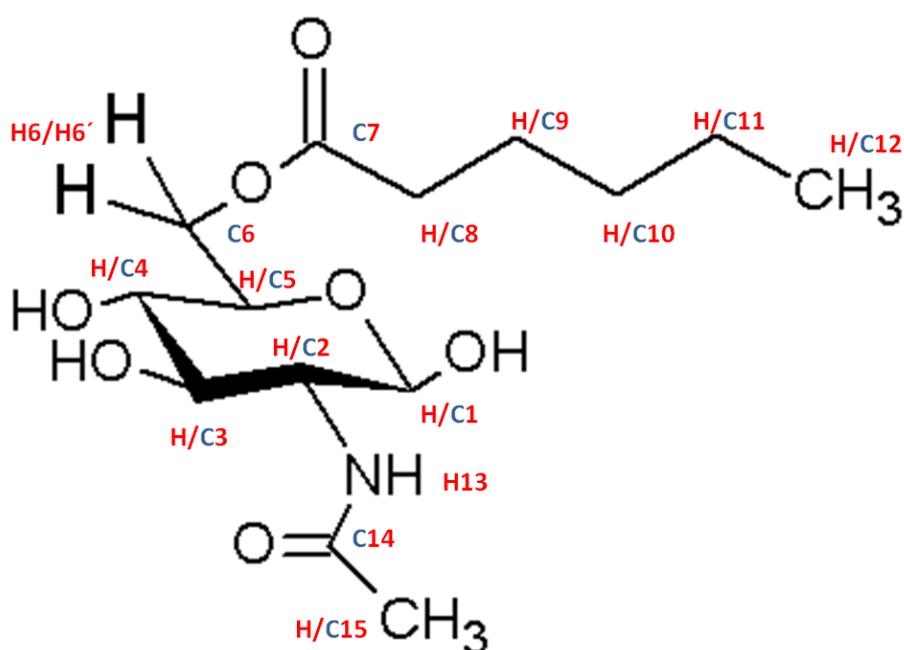
CH-Number	¹ H [ppm]	¹³ C [ppm]	Integral	J(H,H) [Hz]	Comment
1	4,894 (dd)	91,62	1	J(H1,H2)=n.b.	Not detectable
1OH	6,547 (dd)	--	1	n.b.	Not detectable
2	3,584 (ddd)	55,07	1	J(H1,H2)=n.b. J(H2,H13)=8,19 J(H2,H3)=10,7	Not detectable
3	3,490 (ddd)	71,20	1	J(H3,H2)=10,7 J(H3,H4)=8,59 J(H3,OH)=5,55	--
3OH	4,729 (d)	--	1	J(OH,H3)=5,55	--
4	3,110 (ddd)	72,06	1	J(H4,H3)= J(H4,H5)=9,85 J(H4,OH)=5,67	--
4OH	5,162 (d)	--	1	J(OH,H4)=5,67	--
5	3,789 (ddd)	70,179	1	J(H5,H4)=9,85 J(H5,H6)=5,95 J(H5,H6')=1,95/1,83	--
6/6'	H6: 4,017 (dd) H6': 4,290 (dd)	64,67	H6: 1 H6': 1	J(H6,H5)=5,95 J(H6',H5)=1,95//1,83 J(H6/H6')=11,68	--
7	--	173,89	--	--	--
8	2,283 (t)	34,36	2	--	--
9	1,522 (tt)	25,10	2,1	--	--
10	1,2 – 1,35	31,58	4,36	--	--
11	1,2 – 1,35	22,74		--	--
12	0,857 (t)	14,75	3,3	--	--
13	7,660 (d)	--	1	J(H13,H2)=8,19	--
14	--	170,30	--	--	--
15	1,822 (s)	23,59	3	--	--

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To elucidate the postulated structure 1D-1H, 1D-13C, 2D1H1H COSY, 2D-1H13C HSQC und 2D-1H13C HMBC measurements were performed. Comparison between the single spectra allowed assignment of the single atoms of the molecule. Proof of the postulated regioisomer gave the 1H13C HMBC. Here a coupling between C7 and H6/6' was detected. The absence of a coupling between C7 and H4, H3 or H1 also proofs the position of the ester bond at C6.

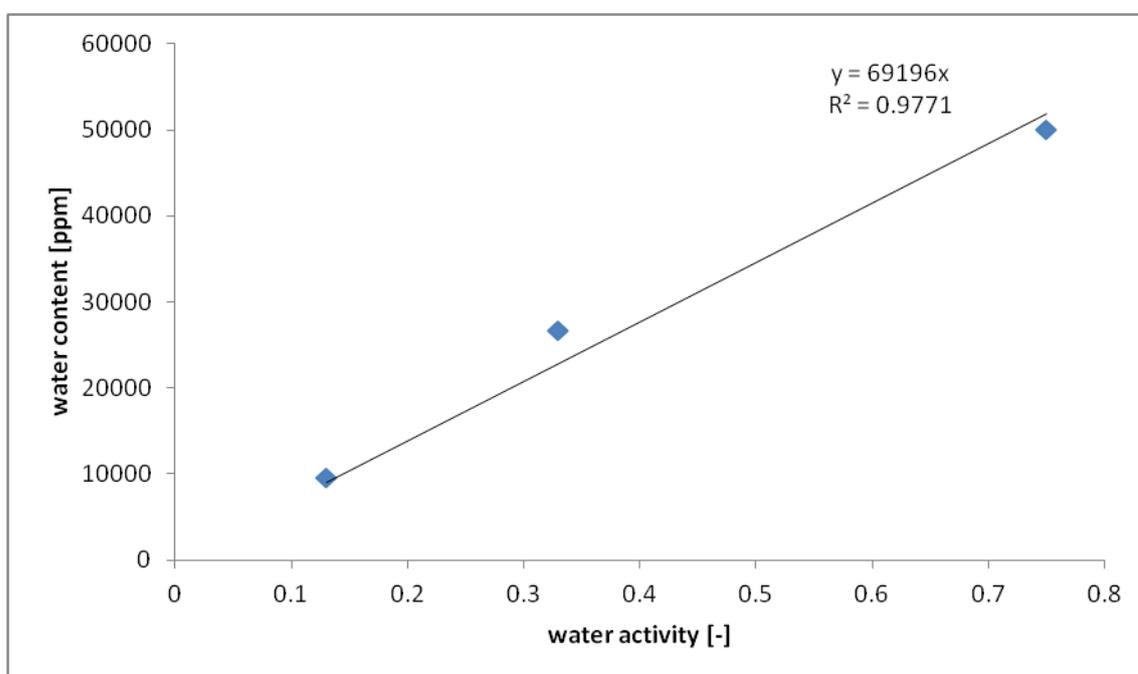
Further the coupling between H13 and C14 and between H2 and H13 verify the amid binding at C2.



C. The effects of substrate ratio, water activity and reaction temperature on the enzymatic synthesis of amino sugar fatty acid esters

Correlation between water content and water activity

By determination of the water content of three 2m2b variants with pre-adjusted water activity an almost linear correlation between water content and water activity of 2m2b could be detected, allowing the approximate estimation of the water activity of 2m2b without further treatment or with additional molecular sieves.



Approximate correlation between known water content and unknown water activity can be achieved using the formula:

$$\text{Water activity [-]} = \text{Water content [ppm]} / 69196$$

Emulsification Test E₂₄

In order to characterize the emulsification capacity of the amino sugar fatty acid esters, mixing of aqueous solutions containing glycolipids with kerosene was tested. The emulsification capacity was determined as

$E_{(t)}$ = height emulsified layer / total height of both phases.

Glycolipid	Concentration [mg/mL]	E ₀	E ₂₄
GlcNAc-6-O-Hex	50	23.3	0
	10	6.7	0
	5	3.3	0
GlcNAc-6-O-Lau	0.5	0	0
GlcNAc-6-O-Palm	0.5	0	0

While GlcNAc-6-O-Hex was able to establish short time emulsions, GlcNAc-6-O-Lau and GlcNAc-6-O-Palm didn't exhibit any emulsification capacity. The reason therefore lies in the fact that both GlcNAc-6-O-Lau and GlcNAc-6-O-Palm are poorly soluble in water, thus only low concentrations of these glycolipids could be tested.

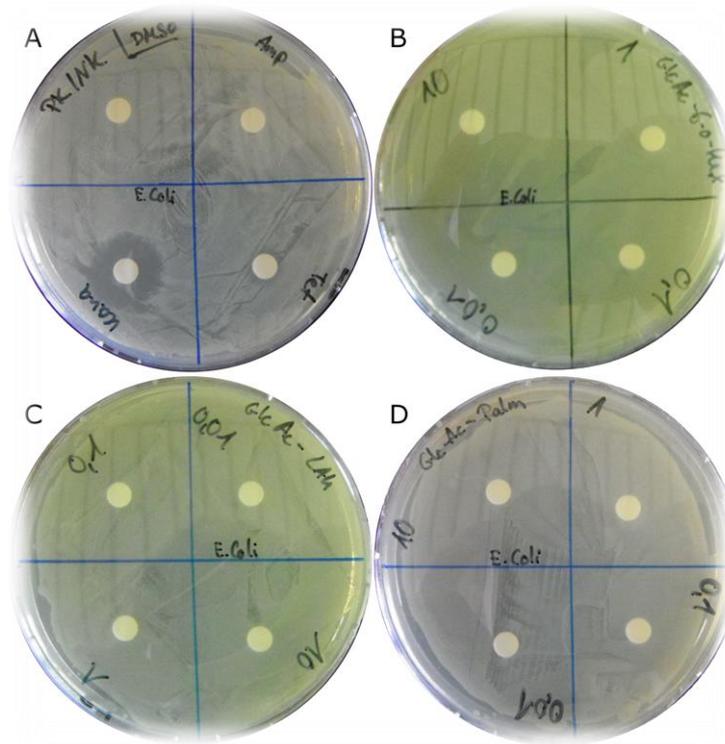
Antibiotic properties

The ability of the novel glycolipids to inhibit bacterial growth was investigated using *Escherichia coli* K12 and *Bacillus subtilis* DSM 10T cultures, grown on LB- (*E. coli*) and YM (*B. subtilis*) agar plates.

Lysogeny broth medium (LB)	
Yeast extract	5 g/L
Tryptone	10 g/L
NaCl	10 g/L
Agar	15 g/L

Yeast malt medium (YM)	
Yeast extract	3 g/L
Peptone	5 g/L
Malt extract	3 g/L
Glucose	10 g/L
Agar	15 g/L

Antibiogramm of *Escherichia coli*



A: Positive control using Kanamycin (Kana), Ampicillin (Amp) Tetracycline (Tet) (all 2 mg/mL) and DMSO

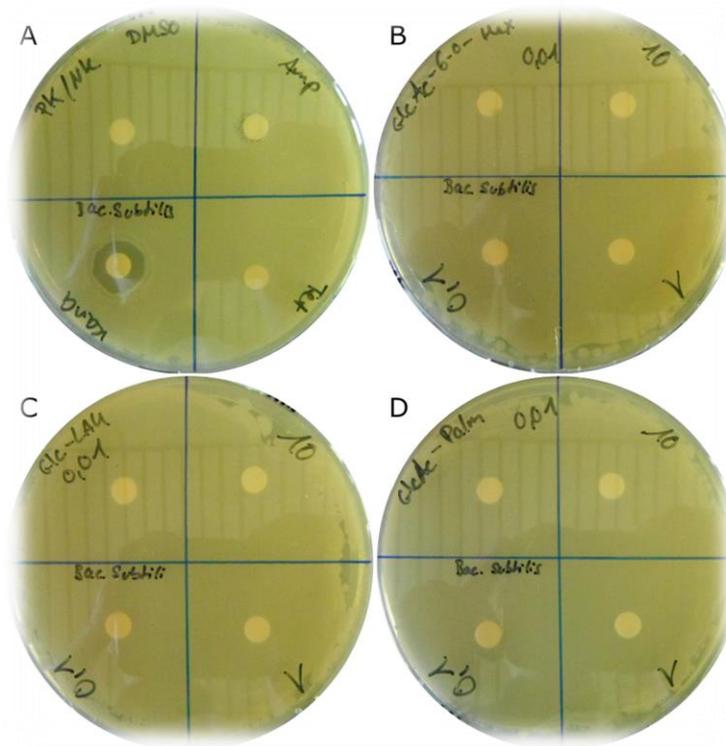
B: Antibiogramm using GlcNAc-6-O-Hex, Concentration 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL

C: Antibiogramm using GlcNAc-6-O-Lau, Concentration 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL

D: Antibiogramm using GlcNAc-6-O-Palm, Concentration 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL

None of the tested amino sugar fatty acid esters was able to inhibit the growth of *E. coli* in any concentration tested.

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A: Positive control using Kanamycin (Kana), Ampicillin (Amp) Tetracycline (Tet) (all 2 mg/mL) and DMSO

B: Antibiogramm using GlcNAc-6-O-Hex, Concentration 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL

C: Antibiogramm using GlcNAc-6-O-Lau, Concentration 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL

D: Antibiogramm using GlcNAc-6-O-Palm, Concentration 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL

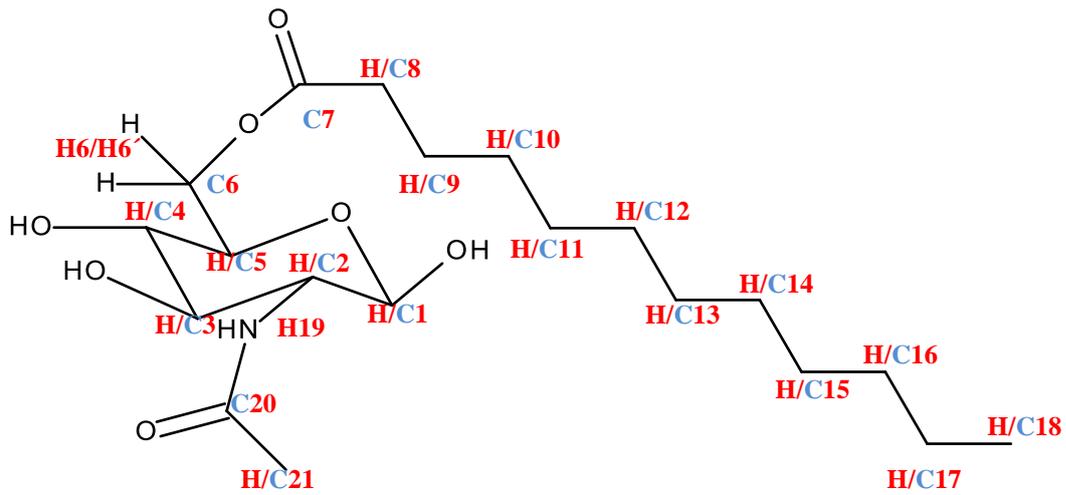
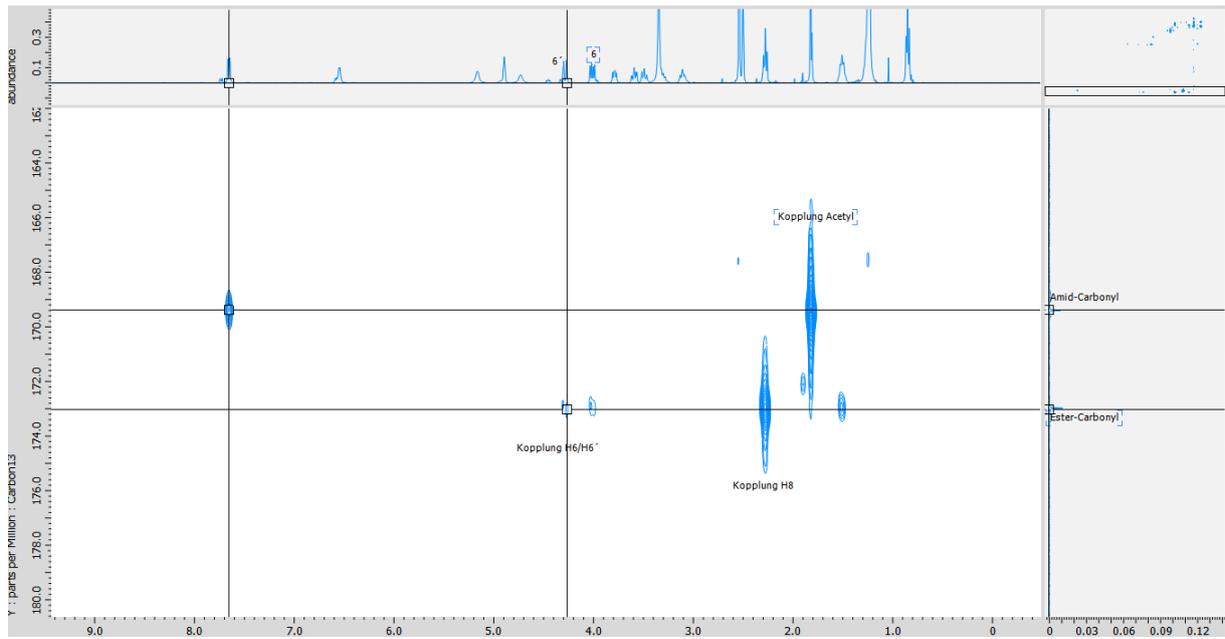
None of the tested amino sugar fatty acid esters was able to inhibit the growth of *B. subtilis* in any concentration tested.

Structure elucidation**NMR Spectra GlcNAc-6-O-Lau**

CH-number	¹ H [ppm]	¹³ C [ppm]	Integral	<i>J</i> (H,H) [Hz]	Multiplicity	Comment
1	4.895	90.72	1	<i>J</i> (H1,H2)= <i>n.b.</i> <i>J</i> (H1,OH1)= <i>n.b.</i>	dd	Reference integral, overlay
1OH	6.547	--	0.96	<i>J</i> (OH1,H1)=3.33	d	--
2	3.593	54.16	0.88	<i>J</i> (H1,H2)=3.22 <i>J</i> (H2,H19)=8.15 <i>J</i> (H2,H3)=10.58	ddd	--
3	3.492	70.30	0.81	<i>J</i> (H3,H2)= <i>n.b.</i> <i>J</i> (H3,H4)= <i>n.b.</i> <i>J</i> (H3,OH)= <i>n.b.</i>	ddd	--
3OH	4.732	--	0,7	<i>J</i> (OH,H3)= <i>n.b.</i>	d	Assigned via comparison with GlcNAc-6-O-Palm
4	3.108	71.16	1.08	<i>J</i> (H4,H3)= <i>n.b.</i> <i>J</i> (H4,H5)= <i>n.b.</i> <i>J</i> (H4,OH)= <i>n.b.</i>	ddd	--
4OH	5.166	--	0.87	<i>J</i> (OH,H4)= <i>n.b.</i>	d	--
5	3.789	69.27	0.88	<i>J</i> (H5,H4)=9.98 <i>J</i> (H5,H6)=6.15 <i>J</i> (H5,H6')=1,61	ddd	--
6/6'	H6:4.012 H6':4.286	63.80	H6:1.11 H6':1.07	<i>J</i> (H6,H5)=6.15 <i>J</i> (H6',H5)=1.61 <i>J</i> (H6/H6')=11.69	H6: dd H6':dd	--
7	--	172.99	--	--	--	--
8	2.278	33.48	2.09	<i>J</i> (H8,H9)=7.46	t	--
9	1.508	24.52	2.12	--	---	--
10	1.15-1.35	22.14; 28.3-29.3; 31,34	17.79	--	--	--
11				--	--	--
12				--	--	--
13				--	--	--
14				--	--	--
15				--	--	--
16				--	--	--
17	--	--	--	--	--	--
18	0.851	13.99	3.41	--	--	--
19	7.655	--	0.97	<i>J</i> (H19,H2)=8.15	d	--
20	--	169.40	--	--	--	--
21	1.823	22.68	3.01	--	s	additional side component

Measurement resulted in low resolution, not allowing assignment of coupling constants.

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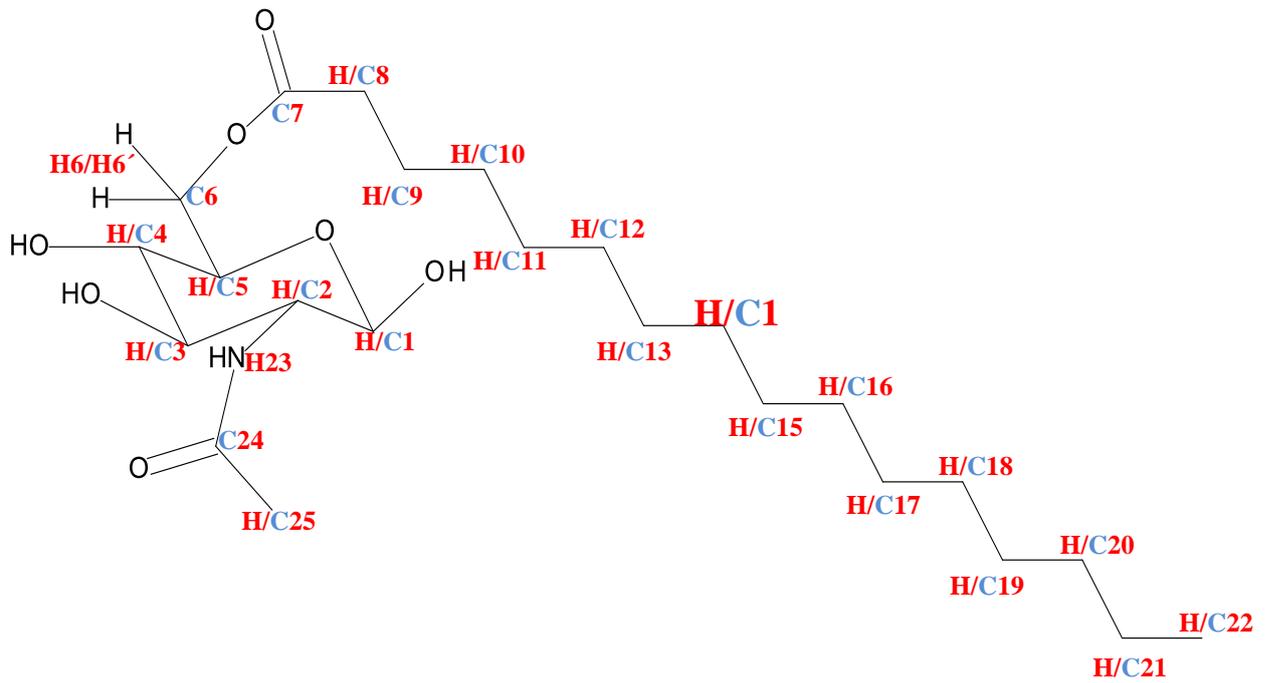
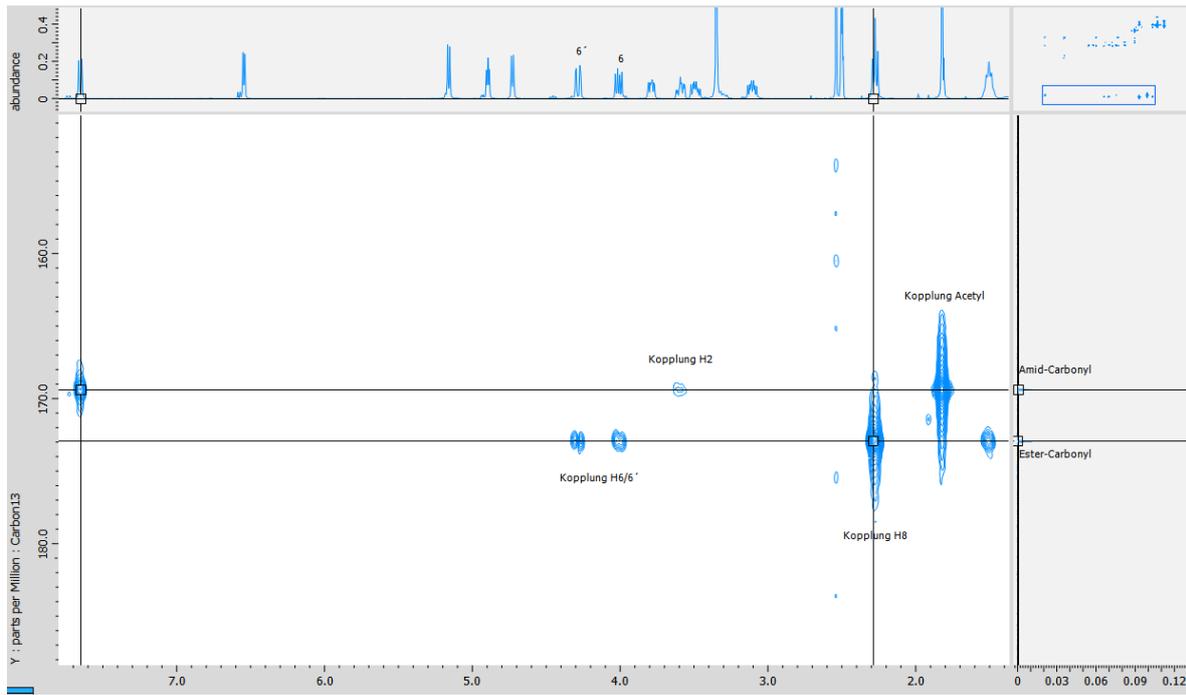


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NMR Spectra GlcNAc-6-O-Lau

CH- Number	¹ H [ppm]	¹³ C [ppm]	Integral	J(H,H) [Hz]	Multiplicity	Comment
1	4.894	90.71	1	$J(H1,H2)=n.b.$ $J(H1,OH1)=n.b.$	dd	Reference integral, similar coupling constants, pseudo triplet
1OH	6.546	--	1.06	$J(OH1,H1)=n.b.$	d	Additional side component
2	3.592	54.14	1.02	$J(H1,H2)=n.b.$ $J(H2,H23)=8.16$ $J(H2,H3)=10.68$	ddd	--
3	3.494	70.30	1.02	$J(H3,H2)=10.68$ $J(H3,H4)=8.46$ $H(H3,OH3)=5.44$	ddd	--
3OH	4.728	--	1	$J(OH3,H3)=5.44$	d	--
4	3.107	71.15	1.03	$J(H4,H3)=8.67$ $J(H4,H5)=9.77$ $J(H4,OH4)=5.74$	ddd	--
4OH	5.160	--		$J(OH4,H4)=5.74$	d	--
5	3.789	69.25	1.05	$J(H5,H4)=9.77$ $J(H5,H6)=6.05$ $J(H5,H6')=1.91$	ddd	--
6/6'	H6: 4.010 H6': 4.286	63.80	H6: 1.08 H6': 1.04	$J(H6,H5)=6.05$ $J(H6',H5)=1.91$ $J(H6/H6')=11.59$	H6: dd H6': dd	--
7	--	172.97	--	--	--	--
8	2.274	33.47	2.19	$J(H8,H9)=7.3$	t	Overlay with side component
9	1.505	24.51	2.22	--	dt	Overlay with side component
10	1.21-1.29	22.14; 28.5- 29.2; 31.33	28.67	--	--	Overlay with side component
11				--	--	
12				--	--	
13				--	--	
14				--	--	
15				--	--	
16				--	--	
17				--	--	
18				--	--	
19				--	--	
20				--	--	
21	--	--				
22	0.848	13.97	3.44	$J(H22,H21)=6.95$	t	Additional side component
23	7.652	--	1	$J(H23,H2)=8.16$	d	Additional side component
24	--	169.41	--	--	--	--
25	1.822	22.67	2.91	--	s	Additional side component

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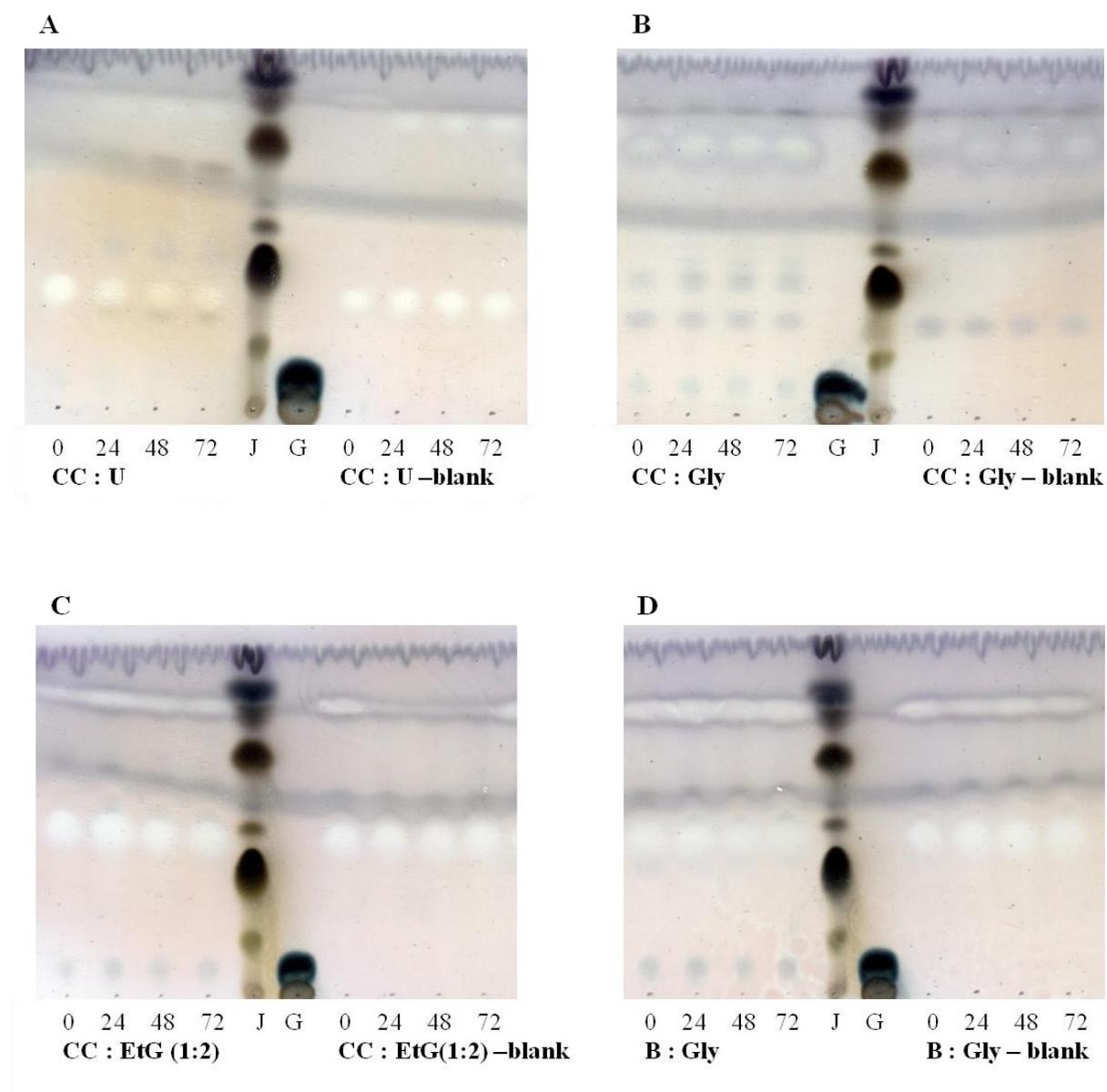
D. Lipase-catalyzed glycolipid synthesis in deep eutectic solvents

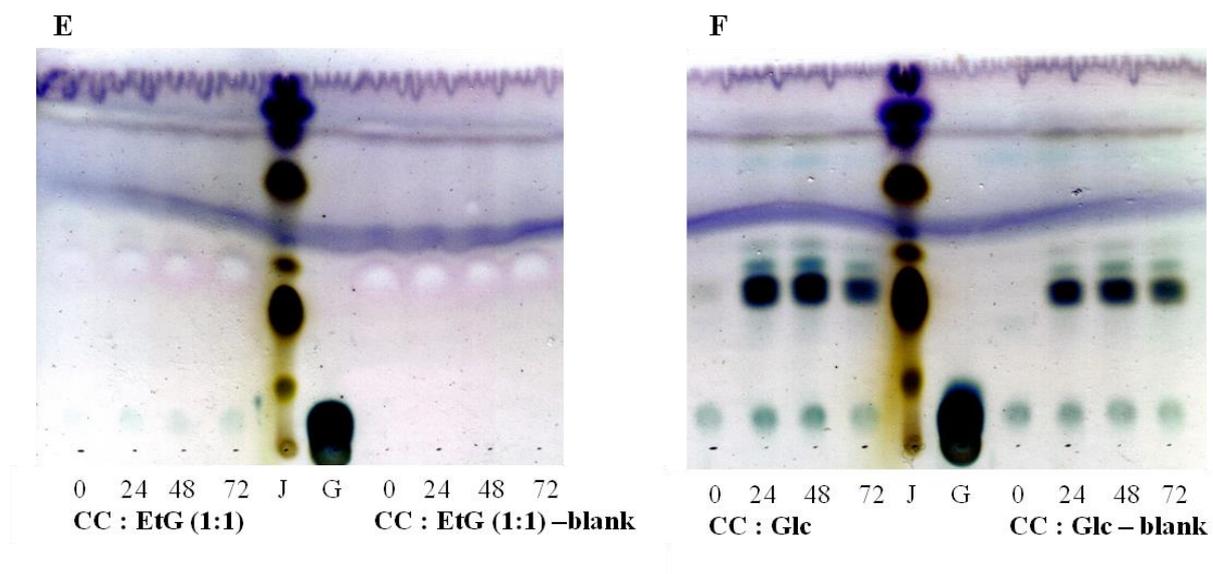
Thin layer chromatography

Numbers indicate time point when sample was withdrawn

J = positive control containing different rhamnolipids

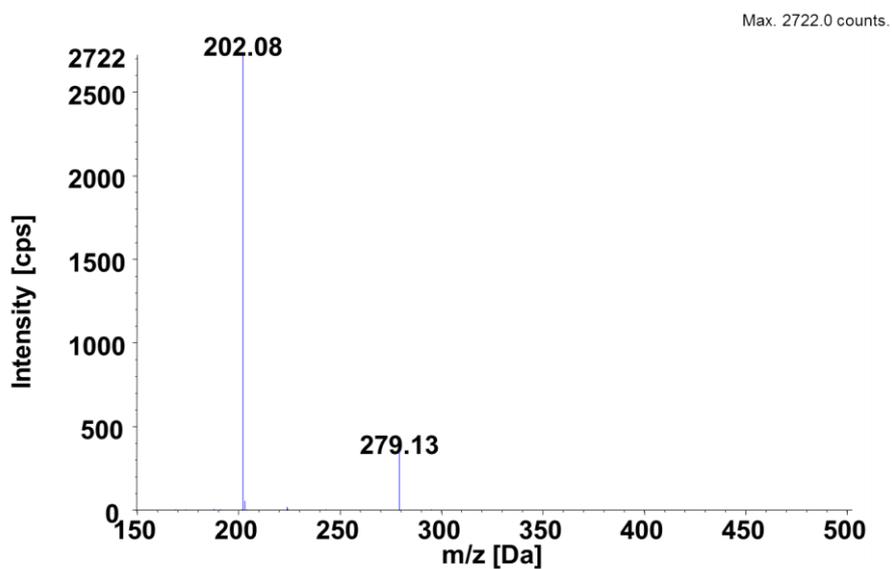
G = Glucose standard (10 mg/mL)

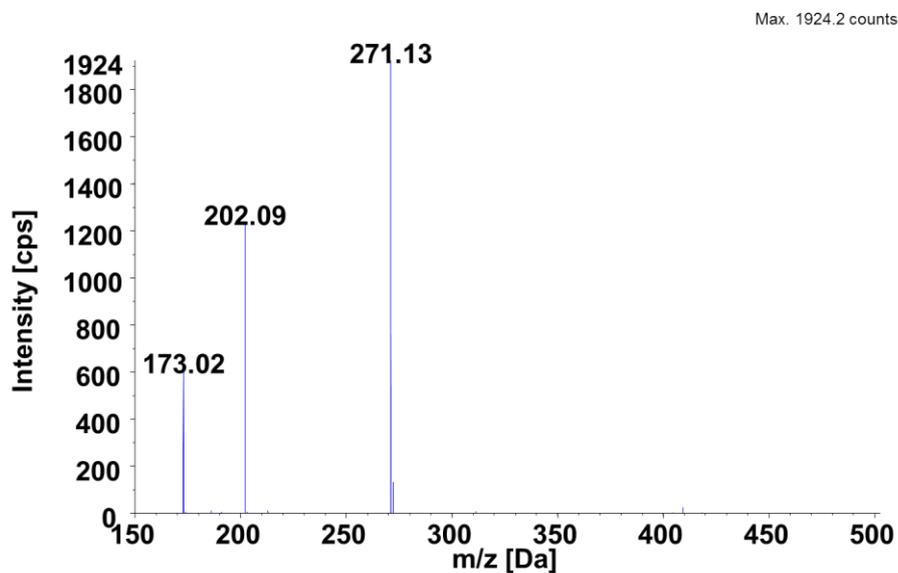
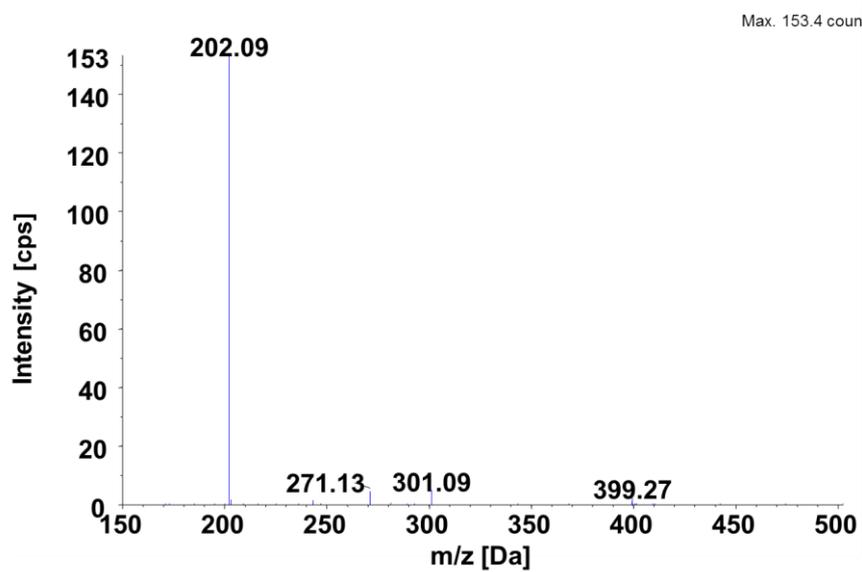




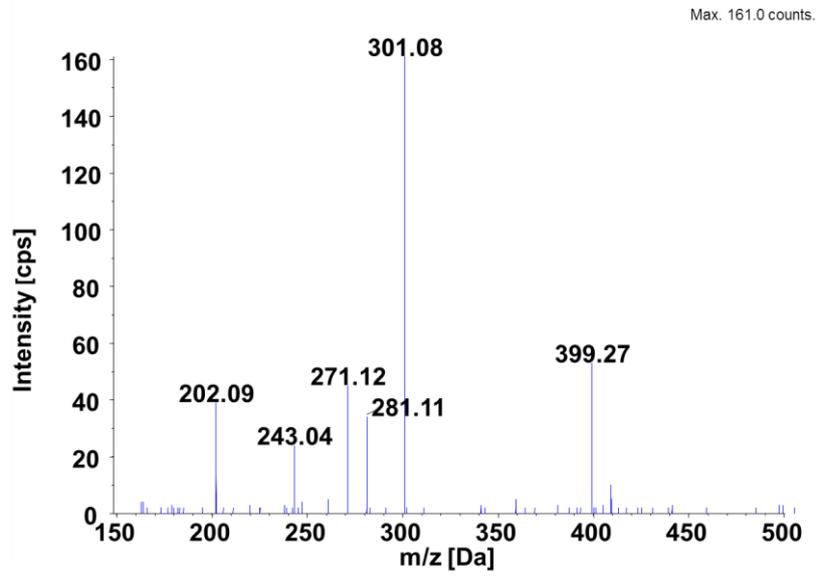
Masses detected via ESI-Q-TOF

Extract: CC : U



Extract: CC : GlyExtract: CC : Glc

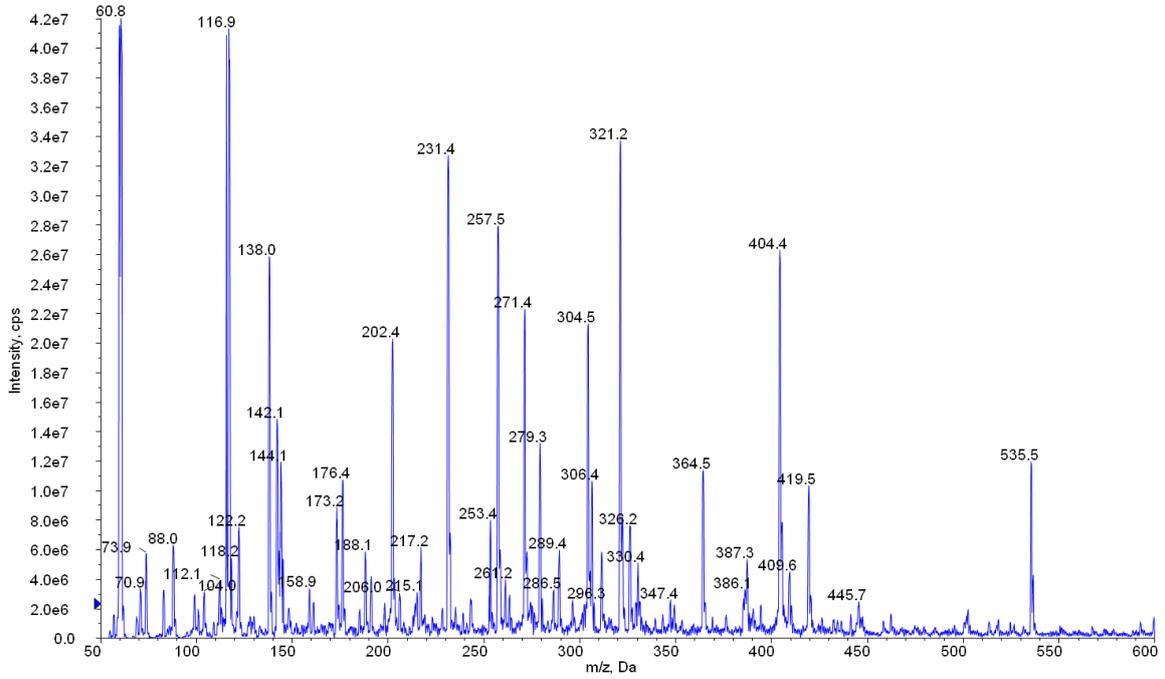
Extract: CC : Glc - blank



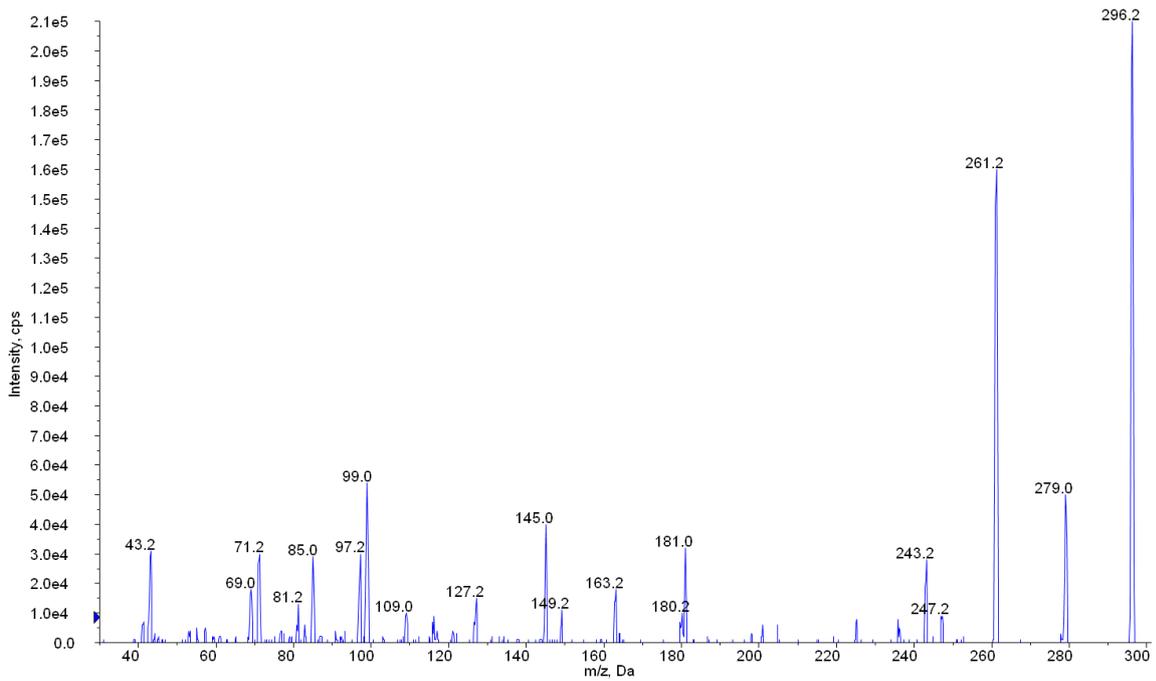
Tandem Mass Spectrometry

Extract: CC : U (diluted 1 : 100000)

Q1 Scans



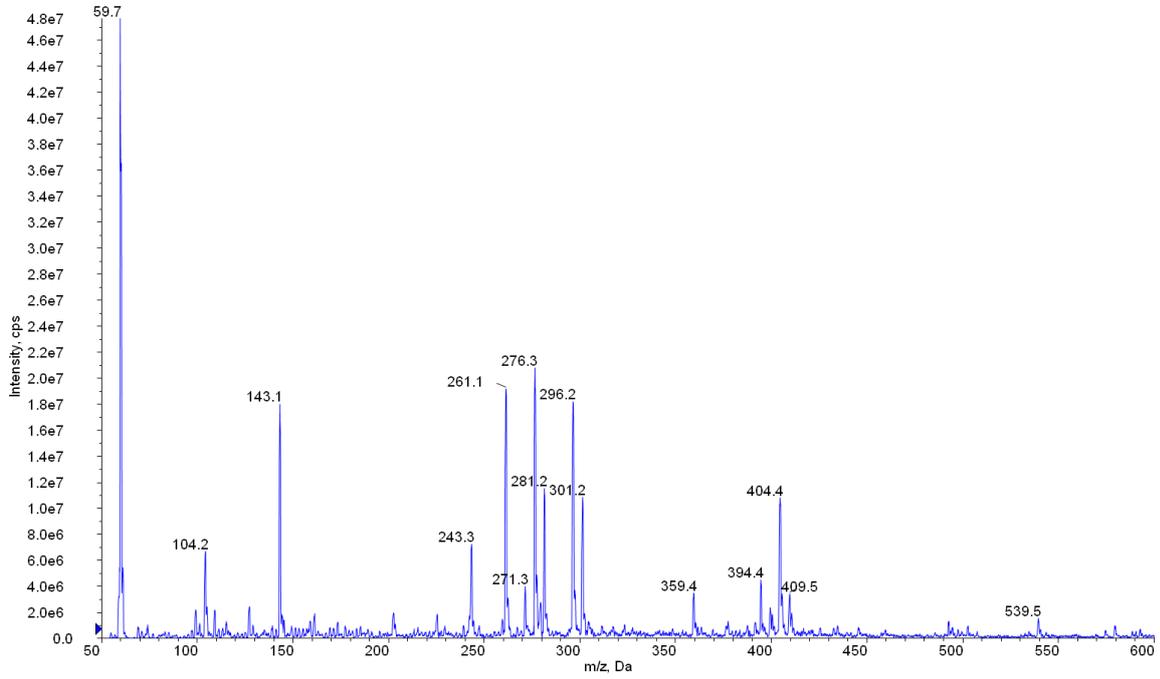
Fragmentation of mass 296.3 amu from Q1 scans



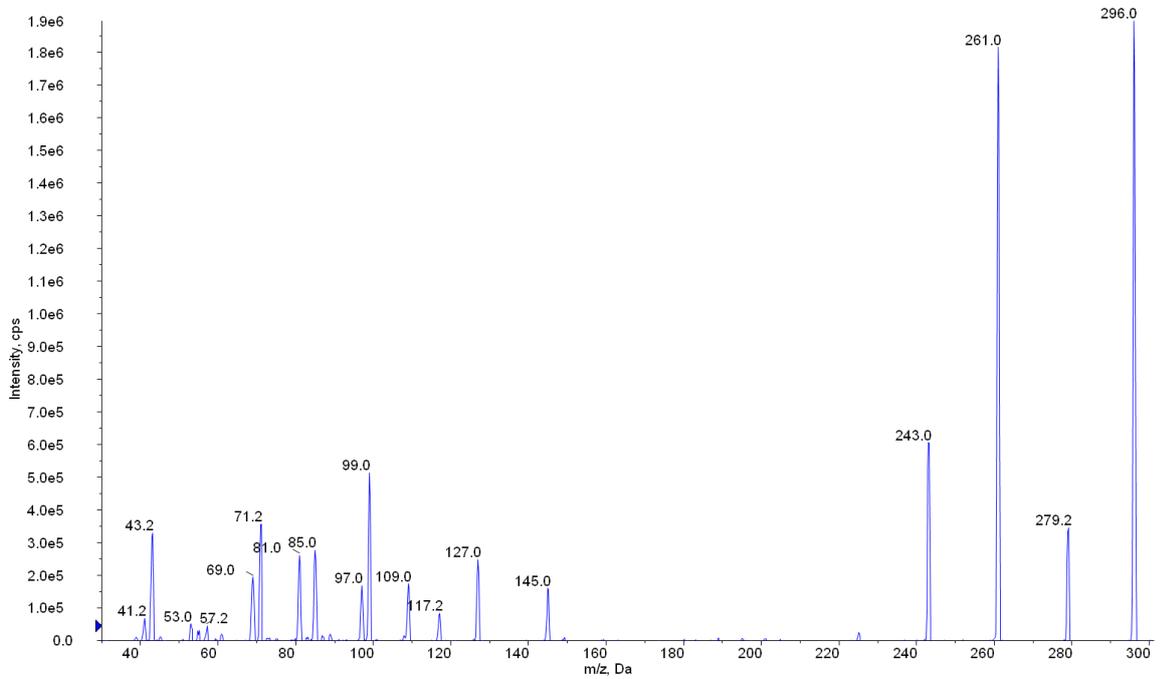
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Extract: CC : Glc (diluted 1 : 500)

Q1 Scan

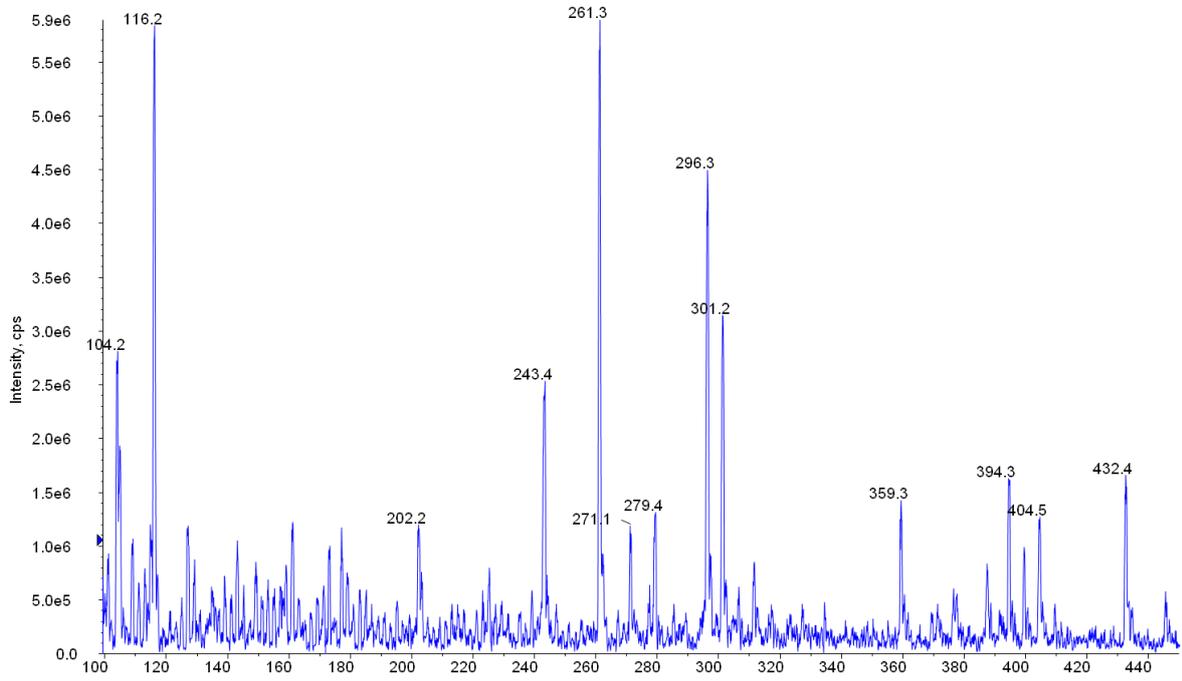


Fragmentation of mass 296.2 amu from Q1 scans

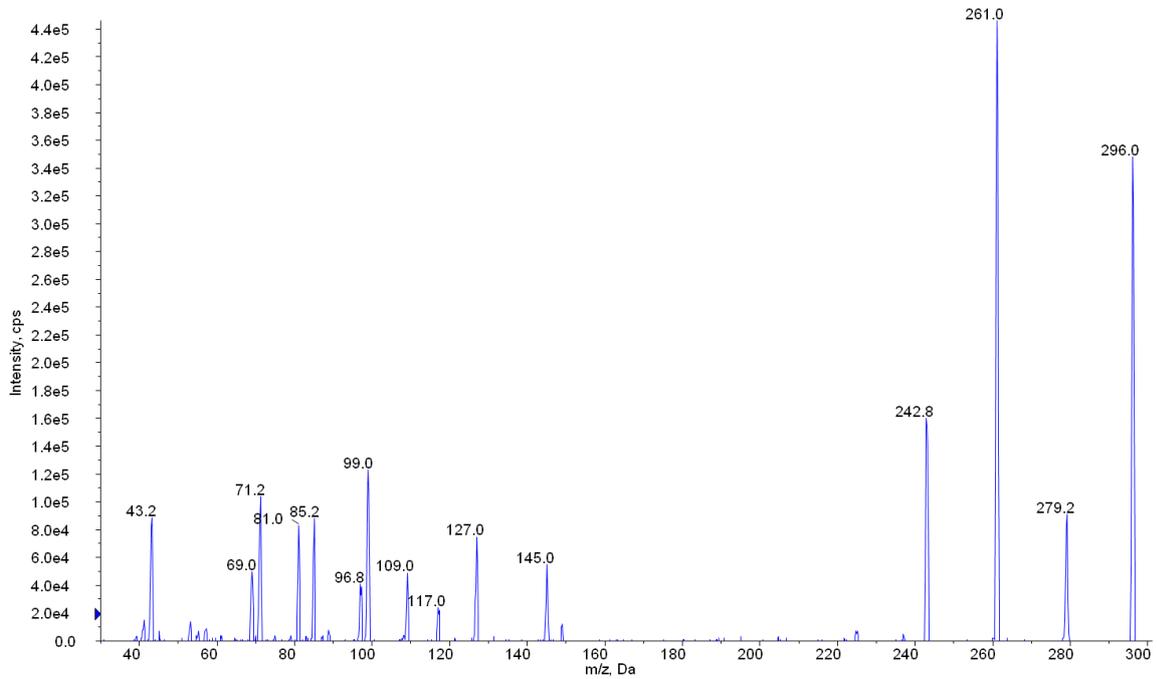


Extract: CC : Glc – blank(diluted 1 : 10000)

Q1 Scan



Fragmentation of mass 296.3 amu from Q1 scans



Nuclear Magnetic Resonance Spectroscopy**glucose-6-*O*-hexanoate**chemical shifts glucose-6-*O*-hexanoate

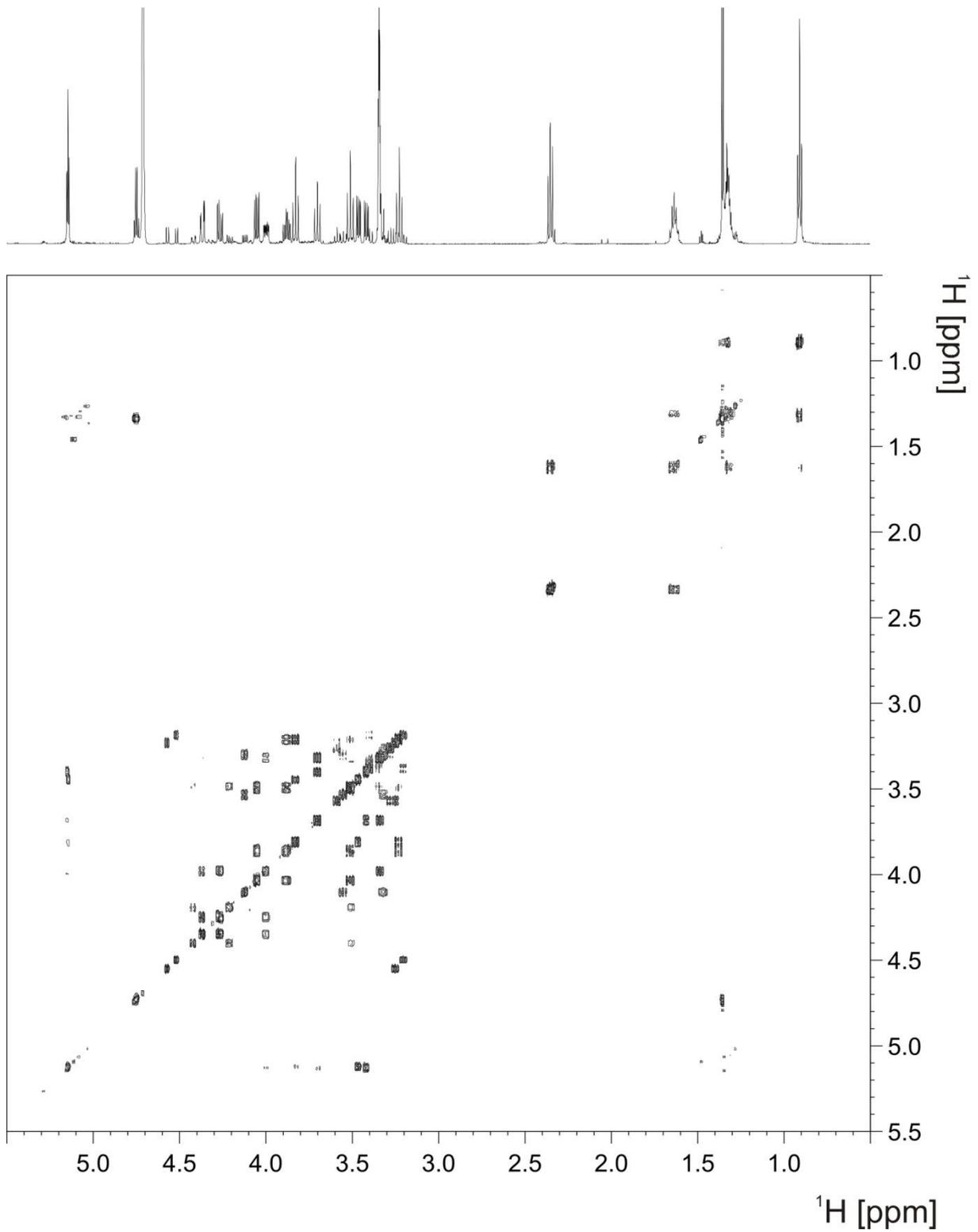
	13C [ppm]	H1 shift [ppm]	Multiplicity	J-coupling [Hz]
<u>Glucose unit</u>				
–C ¹ H–O–	92.5	5.14	dd	3.8
–C ² H–	72.2	3.42	dd	3.8; 9.6
–C ³ H–	73.4	3.70	t	9.3
–C ⁴ H–	70.2	3.33	t	9.2
–C ⁵ H–	69.2	4.00	ddd	10.1; 5.2; 2.1
–C ⁶ H ^a –	63.5	4.27	dd	11.8; 5.2
–C ⁶ H ^b –	63.5	4.37	dd	11.8; 2.2
<u>Acyl moiety</u>				
– ⁶ CH ₃	13.5	0.9	t	6.9
– ⁵ CH ₂ –	31	1.33	m	n.d.
– ⁴ CH ₂ –	22.11	1.32	m	n.d.
– ³ CH ₂ –C–CO–O–R	24.3	1.64	m	7.5; 14.9
– ^α CH ₂ –CO–O–R	33.8	2.35	m	7.5
– ^ω CH ₂ –CO–O–R	174.5			

4,6-methyliden glucose

chemical shifts of 4,6-methyliden glucose, the acetal formed by acetaldehyde, the tautomer of vinylalcohol, and glucose. This compound was likely formed as a side product of the reaction.

	13C [ppm]	H1 shift [ppm]	Multiplicity	J-coupling [Hz]
<u>Glucose unit</u>				
–C ¹ H–O–	93.7	5.14	dd	3.9
–C ² H–	73.7	3.46	dd	3.9; 9.3
–C ³ H–	71.1	3.83	t	9.3
–C ⁴ H–	81.5	3.23	t	9.5
–C ⁵ H–	62.6	3.88	ddd	15.0; 5.0; 5.0
–C ⁶ H ^a –	69.1	4.05	dd	10.3; 5.0
–C ⁶ H ^b –	69.1	3.51	t	10.3
<u>Acetaldehyde unit</u>				
– ² CH ₃	20.4	20.4	d	5.0
– ¹ CH ₁ –	100.1	100.1	q	5.0.

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^1H -DQF-COSY acquired at 19.7°C with 16384 points in the direct and 1024 increments in the indirect dimension.

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VII. ABBREVIATIONS

2m2b	2-methyl-2-butanol
ACN	acetonitrile
CC : Glc	choline chloride : glucose
CC : Gly	cholin chloride : glycerol
CC : U	choline chloride : urea
cps	counts per second
DES	deep eutectic solvent(s)
DMSO	dimethyl sulfoxide
E ₂₄	emulsification index
ESI-Q-ToF MS	electrospray ionization – quadrupole – time of flight mass spectrometry
GC	gas chromatography
GlcNAc	N-acetyl glucosamine, 2-(acetylamino)-2-deoxy-D-glucose
GlcNAc-6-O-Hex	2-(acetylamino)-2-deoxy-6-O-hexanoate-D-glucose
GlcNAc-6-O-Lau	2-(Acetylamino)-2-deoxy-6-O-laurate-D-glucose
GlcNAc-6-O-Palm	2-(Acetylamino)-2-deoxy-6-O-palmitate-D-glucose
GlcNBu	N-butyryl glucosamine, 2-(butyrylamino)-2-deoxy-D-glucose
GlcNBu-6-O-Hex	2-(butyrylamino)-2-deoxy-6-O-hexanoate-D-glucose
h	hours
HPLC	high performance liquid chromatography
LiCl	lithium chloride

VII. ABBREVIATIONS

MeOOct	methyl n-octanoate
MeOPalm	methyl n-palmitate
mg	milligram
MgCl ₂	magnesium chloride
mL	milliliter
mN/m	millinewton per meter
MTBE	methyl tert-butyl ether
mU/mg	milliunit per milligram
NaCl	sodium chloride
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
pNP	4-nitrophenol
R _f	retention factor
Rpm	revolutions per minute
t-butanol	tert-butanol
TLC	thin layer chromatography
U	unit (=μmol product generated per min)
VinOHex	vinyl n-hexanoate
VinOLau	vinyl n-laurate
VinOPalm	vinyl n-palmitate

VIII. CURRICULUM VITAE

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