Synthesis and biological study of deoxy glycosides with potential antimicrobial activity against Bacillus spp.

João Pedro de Almeida Pais

Mestrado em Química

Especialização em Química, Saúde e Nutrição

Dissertação orientada por: Professor Doutora Amélia Pilar Grases Santos Silva Rauter

Doutor Ricardo Pedro Moreira Dias

2011
Acknowledgements

This first part goes to the people that helped me through this work, my sincerest gratitude to my supervisor, Professor Amélia Pilar, who has supported me throughout the entire work with her knowledge and expertise and it’s always there to help me go further. Also, to my co-supervisor, Dr. Ricardo Dias, a great thank you for all the things I learned from him, for all the brain-storming, the endless discussions and for the guidance in this new realm of science, and also in life. Also, all this work wouldn’t have been done without the always present support and assistance of my co-worker Dr.ª Alice Martins, a sweet and supportive person to whom I owe a great deal of encouragement and help. I simply couldn’t wish for a better or friendlier workgroup, always there to help me.

I would also like to thank to all my co-workers for the help, advice and support given throughout this experimental work, especially to Patricia Serra, with whom I shared some of the work, but also for the talks and cooperative environment that was always present.

Thanking the family and friends is always difficult because words cannot express the importance of the people that are always there, no matter what, even if it is a phone-call away. They are what life is about.

Thanks to Gonçalo for all the indispensable help and presence in the different stages of the work, even among despair he took my grouchiness, supported me and helped me through. Thanks to Mónica, for all the support and presence, and for making me laugh just by being herself.

Thanks to my sister and my “brother-in-law” for all the relaxation they’ve given me, the support and friendship always present even while mocking me for everything.

A very special thanks for my parents because I certainly wouldn’t be here if it wasn’t for them, for all the support, dedication and advice, for always being there, for the fatherly hugs and the motherly advices and good listening skills, and for everything else you’ve done for me, including making me what I am today. I’m proudly your son.

Finally, to an element which is both friend and family in equal parts, “Drª” Ângela Veloso, for the support in every situation, the motivation, the laughs, the cries,
the presence and the comprehension, even thought our enormous thinking differences. Thank you for all motivation and emotional support and for the love we share.

Thanks also to everyone not mentioned above, for providing a life full of support and help whenever needed.
Abstract

In previous work, a series of 2,6-dideoxy glycosides have demonstrated antibacterial potential mainly against *Bacillus* species, *Enterococcus faecalis* and *Listeria monocytogenes*. The present research is directed towards the synthesis of new 2,6-dideoxy glycosides and analogs, in order to build a small library of compounds providing novel structure-activity relationships, aiming to understand the mode of action of such compounds. In a parallel pathway, the study of the biological effect of such compounds and the induced metabolic changes will ultimately allow a target-guided synthesis, where the converging pathways will meet to give a novel class of antibiotics. The first steps were given using a phenotypic approach to further understand the changes in the bacterial metabolism induced by this type of compounds. Meanwhile, the synthesis of novel derivatives using the most active compound as scaffold was performed, and the assessment of their antibacterial capacity provided new data for a solid structure-activity relationship study.

In this work we present a synthetic strategy for 2,6-dideoxy glycosides based on reaction of 6-deoxyglycals with a variety of alcohols catalysed by triphenylphosphane hydrobromide. The antibacterial activity exhibited by these new compounds was evaluated by agar dilution plates and microdilution methods. In addition, a phenotypic microarray assay was also performed, in order to further understand the biological effects of the synthesized compounds over *Bacillus cereus*.

The first results obtained suggest that the mechanism of action is associated with the cell membrane or its components, confirming dodecyl 2,6-dideoxy-α-L-arabinohexopyranoside as the compound with the best antimicrobial activity over *B. cereus* strains.

Once the rise of bacterial resistance is a real problem of public health, the demand for new antibiotics with new mechanisms of action is growing, to which the present research intends to answer.
List of figures

Figure 1 – General representation for the compounds discussed in the present work. 1
Figure 2 – 3D molecular structure of β-D-glucose. 3
Figure 3 – Schematic representation of mutarotation of D-glucose in water. 4
Figure 4 – Schematic representation of equilibrium chair-boat-chair conversions of β-D-glucose. 5
Figure 5 – Schematic representation of the electrostatic model (a) and the hyperconjugation model (b). 6
Figure 6 – Chemical reactions associated to photosynthesis (a) and respiration (b). 7
Figure 7 – 3D representation of a nucleic acid. 8
Figure 8 – 3D representation of a fatty alcohol (a) and a fatty acid (b). 9
Figure 9 – 3D representation of a glycerophospholipid. 10
Figure 10 – Chemical structures of some representative quorum sensing signalling molecules for Gram negative bacteria (a) and for Gram positive bacteria (b). 10
Figure 11 – Structure of the 2-deoxy-arabino-glycosides previously studied. 12
Figure 12 – Structure of the 2-deoxy-α-arabino-glycosides first assessed for antibacterial study. 24
Figure 13 – Structure of the 2-deoxy-α-D-threo-pentapyranosides assessed for antibacterial activity. 25
Figure 14 – Representation of the D-sugar moiety used in the present work. 26
Figure 15 – Structure of the 2-deoxy-L-arabino-hexopyranosides assessed for antibacterial activity. 27
Figure 16 – Growth curve for B. cereus ATCC 14579 in the presence of compound 23. 30
Figure 17 – Growth curve for B. cereus ATCC 14579 in the presence of compound 25. 30
Figure 18 – Growth curve for B. cereus ATCC 14579 in D1 well with and without compound 7B. 33
Figure 19 – ABC transporters metabolic map for B. cereus ATCC 14579 with signalized results. 36
List of tables

Table 1 - Antimicrobial activity expressed by the diameter of the inhibition zones ± standard deviation (mm) for compounds 1–8 by paper disk diffusion method……………………………………13
Table 2 - Antimicrobial activity expressed in MIC and MLC (lg/mL)a of compounds 2 and 4–7 compared to chloramphenicol, using the dilution method……………………………………13
Table 3 - Stock solution for PM additive solution…………………………………………………………19
Table 4 - PM additive solution (12X)……………………………………………………………………19
Table 5 - 1x PM Inoculating Fluids from stock solutions………………………………………………….20
Table 6 - Antimicrobial activity expressed in MIC (µg/mL), obtained using both the microdilution (lm) and solid media (sm) methods………………………………………………………………..25
Table 7 - Antimicrobial activity expressed in MIC (µg/mL), obtained using both the microdilution and solid media methods……………………………………………………………………26
Table 8 - Antimicrobial activity expressed in MIC (µg/mL), obtained using both the microdilution and solid media methods……………………………………………………………………28
Table 9 – Absorbance values for D1 well and the difference between D1 and the control (A1)……………………………………………………………………………………………..29
Table 10 – Corrected absorbance values for D1-A1………………………………………………………32
Table 11 – A1 absorbance values and calculated variance and standard variation values………34
Table 12 – Application of cut-off values to the corrected absorbance values of D1 well………35
Table 13 – Metabolic pathways with higher number of compounds associated………………….38
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>13C-NMR</td>
<td>Carbon-13 Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ABC transporters</td>
<td>ATP-binding cassette transporters</td>
</tr>
<tr>
<td>Hax.</td>
<td>Axial proton</td>
</tr>
<tr>
<td>br.</td>
<td>Broad</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius degrees</td>
</tr>
<tr>
<td>(delta)</td>
<td>Chemical shift</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dd</td>
<td>Double doublet</td>
</tr>
<tr>
<td>ddd</td>
<td>Double doublet</td>
</tr>
<tr>
<td>qd</td>
<td>Double quartet</td>
</tr>
<tr>
<td>td</td>
<td>Double triplet</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>Heq</td>
<td>Equatorial proton</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>Ha,Hb</td>
<td>Geminal proton A and B</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Coherence</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LBA</td>
<td>Luria-Bertani Agar</td>
</tr>
<tr>
<td>ODₘₐₓ.</td>
<td>Maximal Optical Density</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimal Bactericidal Concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal Inhibitory Concentration</td>
</tr>
<tr>
<td>min.</td>
<td>Minutes</td>
</tr>
<tr>
<td>MH</td>
<td>Muller-Hinton</td>
</tr>
<tr>
<td>MHA</td>
<td>Muller-Hinton Agar</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
</tr>
<tr>
<td>AHL</td>
<td>N-Acyl homoserin lactones</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>1H-NMR</td>
<td>Proton Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>quin.</td>
<td>Quintuplet</td>
</tr>
<tr>
<td>Rf</td>
<td>Retention factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>s</td>
<td>Singlet</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-Activity Relationship</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
</tr>
<tr>
<td>TPHB</td>
<td>Triphenylphosphane.Hydrobromide</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>σ</td>
<td>Variance</td>
</tr>
</tbody>
</table>
## Contents

Acknowledgements ...................................................................................................................... iii
Abstract ......................................................................................................................................... v
List of figures ............................................................................................................................... vii
List of tables .................................................................................................................................. ix
List of schemes ........................................................................................................................... x
List of Abbreviations .................................................................................................................. xi
List of compounds ....................................................................................................................... xiii
Introduction ................................................................................................................................... 1

Carbohydrates ........................................................................................................................... 2
  Carbohydrates physical and chemical properties ..................................................................... 4
  Biological Role .......................................................................................................................... 7
Lipids and Fatty acid derivatives ................................................................................................. 9
State of the Art ........................................................................................................................... 11
Present Work ............................................................................................................................. 14

Materials and Methods .......................................................................................................... 17
  Chemical synthesis materials and methodology ................................................................ 17
  Anti-bacterial activity assessment ....................................................................................... 17
  Bacterial strains and growth conditions ............................................................................... 18
  Medium Preparation ............................................................................................................... 18
  Compound Preparation ......................................................................................................... 18
  Microdilution Method .............................................................................................................. 19
  Solid media method ................................................................................................................ 19
  Phenotypic Array .................................................................................................................... 20
    Data analysis ....................................................................................................................... 22

Results and Discussion .......................................................................................................... 25
  Structure-Activity Relationship (SAR) study ........................................................................ 25
  Phenotypic microarray study ............................................................................................... 32

Conclusions and Future Work ................................................................................................ 41

Experimental .......................................................................................................................... 43
  Synthesis of dodecyl 3,4-di-O-acetyl-2,6-dideoxy-L-arabinohexopyranosides ......................... 43
  Synthesis of dodecyl 2,6-dideoxy-L-arabinohexopyranosides .............................................. 44
Synthesis of dodecyl 4-acetyl-6,2,3-trIDEOxy- α-L -arabino-2-enopyranoside .....................46

Synthesis of dodecyl 6,2,3-trIDEOxy- α-L -arabino-2-enopyranoside.................................46

Synthesis of decyl 3,4-di-O-acetyl-2,6-dIDEOxy- L-arabino-hexapyranosides .....................48

Synthesis of decyl 2,6-dIDEOxy-L-arabino-hexapyranosides ........................................48

Synthesis of 1H,1H Perfluorodecyl 3,4-di-O-acetyl-2,6-dIDEOxy- L-arabino-hexapyranosides .................................................................51

Synthesis of 1H,1H Perfluorodecyl 2,6-dIDEOxy- L-arabino-hexapyranosides .................51

Synthesis of 2-octyl-dodecyl 3,4-di-O-acetyl-2,6-dIDEOxy- L-arabino-hexapyranosides .....53

Synthesis of 2-octyl-dodecyl 2,6-dIDEOxy- L-arabino-hexapyranosides .........................54

References ................................................................................................................................55

Appendix .....................................................................................................................................61
Introduction

The rise of bacterial resistance is a real problem of public health and the demand for new antibiotics with new mechanisms of action is growing. The present study intends to develop the synthesis of new antibiotics from sugars, related to those previously reported by our group, who reported the synthesis of some deoxy glycosides, their physical properties such as their stability, melting temperature, surface activity, micellar formation, and more important their biological activities such as antibacterial activity and cytotoxicity. From the antibacterial assays some compounds demonstrated important inhibition to some Gram positive bacterial species, namely the *Bacillus* gender. Taking this into account, this work intends to deepen the studies for potencial and related new antibiotics, as well as the discovery of the mechanism of action of this type of compounds.

A number of compounds were assessed which allowed some structural guidance to the ongoing research, regarding both the sugar moiety and the saturated carbon chain. The compounds tested are 2-deoxy glycosides (fig. 1). From the structural information and bioactivity results it was possible to conclude that deoxygenation of the 6\textsuperscript{th} position in the sugar moiety increased the antibacterial activity, as well as the elongation of the aliphatic chain (the 12 carbon chain compounds tested revealed better activity than the 8 carbon chain compounds).(Rauter et al., 2005; Silva et al., 2008). These studies, together with other reports opportunely referred, in which similar compounds were used, and from which some more structural guidance could be drawn, were the starting point for this work. From all data gathered, but mainly from the work developed and the knowledge of our work group in this field, a narrow structure-activity study was accomplished, as a mean to guide the synthesis of new molecular entities, the biological studies and the study of the mechanism of action for this type of compounds.

![Figure 1 - General representation for the compounds discussed in the present work.](image-url)
compounds, which gained shape in the present study. So, we intend to develop new compounds in order to get information on the structure-activity relationship to further direct the synthesis towards increasing the biological activity and improving the physical properties of the target molecules. This synthetic work aims to find the appropriate structure for a potential drug, but without discarding the hypothesis of its application to surfaces disinfection and to several other uses in the industry that already explore similar compounds. Also, a solid structure-activity relationship data will help determining the mechanism of action of this type of compounds and the discovery of the biological target or targets responsible for the antibacterial activity, and, consequently, the compound structural features which better fit those targets and lead to improved biological activities.

The compounds discussed in the present work (fig. 1) are glycosides, once they are constituted by mainly two structural pieces, a carbohydrate moiety, and an aglycone, which in this case is a lipid or derivatives, also referred to as the lipidic moiety of this kind of molecules. So, an introduction to each of the moieties will follow, in order to better understand their roles in the molecular structure, as well as their separate biological functions and relevance in the natural world. Finally, insights onto the effects to be expected from the discussed compounds will be given.

**Carbohydrates**

Carbohydrates and their derivatives constitute one of the most abundant types of biomolecules, being involved in a wide variety of biological processes, namely in essential biological roles for all types of life forms, making these compounds essential to life.

Saccharidic units are present in compounds such as monosaccharides, oligo- and polysaccharides and in association with a wide variety of molecules, creating different groups of compounds such as glycoproteins, proteoglycans, glycopeptides and many other forms of glycoconjugates, each with distinctive biological and physicochemical characteristics.

As seen, such biological significance is highly associated to their poly-functionality and enormously complex chemistry, which promote the complexity of biological systems which are known to all life forms. Such complex and versatile
chemistry is of advantage to the biological systems, acting also as a source of important biological targets, but in the lab it renders glycochemistry a demanding realm of synthetic chemistry.

By looking into their molecular structure (fig. 2), we can better understand the versatility of such compounds, once they possess several functional groups, each with their particular reactivity and nature, normally consisting of several secondary alcohol functions as well as a primary alcohol and hemiacetal functions. Such groups give unique chemical properties to carbohydrates, which study has already begun 120 years ago by the hands of Emil Fisher (Stick, 2001).

In chemistry, the use of these compounds is also essential, but they may represent a though challenge, because even the simplest monosaccharide unit has multiple chiral centers (fig. 2), resulting in a variety of isomers, and conjugation possibilities, all of them with slightly, sometimes drastically, different properties. This variety represents the difficulties chemistry faces, once it is a synthetic challenge to conjugate or modify one functional group specifically, while maintaining the stereoisomeric structure and achieving this in good yields.

Nevertheless, carbohydrates nowadays are a growing field in chemistry, being used in several industries such as food, textile, agrochemical, and in pharmaceutical industry. Glycoscience is continuously in development as a branch of chemistry, but new aspects need now to be considered, with the increasing significance of life sciences in general, and the understanding of the true relevance of carbohydrates can only be achieved by correlating several scientific disciplines such as organic chemistry, biochemistry and cellular and molecular biology (Collins, 1995). With this goal, a branch of the glycosciences has emerged by the name of glycobiology, and has already essential role in the glycosciences.
Carbohydrates physical and chemical properties

As it was said, this type of compounds has unique chemical and physical properties which differentiate them from the other types of organic compounds. The study of such properties was a long time ago, with the discovery of the structure of the glucose, by Emil Fisher, but many discoveries were yet to be made for many other very important names such as Haworth and Lemieux (Stick, 2001).

One of this crucial properties is in the phenomenon of mutarotation. It was observed that the optical rotation of a monosaccharide solution changes over time, until it reaches an equilibrium, but the solution is no longer pure. It was discovered that, in a solution, a free monosaccharide in its more stable, cyclic, hemiacetal or hemiketal form, is actually in an equilibrium with its linear aldose or ketose form (fig. 3).

![Figure 3: Schematic representation of mutarotation of D-glucose in water](image)

Also, upon cyclisation, it can occur in different conformations either in hexa- or pentacyclic rings (figure 3). Hence, when free sugars are used in chemical reactions, we may expect a range of different compounds to emerge, although normally with one or two largely main products, which result from the most stable forms of the starting monosaccharide (Stick, 2001).

Upon cyclisation, monosaccharides can occur in different conformations either in hexa- or pentacyclic rings (figure 3). Hence, when free sugars are used in chemical reactions, we may expect a range of different compounds to emerge, although normally
with one or two largely main products, which result from the most stable forms of the starting monosaccharide (Stick, 2001).

Other important characteristics are linked to their biological role and tridimensional configuration. As shown above, sugar units can adopt very different configurations, but even in the generally most stable hexopyranoside form, conformational isomers are present. The most stable hexopyranoside conformations are the chair conformations ($^4C_1$ and $^1C_4$) and the less stable is the boat conformation, which are always present in an equilibrium, as represented in figure 4. These different conformations result in completely different tridimensional orientations of the hydroxyl groups or their substituents in the sugar moiety, assuming equatorial or axial positions (fig. 4) which, for the given example, renders the $^4C_1$ boat conformation as the most stable, and consequently the most abundant, once all the functional groups are in equatorial position (Stick, 2001).

![Figure 4: Schematic representation of equilibrium chair-boat-chair conversions of β-D-glucose](image)

The change between axial and equatorial positions is much more frequent when regarding the anomic position, once, due to mutarotation, upon cyclization, the hydroxyl group of the anomic position can assume either position, depending on which side the hydroxyl group attacks the carbonyl group. In a Fischer projection, if the resulting anomic chiral center has its hydroxyl group on the same side as the oxygen of the configurational (D- or L-) reference atom, then it is the $\alpha$-anomer. If it is directed in the opposite direction it is the $\beta$-anomer. This anomeric configuration results in an equatorial or axial position of the hydroxyl group or, more importantly, of the aglycone, which can lead to completely different biological activities, or a much better bioactivity of one of the anomers. Hence, the stereoselective synthesis of a single anomer represents a major challenge for synthetic chemists. So, there are several synthetic strategies to try to maximize one of the anomers, for example, by promoting stereochemical hindrance with protecting groups in the neighborhood of the anomic
position, by using certain solvents, such as acetonitrile, and by using acyl protecting groups on position 2 which promote the neighboring group participation.

The α anomer is usually in a higher proportion by a phenomenon which is called the anomeric effect (fig. 5). The cause for this effect is not yet proven, but two major theories emerged: the electrostatic model, which states that the preference for the α-anomer arises from the favorable local dipole–dipole interaction (fig. 5-a), and the hyperconjugation model, which is based on the stabilization gained from electron delocalization from the oxygen lone pairs to the vacant antibonding orbital of the anomeric carbon (fig. 5-b) (Mo, 2010) (Stick, 2001).

![Figure 5: Schematic representation of the electrostatic model (a) and the hyperconjugation model (b)](image)

The discussion regarding the cause of the anomeric effect is still undisclosed, and is target of several studies, the most recent ones, experimentally disproving the hyperconjugation model (Mo, 2010).

As we can see, a carbohydrate chemist must take into account all these effects and come out with a way to produce the pretended compound, while maintaining the desired configurations on the sugar moiety, dealing with the potentially poor reactional specificity, once a sugar moiety has a variety of free, reactive, hydroxyl groups, resulting in unwanted side-products. So, most carbohydrate chemistry is based on several steps that use specific protecting groups in order to limit the reactivity of some hydroxyl groups, making the main objective reaction as selective as possible. Meanwhile, after such protection, is important to easily deprotect the hydroxyl groups, either for future reactions or because they are important in the biological activities. Summing up to this necessary synthetic game, between most of the synthetic steps, the purification of the obtained products is necessary, inevitably producing losses and resulting in lower yields.
Biological Role

The growing attention given to the importance of carbohydrates and their derivatives has created a huge amount of information that suggests that they are involved in a big variety of biological processes, from the most trivial to the most crucial for the survival of life as we know it. Carbohydrates are essential to energy regulation, structural support and even cellular communication for all forms of life. The understanding of this importance has resulted in important developments in medicinal chemistry and the way certain diseases are approached, as well as big opportunities for the development of new carbohydrate-based therapeutics.

Carbohydrates are used, mostly, conjugated with other types of compounds, or in polymeric or oligosaccharidic units. A very good example of this is their structural function, being the most abundant polymers on the planet. For instance, structural support for the plants is cellulose, by being the main component of the cell wall. Cellulose is a polymer of D-glucose units linked by β-1,4-glycosidic bonds. This same monomer, when in α-1,4-glycosidic bonds, forms another polymer, starch, whose primary function is the energy storage in plants.

In the animal realm, one of the most abundant polymers is chitin, a N-acetyl-D-glucosamine polymer, used mainly for insects and crustaceans in the construction of their hard shells, and also by the fungi realm, constituting the cell wall of many mushrooms. In higher animals, carbohydrates are mainly used in the extracellular matrix that holds together cells. Bacterial cell wall is also manly constituted by a carbohydrate polymer, murein, constituted by two aminosugars, N-acetyl-D-glucosamine and N-acetyl-D-muraminic acid, and it is an example of a class of carbohydrate derivatives, called peptidoglycan.

Many types of compounds intervene in the energetic biological pathway for the biological systems, but are the carbohydrates that rest at the core of it. Glucose is the key product of photosynthesis, the process that made Earth an habitable planet by converting solar energy to chemical energy and producing oxygen (fig. 6-a), as well as it is the starting material for the respiration processes consuming the oxygen (fig. 6-b), and which decompose glucose into carbon dioxide, releasing the chemical energy previously stored (Lehninger, 1995).

\[
\begin{align*}
\text{(a)} & \quad \text{light} + 6\text{H}_2\text{O} + 6\text{CO}_2 & \rightarrow & \quad \text{C}_6\text{H}_{12}\text{O}_6 (\text{glucose}) + 6\text{O}_2 \\
\text{(b)} & \quad \text{C}_6\text{H}_{12}\text{O}_6 (\text{glucose}) + 6\text{O}_2 & \rightarrow & \quad 6\text{H}_2\text{O} + 6\text{CO}_2 + \text{energy}
\end{align*}
\]

*Figure 6: Chemical reactions associated to photosynthesis (a) and respiration (b)*
Carbohydrates are not only present in the energy production but also in its storage. Polysaccharides such as starch, in plants, and glycogen for animals, mainly mammals, are the way to store glucose and other sugars as energetic reserves. This is an important strategy for the survival of life, once it’s a life fact that food won’t always be available.

Carbohydrates are also structural components of nucleic acids, a class of biomolecules that constitute the building blocks of DNA and RNA, as well as a diverse set of other biological functions. The nucleotides are made of 3 units, a heterocyclic aromatic amine, a pentose sugar and a phosphoric acid (fig. 7). DNA, a polymer formed by nucleotides, has the unique function as the carrier of the genetic information, the base code for every type of life. Also, the various types of the messengers of RNA are constructed on the same or derivatives of DNA’s nucleotides. Their biological functions are to take the information contained in DNA to the ribosome where it is “translated” for the synthesis of proteins with the correct sequence of aminoacids. Nucleotides also have other biological functions such as enzyme cofactors and energy storage as adenosine triphosphate (ATP) (Lehninger, 1995).

Another very important role played by carbohydrates is in cellular communication and recognition. As it has been said, many carbohydrates exist as conjugates, in this case, covalent links to lipids or proteins expose carbohydrate moieties at the cells surface. These glycoproteins and glycolipids expressed at the cellular surface are crucial to a variety of biological functions like cell signaling transduction, cellular recognition, and cellular adhesion. For instance, changes in glycoconjugates influence the concentration of hormones and blood cells in circulation. The recognition of these carbohydrates, that constitute a structure named glycocalix, it’s made by proteins named lectins. This recognition mediates not only cell to cell recognition, communication and adhesion, but also the adhesion of bacterial and viral pathogens, that identify the desired tissue using also lectins (Sharon, 2007).
So, as we can see, the carbohydrate moiety is a remarkable target for synthetic chemistry involved in the pharmaceutical industry, once such broad spectrum of biological presence and involvement in the biological processes, makes of carbohydrate drug research an ever-growing science, especially as new discoveries in the biological systems demonstrate an increasing importance for these types of compounds. Also, the compounds discussed in the present work are in this category, belonging to the glycobiology research aiming to the development of new compounds and to achieve the pharmacological market.

**Lipids and Fatty acid derivatives**

The glycosides here discussed are not only part of the carbohydrate realm, but also of another group of compounds, the lipids. The aglycone of such compounds is, in most cases, an saturated aliphatic chain, from 8 to 12 carbon atoms, conferring to the molecule properties such as lipophilicity, micelle formation and surfactant capacity.

Lipids are a very broad group of compounds, occurring naturally in all biological systems performing several biological functions. They are the main constituent of cellular membranes in living organisms; nevertheless such compounds are also important in energy storage, where they also play a major role and in cellular communication (Lehninger, 1995).

They are normally found conjugated with a wide variety of different compounds, so, lipids can be divided in several categories relating to their molecular components, for example, the fatty acyl group comprehends the fatty acids and their derivatives such as esters or alcohols, which will be used in the present work. This group is characterized by a hydrocarbon chain with a terminal polar functional group, such as a carboxylic acid (ester) or an alcohol (fig. 8).
The lipids that are the main components of cellular membranes are the glycerophospholipids, being of great importance, but they are also involved in metabolism and cellular signaling functions. As the name suggests, this type of lipids is constituted by a glycerol unit or units, which is conjugated with at least one long-chain fatty acid, and the third hydroxyl group is phosphorylated which can be further substituted, regularly by carbohydrate moieties (Lehninger, 1995).

Another type of lipids to which the compounds to be discussed may be more closely related are the saccharolipids. They are compounds where a fatty acid group is directly linked to a carbohydrate structure, that can go from a single monosaccharidic unit to a oligo- or polisaccharide. This type of lipids occurs naturally mainly in the cellular membrane of some bacteria.

As lipids are present in all kinds of organisms and intervene several biological roles, in microorganisms they can assume another level of importance, once bacterial families can have completely different lipid profiles, as well as some unique types of lipids than cannot be found anywhere else. This specificity is important when an understanding of a certain metabolic pathway or biological effect is needed, once it can be performed by distinct compounds in distinct bacterial species. One good example is the quorum sensing mechanism for Gram positive and Gram negative bacteria, because once, as far as it is known, in the first case the bacterial signaling is performed by oligosacharides, but in the later the signaling is performed by lipid compounds, the N-acyl homoserin lactones (fig. 10).

![3D representation of a glycerophospholipid](image)

*Figure 9: 3D representation of a glycerophospholipid*

![Chemical structures of some representative quorum sensing signalling molecules for Gram negative bacteria (a) and for Gram positive bacteria (b) Adapted from (Williams, Winzer, Chan, & Camara, 2007)](image)

*Figure 10 - Chemical structures of some representative quorum sensing signalling molecules for Gram negative bacteria (a) and for Gram positive bacteria (b) Adapted from (Williams, Winzer, Chan, & Camara, 2007)*
Regarding the glycolipids presented in this work, the lipid moiety has an essential role in the biological activities, once the carbon chain length is a determinant factor for the bioactivity, as well as the nature of the lipid, for example regarding its insaturation (Kubo, Muroi, & Kubo, 1995; S. Matsumura, Imai, Yoshikawa, Kawada, & Uchibori, 1990). These observations were also verified in the work group and along the development of the present work (Silva et al., 2008).

State of the Art

Joining these two families of compounds into a monosaccharolipid is an idea that has roots in the late 70’s until the 80’s, where several compounds of a similar nature (Kabara, 1984; S. Matsumura et al., 1990; S. Matsumura, Kawamura, Yoshikawa, Kawada, & Uchibori, 1993), for example dodecyl glucoside, were screened for biological activities and bacterial inhibition was present for a collection of strains tested, demonstrating an increased antibacterial activity for Gram positive bacteria. Also, in some comparison studies regarding the effect of the aglycone, comparing the bioactivity of the free aliphatic moiety and that of the glycolipid, it was observed that the free lipid had some antibacterial activity but inferior to that of the glycoconjugated compound.

The studies of these compounds evolved into several patents (US5138043; US Patent Application 20020004467), were they can be found in several formulations, mainly regarding surface cleaning products and cosmetics, but no further study of their mechanism of action, or a structure activity relationship was found. These are the objectives behind the present work.

In our workgroup also some studies were performed regarding this family of compounds, which emerged from a compound screening for biological activities, and again antibacterial activity mainly regarding Gram positive bacteria was found. With the progression of the study, the bacterial activity for these structurally different compounds was found to be mainly into a more restrict group of bacterial strains (Silva et al., 2008).

A study of the physical-chemical properties of such compounds was made, regarding the micelle formation capacity and surface tension, as well as the study of their antibacterial activity along with a study of their toxicity. A comparison between the CMC (critical micelle concentration) and the antibacterial activity values was made,
in order to understand if they were correlated, but the conclusions were that in some bacterial strains, it could be observed a correlation between the values, where the biological activity increased with the decreasing of CMC, but such effect was not observed in all of them (Silva et al., 2008). There are also examples in the literature comprising antibacterial activities and CMC studies for related compounds, but no conclusions were presented regarding a possible relationship between the two properties (S. Matsumura et al., 1990; S. Matsumura et al., 1993).

The main conclusions of these studies were focused in the relation between the structures of the compounds and the biological activity they exhibited. Some variations were the same in all the studies, specially the variation in length of the aliphatic chains used, which varied from 8 to 12 carbon atoms. Also the conclusions regarding this alteration are common to all studies, once they all agreed that the compounds containing a 12 carbon atom length chain were the most active ones. About the structural differences in the sugar moiety, several sugar configurations were tested, but no general conclusion can be drawn from them.

As referred, the experience of our research group provides information relative to both the sugar moiety and the aglycone and their relation with the biological activity observed. To better understand the state of art of this research, the results published are presented next: the structures of the compounds assessed are depicted in figure 11 and the results from the biological activities in tables 1 and 2.

*Figure 11 - Structure of the 2-deoxy-arabino-glycosides previously studied
*Adapted from (Silva et al., 2008)*
Also, in addition to this information, the surface-activities and the cytotoxicity of these compounds were evaluated. Unfortunately, the most active compounds, namely compound number 7, were regarded as highly toxic. Also, the information regarding the surface–activity for these compounds, specially the critical micelle concentration (CMC) was expected to be correlated with the exhibited antibacterial activity but the conclusions were that such relationship was not present for every case, as it was already referred.

Conclusions regarding the structure-activity relationship were intimately linked to the fact that compound 7 had shown the higher antibacterial activity of all the compounds, and also associated to a greater specificity in the strains assessed, showing
a higher bacterial growth inhibition in *Bacillus* species. Relative to the structure responsible for the antibacterial activity, the main conclusions were that the 12 carbon atoms aliphatic chain was preferred as well as the 2,6-dideoxygenation pattern. Also a higher biological activity was achieved by the α anomers among all compounds tested.

Hence, part of the work here presented followed the same research line, where several different structures were tested for antibacterial activity aiming to establish a structure-activity relationship study, providing guidance for future work.

**Present Work**

The synthetic approach used to obtain new potentially antibacterial glycosides was identical to the one previously reported by our group (Silva et al., 2008), and the molecular scaffold of choice was the one that exhibited the highest activity, namely the 2,6-dideoxy-α-L-glycoside.

Once the glycosyl donor is defined, the next step in the approach is the creation of a linkage with the glycosyl acceptor, in this case, a desired lipid. This linkage can be achieved mainly by two synthetic approaches: using a specific anomeric substituent which can be activated to become a good leaving group, or using a precursor for the sugar structure, that by reaction with the desired aglycone, gives rise to the glycolipid.

For the present work, a glycal was used as a glycosyl donor. This type of compounds are unsaturated at the anomeric position, which renders capable to react with the desired aglycone, in the presence of a catalyst. The glycal of choice was 3,4-di-O-acetyl-1,5-anhydro-6-deoxy-L-arabino-hex-2-enitol for reaction with an alcohol to give the 2,6-dideoxygenated glycoside. Such reaction does not occur without the help of the catalyst, that will play a determining role in the synthesis.

According to the literature (Hou & Lowary, 2009; Wandzik & Bieg, 2006) and to the experience of our group (Rauter et al., 2006), the selected catalyst was triphenylphosphane hydrobromide, which acts effectively in mild conditions and results in the formation of the α and β anomers of the glycolipid, which for the present case is an advantage, once the present work has an exploratory nature and both anomers should be tested for activity. The general ratio between the anomers obtained, mostly α/β: 5/1 according to our group’s experience (Rauter et al., 2005), is also an advantage once,
from previous work, the α anomer was considered the most active one, so it is also the desired main product in the synthetic approach to be performed.

In the present work we focus on modifying the aglycone to better understand its importance for the biological activity exhibited by this family of compounds.

Also, the present work aims to understand the mechanism of action for this type of compounds, and the biological targets and effects that are better observable in the *Bacillus* species. So, the range of bacterial strains tested for this assay was focused on the *Bacillus* family aiming to the potential use of the optimized compounds against *B. anthracis*, a known pathogen capable of being used as a biological weapon.

This effort to understand the mechanism of action for this type compounds is not found in the literature, despite of the existence of reports concerning similar compounds with antibacterial activity (S. Matsumura et al., 1990; S. Matsumura et al., 1993; S. Matsumura, Kawamura, Y., Yoshikawa, S., Kawada, K., & Uchibori, T., 1998). As a consequence, a robust study must be made in order to prevail in the absence of literature support. For this, the bacterial strain selected were two *Bacillus cereus* strains (ATCC 14579 and ATCC11778) the first being one of the best genetically described *Bacillus* strains, allowing several genetic and phenotypical approaches to be made with some theoretical support, and the second being one of the most closely related to B. anthracis, genetically speaking. Also a *Bacillus subtilis* (ATCC 6633) was used, once it is a reference species for the *Bacillus* family, an *Escherichia coli* (ATCC 8739) strain as the most used Gram-negative representative bacteria and a *Staphylococcus aureus* (ATCC 29213) strain, as a general representative for Gram-positive bacteria, which also served as an indicator of the compounds specificity, once in the studies regarding similar compounds showed bioactivity to most Gram-positive bacteria, including *S. aureus*. 
Materials and Methods

Chemical synthesis materials and methodology

Reagents for synthetic procedures were purchased to Sigma-Aldrich and the reactions performed were controlled by Thin-Layer Chromatography (TLC), using aluminum plates with a coating of silica gel Alugram Xtra Sil G/UV F254 (Macherey-Nagel). The detection of products in TLC was made by observation under UV light (254 nm) (Camag) and/or by spraying the plate with a solution of sulfuric acid in pure ethanol 10% (v/v) and heated to 120 °C with a hot-air pistol. The verification of the neutralization processes was made using pH paper (Filterlab, RL911R07).

The solutions were concentrated in a rotary evaporator (Buchi.V220) at 40 °C under reduced pressure. The separation and purification of products was made by column chromatography, using silica gel 60 G (0.040-0.063 mm, SDS). The quantity of stationary phase to use was approximately 50 times the mass of the dry residue. All the solvents used (Carlo Erba, VWR, Panreac) were previously distilled and maintained in molecular sieves 4 Å. Glassware was previously dried in a stove at 100 °C.

All the structure identification of compounds was performed by ¹H-NMR, COSY, ¹³C-NMR and HMBC obtained from a BRUKER Avance 400 (400.13 MHz for ¹H and 100.62 MHz for ¹³C) apparatus. The solvent used was deuterated chloroform (CDCl₃) (1% v/v Me₄Si, Sigma-Aldrich). The chemical shifts (δ) are expressed in ppm, and Me₄Si was used as standard. The data was analyzed using ACD/Labs software (ACD/NMR Processor Academic Edition Version 12.01).

Anti-bacterial activity assessment

All procedures used in the biological activity assessment are according to the Clinical Laboratory and Standards Institute (CLSI) specifications (CLSI, 2005, 2006).
**Bacterial strains and growth conditions**

The bacterial strains used for this work were *B. cereus* ATCC 11778, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739 and *S. aureus* ATCC 29213 from the American Type Culture Collection (ATCC). The strains were preserved as lyophilized cells at 4 ºC or in a solution of 10 % glycerol and LB at -70 ºC. Pré-inoculum were performed before each assay, in the same growth medium used in the assay (20 ml), and placed in an incubator at 30 ºC, with orbital stirring at 200 rpm, in overnight.

**Medium Preparation**

All the Muller-Hinton cation adjusted broth (MH) used was made according the instructions of the manufacturer, dissolving 21 g of dry Muller-Hinton medium (Fluka, 90922) and adding 1 L of purified water (Milli-Q). For the preparation of Muller-Hinton Agar broth (MHA), 15 g of agar were added to the dry MH medium.

Luria-Bertani (LB) broth was made with 10 g of Tryptone, 5 g of yeast extract and 10 g of NaCl, dissolved in 1L of purified water (Milli-Q). For the preparation of Luria-Bertani Agar broth (LBA), 15 g of agar were added to the previous formulation.

The growth mediums were sterilized at 121 ºC during 15 minutes and stored at room temperature.

**Compound Preparation**

A stock solution of all the compounds tested was made, by dissolving the compounds in DMSO in order to obtain a solution of 4 mg per milliliter of DMSO. Those solutions where then stored at -70 ºC to minimize compounds degradation.
**Microdilution Method**

Overnight cultures of the bacterial strains were diluted to the absorbance of 0,08 to 0,10 at the wavelength of 625 nm, using sterilized medium as a reference (inoculum standardization to 0,5 McFarland Unit). Growth medium was placed in a microplate and the desired amount of compound was diluted in the media by serial dilution. This growth media was inoculated with 10 µl of 1/10 dilution of the 0,5 McFarland solution. The plate was sealed with parafilm and placed in the microplate reading apparatus (Anthos Zenith 3100 Microplate Multimode Detector) for readings at 595 nm, every 10 minutes, with agitation before each reading, at the temperature of 35 ºC during 72 hours.

At least one compound and one bacterial strain were maintained in all assays, in order to serve as inter-experimental control.

The data was collected by Beckman Coulter Multimode Detection Software (V 2.1.0.17) and analyzed using Microsoft Office Excel.

**Solid media method**

Overnight cultures of the bacterial strains into use were diluted to an absorbance of 0,08 to 0,10 at the wavelength of 625 nm, using sterilized medium as a reference (inoculum standardization to 0,5 McFarland Unit). Liquefied growth medium was placed in the plates and the desired amount of compound was diluted in the media. After the drying of the medium, it was inoculated with 10 µl of drops 1/10 dilution of the 0,5 McFarland solution. After the drying of the drops, the plates were placed in an incubator, at 35ºC during 24 hours. Visible growth of colonies was verified afterwards, and the results registered.
Phenotypic Array

Phenotype MicroArrays Biolog® technology was used for assessment of the impact of compound 17 at the concentrations of 16, 8 and 4 µg/ml in the metabolism of 95 different carbon sources by *B. cereus* ATCC 14579 strain. The procedures undertaken were according to the recommendations of the manufacture (Bochner *et al.*, 2001). The pre-inoculum was prepared similarly to the procedure used for the microdilution assays: 10 ml of LB broth was inoculated and placed in an incubator at 35 ºC, with orbital stirring at 200rpm in overnight. Several preparations of the solutions to use must be done, once the growth medium for this experiment is specific to each PM plate used.

The solutions needed to assemble the Phenotype microarray - PM additive solution were prepared according to the manufacturer recommendations and summarized in the Table 3:

Table 3 - Stock solution for PM additive solution

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ingredient</th>
<th>1x Conc.</th>
<th>40-120x Conc.</th>
<th>Formula Weight</th>
<th>Grams/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>MgCl₂, 6H₂O</td>
<td>2mM</td>
<td>240mM</td>
<td>203.3</td>
<td>4.88</td>
</tr>
<tr>
<td></td>
<td>CaCl₂, 2H₂O</td>
<td>1mM</td>
<td>120mM</td>
<td>147.0</td>
<td>1.76</td>
</tr>
<tr>
<td>C</td>
<td>L-arginine, HCl</td>
<td>25uM</td>
<td>3mM</td>
<td>210.7</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>L-glutamic acid,</td>
<td>50uM</td>
<td>6mM</td>
<td>169.1</td>
<td>0.101</td>
</tr>
<tr>
<td>D</td>
<td>L-cystine pH8.5</td>
<td>25uM</td>
<td>1mM</td>
<td>240.3</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>5'-UMP, 2Na a</td>
<td>25uM</td>
<td>1mM</td>
<td>368.1</td>
<td>0.037</td>
</tr>
<tr>
<td>E</td>
<td>yeast extract</td>
<td>0.005%</td>
<td>0.6%</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>F</td>
<td>Tween 80 b</td>
<td>0.005%</td>
<td>0.6%</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Water miliQ</td>
<td>Until 100ml</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*If necessary, adjust pH to 8.5 with saturated NaOH solution and check with pH paper.*

The aforementioned solutions where sterilized in autoclave at 121 ºC during 15 minutes, and afterwards stored at 4ºC until use (maximum shelf time of 48H). The
assemblage of the PM additive solution was prepared by combining the previous solutions according to Table 4 and stored at 4°C.

Table 4 - PM additive solution (12X)

<table>
<thead>
<tr>
<th>Solution</th>
<th>For PM 1,2</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>10 ml</td>
</tr>
<tr>
<td>C</td>
<td>10 ml</td>
</tr>
<tr>
<td>D</td>
<td>30 ml</td>
</tr>
<tr>
<td>E</td>
<td>10 ml</td>
</tr>
<tr>
<td>F</td>
<td>10 ml</td>
</tr>
<tr>
<td>Water</td>
<td>30 ml</td>
</tr>
<tr>
<td>MiliQ</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Serial dilutions of compound 7 in DMSO were made from the stock solution to 1 ml solutions with the final concentrations of 320 µg/ml, 160 µg/ml and 80 µg/ml.

The 10 ml of overnight pre-inoculum previously prepared, were centrifuged at 3000g, 4°C for 5 minutes and resuspended in 1ml of IF-0a medium, by mixing carefully with a micropipette with cut tip. This cellular suspension was then standardized to 0,5 McFarland Unit in 5 ml of IF-0a (OD_{600nm adjusted to 0,088})

The PM Inoculating Fluid used in the PM microplates was the result of mixing the PM additives prepared before with the following solutions, according to the presented in Table 5.

Table 5 - 1x PM Inoculating Fluids from stock solutions

<table>
<thead>
<tr>
<th>PM Solution</th>
<th>Stock</th>
<th>Control (ml) for 2 plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF-0a (1.2x)</td>
<td>GN/GP</td>
<td>40.0</td>
</tr>
<tr>
<td>Dye mix F (100x)</td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td>PM additive (12x)</td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>Cells (13.64x)</td>
<td></td>
<td>3.52</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>48.0</td>
</tr>
</tbody>
</table>
100 µl of the PM inoculating fluid was added as well as compound 17 solutions to each well of the 4 PM1 microplates used (corresponding to control experiment and compound 17 at the final concentrations of 16, 8 and 4 µg/ml). After that, the plates were marked and sealed using parafilm. Then at point 0, data acquisition of absorbance at 590nm was performed for each plate. The plates were then incubated at 35ºC, under orbital agitation, covered with aluminium foil in order to minimize the auto-degradation of the dye.

The experimental readings were taken after half an hour’s time, and then hourly for the rest of the available time. At each reading point, data acquisition was undertaken using the microplate reader apparatus (Anthos Zenith 3100 Microplate Multimode Detector).

Data analysis

The data obtained thru the Phenotype MicroArrays Biolog® technology was analysed taking into account the following assumptions: 1) An internal standard (A1) was present in all the plates, in order to verify the auto reduction of the tetrazolium dye, 2) The variance of absorbance observed was assumed to be result of the irreversible reduction of a dye, so, any decreases in the read absorbance lack any biological meaning. 3) The data obtained was grouped in two main periods, which corresponds to an initial phase of response to the compound 17 (from 0 to 5 hours of incubation), and to a late phase of response (from 16 to 27 hours of incubation). 4) The compound was only added after 1 hour of incubation (baseline determination), the experimental handling needed to the addition of the compound can cause the experiment susceptible to modifications, therefore when data discrepancy observed at the next reading point, data will not be used for analysis. 5) Each well per plate was assumed to have only the stated carbon source by the manufacture.

Data normalization: In order to normalize data obtained for the consumption of each tested carbon source regarding bias related to the experimental design; to the absorbance measured in each well of each microplate was subtracted the absorbance value of A1 for each corresponding time point for each microplate. The resulting values were assumed to be zero, when negative values were obtained.

Assessment of experimental baseline variance: Once the A1 wells were independent replicates in our experiment (4X), they were chosen to calculate the
standard deviation (SD) related to the experimental procedure, for each temporal point. The highest SD value observed along all temporal points, was used as baseline.

Cut-offs determination and hits classification: Assuming a normal distribution in the scattering results, and the 2σ value comprehends 95.45% of the observed dispersion for the experiment, the 2σ value was used as cut-off for defining a significant hit for the classification of the module value of the difference observed between the experimental values for compound 17 and control experiment for each tested carbon source in each of the tested time periods, assuming an error < 5%.

Further cut-offs were defined, the effects observed due to the presence of our compound can be drastic, mild or even subtle. Therefore, a cut-off corresponding to a 5σ value was used for classification as drastic changes, assuming an error < 0.001%. For potentiation effect, a smaller cut-off was needed, due to be a much more subtle observed effect, therefore a σ value was used. Hit classification was made along the temporal interval. Additional to the mathematical interpretation, a careful analysis of data for each carbon source was performed by visual inspection of the respective graphic.

After the mathematical and graphic representation analysis, several different results arose, which were divided into five categories depending on the effect shown: highly inhibitory effect, when the difference between the effect of the compound 17 and control was higher than 5σ cut-off value; inhibitory effect, when the difference observed was between the 5σ and 2σ values; no effect, when there was bacterial growth in the absence of compound (Abs>2σ) and the difference between the values in the absence and presence of compound is < 2σ; no growth, when the value with and without the presence of compound is < 2σ; and potentiation effect, when the value without the compound is < 2σ and the difference of value between the presence and absence of compound is superior to σ.

Determination of main metabolic pathways impact by the compound:

For each hit, a search was made using the KEGG database (Kyoto Encyclopedia of Genes and Genomes, available at http: www.genome.jp/kegg/), thru the KEGG Ligand, , in order to identify what the metabolic pathways associated to the classified carbon sources. The objective of this task was to understand if the identified hits were clustered in one or a group of metabolic pathways, providing us some insight about the possible targets for the compound 17. Therefore a list of hits with inhibitory and
potentiating effects was associated to the metabolic pathways relevant in *B. cereus* to determine the most common metabolic pathways among this set of compounds.
References


Mo, Y. R. (2010). Computational evidence that hyperconjugative interactions are not responsible for the anomeric effect (vol 2, pg 666, 2010). Nature Chemistry, 2(9), 789-789. doi: Doi 10.1038/Nchem.826


