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Absolute Configuration Determination of Sugar: A Review of the Different Methods

João Rufino de Freitas Filho [a*], Jucleiton José Rufino de Freitas [a], Juliano Carlo Rufino de Freitas [a], Mônica Freire Belian [a], Claudio Augusto Gomes da Camara [a], Pedro Ramos de Souza Neto [a], Alécia Regina Andresa Silva [a], Alex Sandro Nascimento da Silva Filho [a], Alice Mariana Souza de Almeida [a], Jonatas Tavares da Silva [a], Mirian Luzinete da Silva [a], Anne Gabrielle Marques da Silva [a], Marcilio Martins de Moraes [a], Clécio Souza Ramos [a] and Ronaldo Nascimento de Oliveira [a]

[a] Departamento de Química, Universidade Federal de Rural de Pernambuco. Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil.

Authors' contributions

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**Corresponding author: E-mail: joaoveronice@yahoo.com.br;*

Review Article

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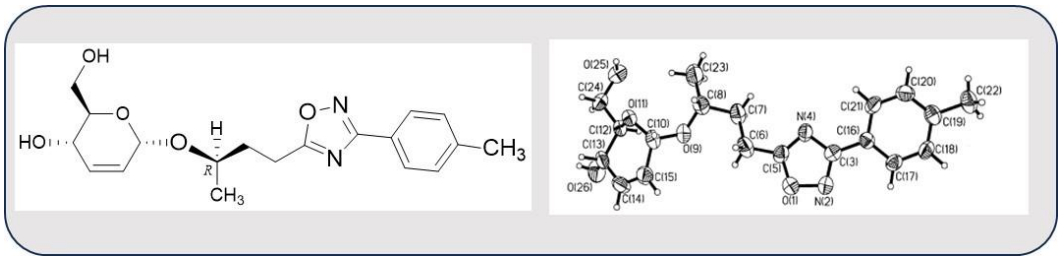
ABSTRACT

Determination of the stereochemistry of the stereocenter from the aglyconic part of carbohydrates is always one of the most important tasks for chemistry carbohydrates researchers. The absolute configuration (AC), a challenge for synthetic chemists, has attracted much attention. During the past few decades, many techniques and approaches have been developed to determine the AC of carbohydrates, including methods of X-ray diffraction (XRD), electronic and vibrational circular dichroism (ECD and VCD), Raman optical activity (ROA), nuclear magnetic resonance (NMR) utilizing anisotropic effects of chiral derivatizing agents, and quantum chemical calculations. On the other hand, none of the currently applied techniques can dominate AC determination, since they each have their respective limitations corresponding to the different structural features. This review summarizes most of the techniques and methods commonly used in AC assignment of carbohydrates (but specifically glycosides), in the last decades (2000-2023).

GRAPHICAL ABSTRACT



Keywords: Chiral molecules; stereochemical assignment; absolute configuration; glycosides.

1. INTRODUCTION

Carbohydrates are the most stereochemically intricate family of biomolecules and present substantial challenges to anyone trying to understand their nomenclature, reactions or branched structures. However, each method has its unique advantages and limitations which should be considered while using them [1].

The carbohydrates and derivatives incorporate several chiral motifs, an additional challenge is the determination of the relative configuration (RC) of constituting stereogenic centers which in turn govern the conformational properties. Furthermore, in addition to RC and conformational stereochemical features, the absolute configuration (AC) of the stereogenic centers has a profound impact on a variety of molecular properties, such as chemical reactivity and catalytic, biological, and pharmacological activities [2,3]. In light of the above considerations, full stereochemical assignment of a given system is of fundamental importance in many different fields, spanning from chemical physics to biochemistry.

On the other hand, the absolute configuration of carbohydrates is already well defined in the literature, making it necessary to elucidate the stereochemistry of the aglycone portion when forming glycosides. A current example in determining the absolute configuration of aglycones is the use of two methods, derivatization and non-derivatization, in which it was possible to determine the absolute value of the configuration of aldose enantiomers in traces of natural glycosides. However, it is worth noting that the availability of identification methods such as GC (Gas chromatography), TLC (Thin-layer chromatography), HPLC-UV (High- Performance Liquid Chromatography-Ultraviolet), UPLC/HPLC-MS (Ultra-high performance liquid chromatography - mass spectrometry) and NMR (Nuclear Magnetic Resonance Spectroscopy) require milligrams of glycosides and are

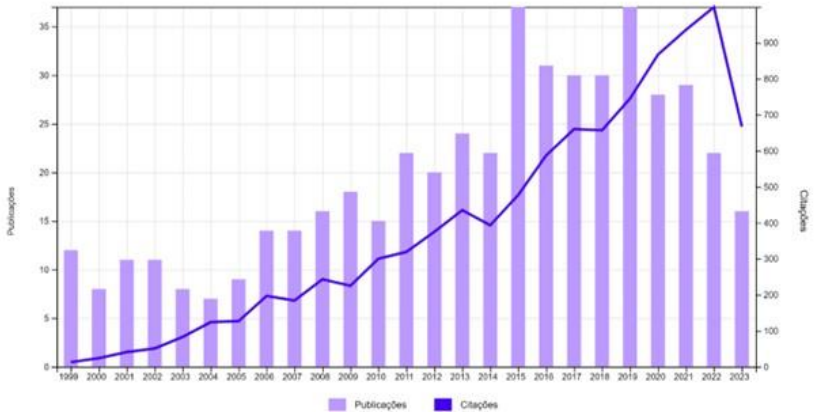


Fig. 1. Number of publications per year and number of citations per year

characterized by a complex derivatization process and low detection resolution, having the same disadvantage of requiring milligrams of sample, which represents a limitation that the other methods address [4].

Other techniques can be used to characterize the absolute configuration, such as by means of optical rotations which, when compared with already established standards, can determine the stereoisomeric position of the products. However, the author highlight the existence of other procedures to determine the absolute configuration of the aglycone, including X-ray diffraction (XRD), electronic and vibrational circular dichroism (ECD and VCD), Raman optical activity (ROA) and nuclear magnetic resonance (NMR) [5].

Fig. 1 we can observe the statistical data regarding the number of publications on the web of Science platform according to the years and citations where the research was based on the search ranging from 2000 to 2023 using the following keywords: "*O*-glycosides" and "absolute configuration".

In this review, an overview of the different methods for the determination of the absolute configuration of carbohydrates, specifically glycosides, and their underlying principles are summarized to serve as a reference for researchers.

2. DESCRIPTION OF THE METHODS FOR THE ABSOLUTE CONFIGURATION DETERMINATION IN CARBOHYDRATE CHEMISTRY

Knowing the importance of characterizing correct the position which group in chiral bond compounds, especially those that will be used in biological activities, the assignment of the absolute configuration must be unambiguous. For many decades, incorrect attributions have accumulated due to a lack of advances and contributions in the field, with limited techniques for stereoisomeric characterization. Thus, advances have been made in techniques that must be associated with the set of corroborations to guarantee the reliability of the results and complete knowledge of molecular stereochemistry. The main methods of stereoisomeric analysis, their advances and applications for determining the absolute configuration will be explored in this topic. Among the most commonly used are X-ray crystallography (XRC), electronic circular dichroism (ECD), vibrational circular dichroism (VCD), optical Raman activity (ORA), nuclear magnetic resonance (NMR), and quantum calculations [6,7].

2.1 X-ray Diffraction

X-ray crystallography allows detailed visualization of the arrangement of atoms in the molecule, which is seen after the incidence and refraction of rays in various directions, so called the resonant scattering and depends on the physicochemical properties of the molecule. This is an important and recurrent technique for assigning the absolute configuration to molecules by providing data such as the distribution of bonds, conformations, and bond angles observed, contributing to the thermodynamic explanation of stability and the possible interactions that allow specific biological activities between the isomers, but it is a tool that has some limitations due to the need for the compounds to be in crystalline form [8-10].

A countermeasure widely used to achieve a crystalline network in amorphous compounds or liquids with relative viscosity is reagent-induced crystallization, like example, Puius et al. [11] determined the absolute configuration of kanamycin A (Fig. 2), an antibiotic from the aminoglycoside class used to treat several diseases.

From the knowledge of the structure of this antibiotic, the understanding of its activities and also the biological activities of successor compounds are achieved. In this way, through X-ray crystallography of kanamycin sulfate monohydrate was carried out using single crystal diffractometric data (Fig. 3) [11].

From the radiographic study, the authors confirmed the agreement with previous studies, observing the veracity of the X-ray crystallography technique. From this, the arrangement of the atoms, the conformation of the rings and bonds involved in the molecule were confirmed, contributing to the understanding of the biological activity of this drug.

2.2 Electronic and Vibrational Circular Dichroism

Optical rotation is the most used method for understanding space structures and occurs as a result of the incidence of polarized light as it travels through the chiral molecule, diverting this incident light to specific directions [12]. Computational calculations are recurrent technique to provides greater reliability in the results, as Stephens et al. [13] uses to discover the AC of three organic compounds, associating optical rotation with the Density Functional Theory (DFT) theory.

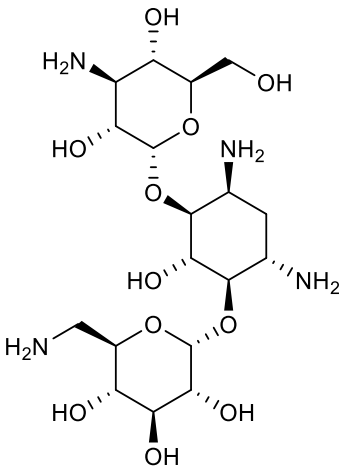


Fig. 2. Structure of kanamycin A

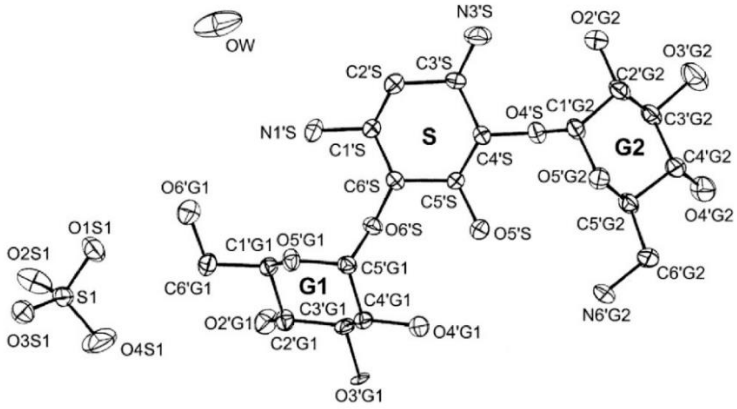


Fig. 3. ORTEP of the molecular structure of kanamycin A

Energy measurements at different wavelengths allowed exploring different regions of the spectrum, such as UV, visible and infrared. This led to the implementation of new techniques that are more sensitive to chirality. As a result of advances in techniques, electronic circular dichroism (ECD) and vibrational circular dichroism (VCD) stand out, which are based on the interaction between polarized light and matter, observing the circular deviation of light to the left or right. Therefore, the molecular structure and its interactions are understood through electronic or vibrational transitions, depending on the technique [14,15].

The combination of different methods allows for different types of observed energies and responses. Energies in the infrared region, observing vibrational transitions, such as VCD, observe a variety of conformations, property that is often not possible to see in other techniques. So, care must be taken because molecules with flexible bonds, that is, those that are easy to change conformations, can interfere with the result. VCD is a more general technique, as it will not look at just part of the structure, but the entire molecule, because the vibration of each bond is observed. Its results are generally obtained through experimental spectra compared with quantum calculation spectra [15].

A survey carried out by authors reported that between 2015 and 2019, around three hundred natural product molecules had their AC determined using VCD along with other complementary techniques, such as computational calculations for comparing theoretical and experimental spectra, infrared spectra, stereocontrolled syntheses and technical chiral, such as chiral HPLC and NMR. With this, different classes of natural products such as terpenes, polyketides, amines, among others, could have their isomer structures confirmed [16].

Monde et al. [17] used the vibrational circular dichroism (VCD) technique was applied to carbohydrates and produced a glycoside characteristic VCD band, which was named the “glycoside band.” The authors confirmed that this vibration was related to anomeric configurations of carbohydrates in the case of the D [17]. The Fig. 4 demonstrates band is defined as a negative, sharp, and intense VCD band at around 1145 cm^{-1}, exhibited by D-sugars with an axial α-glycosidic linkage in the $^4C_1$ conformation.

2.3. Raman Optical Activity

The Raman Optical Activity (ROA) technique details molecular structure after scattering of incident radiation, known as vibrational Raman scattering. ROA is advantageous because a wide range of molecules can be used as VCD, as it also observes vibrations and is sensitive to the chirality of the structure. Occurs in two different behaviors, the first aspect is incident circular polarization (ICP), where the light is circularly polarized in both directions, left or right, and the second is scattered circular polarization (SCP), where an incident light of fixed polarization is used to measuring the intensity of a circularly polarized component of scattered light [18].

Dudek et al. [19] used the Raman spectroscopy and Raman Optical Activity as unique tools to study complex structures of carbohydrates. according to the authors RS and ROA spectra are presented together with an expanded discussion on various structures and conformations of studied carbohydrates in the solution taking into account particular regions, i.e. (1) low wavenumber region (250–600 cm^{-1}), (2) anomeric region (600–950 cm^{-1}), (3) fingerprint region (950–1200 cm^{-1}) and (4) CH_2 and COH deformations region (1200–1500 cm^{-1}) (Fig. 5). Like this, the following information can be obtained about the absolute configuration of the anomeric carbon, and the orientation of the anomeric hydroxyl group, the ring structures and the relative orientation of substituents and the conformation of the exocyclic CH_2OH, respectively.

2.4. Nuclear Magnetic Resonance

The use of Nuclear Magnetic Resonance (NMR) has grown not only for the structural elucidation of unknown molecules but also for the assignment of absolute configuration with specific enantiomerically pure reagents acting as chiral derivatization agents by forming covalent bonds or chiral solvation agents that make intermolecular bonds with the substrate. This determination through chiral derivatization allows the derivatized materials to assume a conformation that will be differentiated through chemical shifts between the enantiomers of the reagents, allowing the stereochemistry to be determined [20].

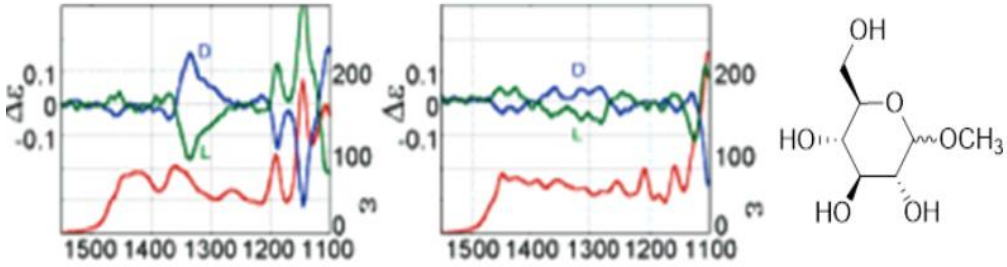


Fig. 4. IR (red) and VCD (blue or green) spectra in DMSO-*d*$_6$ (c 0.16 M, *l* 72 μm) of methyl D- and L-glucopyranosides

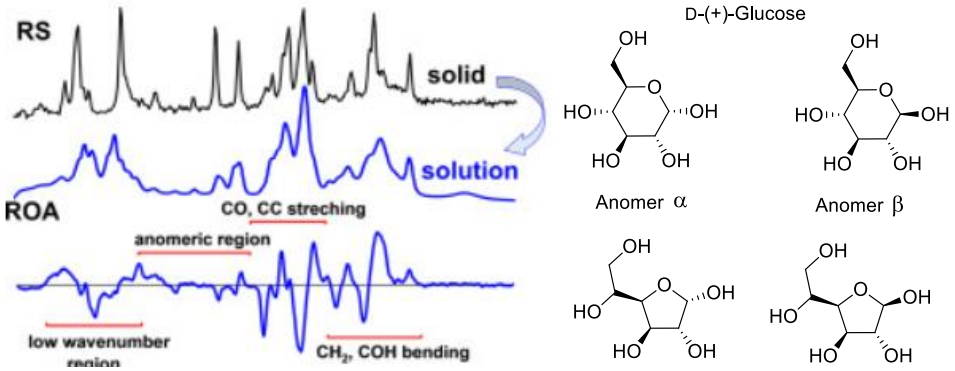


Fig. 5. Raman and ROA spectra of pentoses and hexoses in aqueous solution.

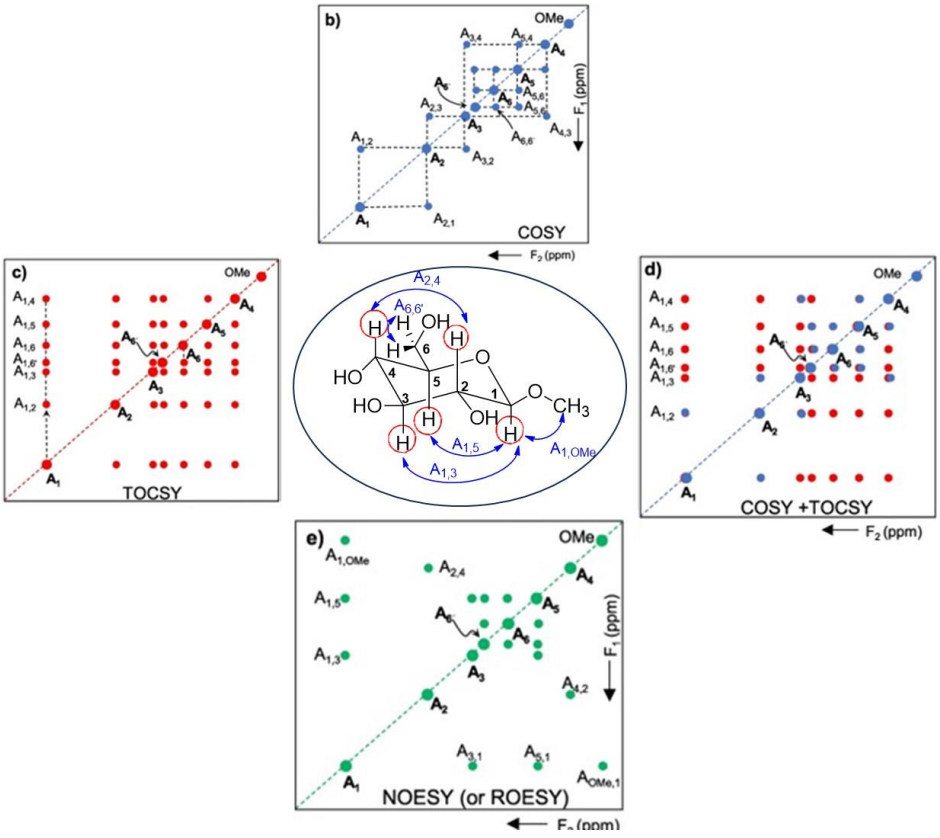


Fig. 6. Determination of absolute configuration performed by liquid-state NMR.

Absolute configuration can be achieved of carbohydrates, second Speciale et al. [21] can be mostly performed by liquid-state NMR, and it is a demanding task because the NMR signals of these biomolecules explore a rather narrow range of chemical shifts, with the result that the resonances of each monosaccharide unit heavily overlap with those of others, thus muddling their punctual identification. Accordingly, in sugars with the axial orientation of H-2, a glucose (Fig. 6), β anomers have the *trans*-diaxial orientation of the H-1/H-2 protons so that they appear as doublets with a coupling constant value of ~7–9 Hz.

The NMR technique allows the determination of the absolute configuration with certain limitations and specific conditions, making it necessary to use other complementary methods such as statistical data, neural networks, empirical optimization, optimization of different parts of the molecule and *ab initio*. In *ab initio*, the functionals of density functional theory, namely PBE and B3LYP, and the second-order Møller-Plesset perturbation theory, Coupled Cluster Singles and Doubles have been used to study spectra, especially displacement, and the spectral simulation techniques must include the chemical shift, the relaxation rate and the prediction of the Overhauser nuclear effect and the coupling constant [22].

3. STRUCTURE ELUCIDATION OF GLYCOSIDES

3.1. Glycosides

The determination of the structure of glycosides is necessary to evaluate its biological activity. Therefore, it is of utmost importance to determine the absolute stereochemistry of monosaccharide components in natural glycosides, especially from natural product glycosides that are newly discovered. However, many natural products with bioactivities are found at very low concentrations, even less than one part per million [23]. Therefore, the discovery of the absolute configuration of their sugars remains a challenge despite the successful purification and isolation.

Recently, there have been notable advances in determining the absolute configuration of natural products. This is largely due to the accuracy and speed of computational predictions of spectroscopic properties. Electronic circular dichroism has been the predominant technique for determining the absolute configuration of molecules that have chromophores. On the other hand, although less common, vibrational circular dichroism (VCD), Raman optical activity, and optical rotatory dispersion (ORD) experiments are gaining prominence, especially for molecules that do not have chromophores [24].

Among various techniques, the application of NMR spectroscopy using microcryosound, together with other "nanomolar scale" techniques such as CD, FTIR, and FTMS, has expanded our ability to analyze minute quantities of compounds, providing a more comprehensive perspective on the discovery of natural products [25]. Nanomolar-scale techniques represent advances in optimizing the signal-to-noise ratio in spectroscopic methods. However, their most significant impact lies in opening up new opportunities to identify new compounds in rare organisms, providing detailed insights into the complexity and chemical diversity present in unique samples [26].

In a study dedicated to the precise identification of antioxidant molecules in *Garcinia buchananii* bark extract, a notable class of compounds identified was flavanone-*C*-glycosides (Fig. 7). Detailed analysis of flavanone-*C*-glucosides was conducted using advanced techniques, including LC-MS/MS, 1D and 2D NMR spectroscopy, and circular dichroism (CD). These analytical approaches allowed for a thorough characterization of these antioxidant compounds, contributing to a comprehensive understanding of the chemical composition of *Garcinia buchananii* bark extract [27].

The data obtained showed that the spectra of C-glycosides **1** and **2** were respectively (2*R*,3*R*)-taxifolin-6-C-β-D-glucopyranoside and (2*R*,3*R*)-aromadendrin-6-C-β-D-glucopyranoside. To elucidate the configuration of the C(2) and C(3) carbon atoms present in the aromadendrin of compounds **1** and **2**, circular dichroism (CD) spectroscopic measurements were carried out using the commercially available reference isomer (+)-(2*R*,3*R*)-taxifolin [28].

Over the years, various methods have been developed for the formation of 1,4-*O*-interglycosidic bond. Among these methods, Kahne sulfoxide glycosylation [29] stands out as a particularly effective technique for synthesizing biologically relevant glycosides [30].

In addition to the X-ray crystallography technique, comprehensive nuclear magnetic

resonance (NMR) methods have been presented to elucidate the configuration of sulfinyl glycosides. One of these methods involves the use of chiral displacement reagents, but the approach is considered less attractive due to the small discrepancies in the chemical shifts observed in the NMR spectra. Another approach is based on correlations between the ^{1}H and ^{13}C NMR chemical shifts and the absolute configuration of the sulfinyl group in the sulfoxylglycosyl [31-33].

The most effective approach to producing glycosyl sulfoxides is the oxidation of glycosyl sulfides. The use of hydrogen peroxide to convert sulfides into sulfoxides has proven to be one of the most attractive methods [34]. The reaction of *t*-butylmagnesium chlorides with diastereomerically pure (*R*)-1,2-*O*-isopropylidene-3,5-*O*-sulfinyl-α-D-glucofuranose (*R*)-4 was found to be stopped at the stage of the corresponding, diastereoisomerically pure 1,2-*O*-isopropylidene-(5-*O*-α-D-glucofuranosyl) t-butanesulfinate (*S*)-10 for which the crystal structure and the (*S*)-absolute configuration was determined by X-ray crystallography [33].

3.2 Unsaturated Glycosides

The characterization of synthetic compounds is the fundamental principle in the synthesis of organic compounds, but some molecules present greater difficulties in their structural elucidation, as is seen in 2,3-unsaturated glycosides due to the presence of several stereogenic centers, making stereoisomeric determination difficult [35-36].

Therefore, given the need to study optically active products in isolation, to be able to compose relevant medicines as pure enantiomers or as racemic mixtures, Srivastava et al. [37], provide a report in which they describe in detail a synthesis process, isolation, and determination of the absolute configuration (AC) of 10 new 2,3-unsaturated glycosides which have a stereocenter in the aglycone portion and which were obtained by the reaction between a glycal and a racemic (4-[3-(aryl)-1,2,4-oxadazol-5-yl]-2-butanols) alcohol (Fig. 9) via Ferrier Rearrangement.

The structures and stereochemistry of all the diastereoisomers formed in the synthesis reaction were elucidated on the basis of ^{1}H NMR spectroscopic analysis. Of the compounds synthesized, the absolute configuration of diastereoisomer **A** was proven using single crystal X-ray diffraction. X-ray crystallography provides precise information on the conformational configuration of molecules, and the crystallographic results Fig. 10 showed that the compound has an *R* configuration (at C-8) and its other pair, the *S* configuration.

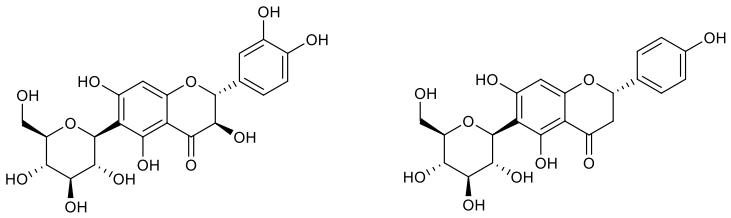


Fig. 7. Chemical structures of compounds 1 and 2

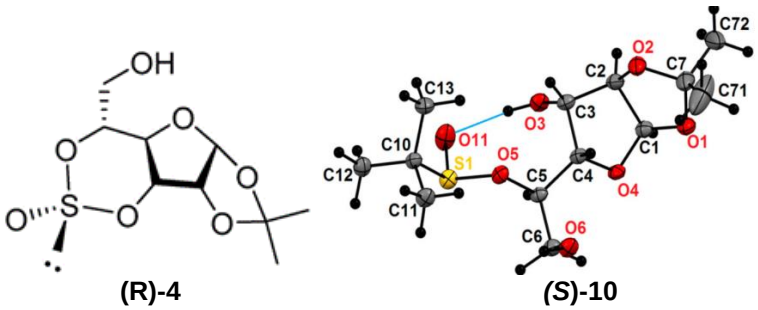


Fig. 8. (*R*)-1,2-*O* -isopropylidene-3,5-*O*-sulfinyl-α-d-glucofuranose (*R*)-4 and molecular structure of (*S*)-1,2-*O*-isopropylidene-(5-*O*-α-d-glucofuranosyl) *t*-butane-sulfinate 10.

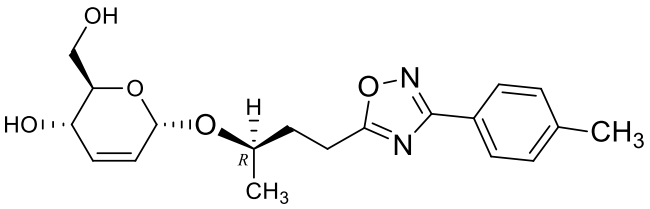


Fig. 9. Unsaturated glycoside containing 1,2,4-oxadiazole as an aglycone.

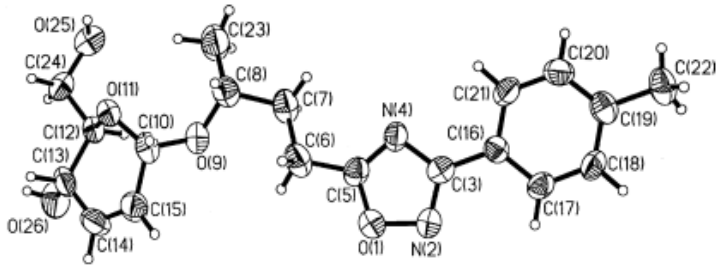


Fig. 10. Ortep diagram of the conformational isomer of compound A

In addition, the stereochemistry of the bonds in C(11), C(13), and C(14) were identified as being (*S*), (*R*), and (*S*), respectively. Calculations of the molecular orbitals using semi-empirical methods (AM1) carried out by the researchers, showed that the anomeric hydrogen is arranged equatorially, given that the molecule has a torsion angle at H(15)-C(15)-C(10)-H(10) of -43.2º, agreeing with experimental results obtained when compared.

Despite continuous advances in techniques for the structural elucidation of organic compounds, quantum computational calculations have been pointed out as strategies that allow us to explore new possibilities for the synthesis and elucidation of molecules of interest, as well as favoring a better understanding of properties that are not easily accessible by experimental methods for large and complex molecular systems [38-40].

C-glycosides have attracted a lot of attention from researchers because they can act as mimics of *O*-glycosides and have relevant biological activities [41]. A strategy for stereoselectively obtaining these compounds has been reported by Gálvez et al. [42] based on the addition reaction of titanium enolates (*S*)- or (*R*)-4-isopropyl-*N*-(2-propanoyl-1,3-triazolidine-2-thione, and (*S)*-4-isopropyl-*N*-(2-pivaloyloxyacetyl)-1,3,-thiazolidine-2-thione [43] to glycals mediated by SnCl. Under these conditions, the appropriate choice of the chiral auxiliary (*R* or *S*) and the C6-hydroxy protecting group enables the controlled obtaining of corresponding *α*- and/or *β*-*C*-glycosides in satisfactory yields and with high diastereoselectivities. The configurational analysis of the new stereocenters of the products formed was initially established through 2D NOESY NMR studies and by analyzing the $^{3}J_{H1-H1'}$ coupling constant of the ^{1}H NMR spectrum, allowing the positioning of the atoms on the asymmetric carbon of the aglycone to be identified. Once the configuration was known, it was confirmed by X-ray analysis (Fig. 11).

In the synthesis of 2,3-unsaturated alkyl *O*-glycosides obtained from the reaction between five optically active alcohols with known configuration (*S*-(4-[3-(aryl)-1,2,4-oxadiozol]-5-yl] butanols) and tri-*O*-acetyl-D-glucal via Ferrier rearrangement, Freitas et al. [44] determined the stereochemistry of the products obtained using ^{1}H NMR spectroscopy in the presence of a europium chiral displacement reagent (tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorate), carried out on racemic and enantiopure samples. The authors point out that after glycosylation, the enantiomers formed interact in different ways with the chiral displacement reagent, and the complexes formed will be diastereoisomers and will therefore show differences in spectra. The NMR and single crystal X-ray diffraction results of the selected compound confirmed the (*S*) configuration on the carbon atom containing the methyl group in the aglycone portion (Fig. 12).

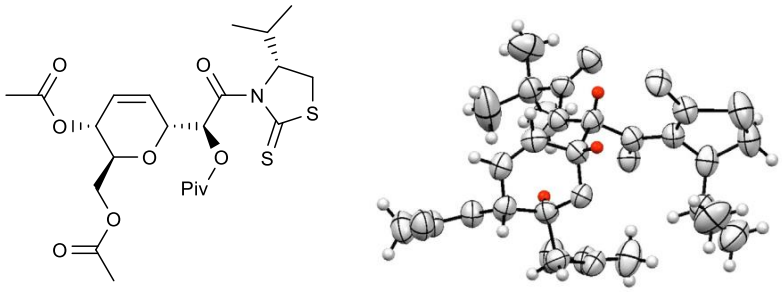
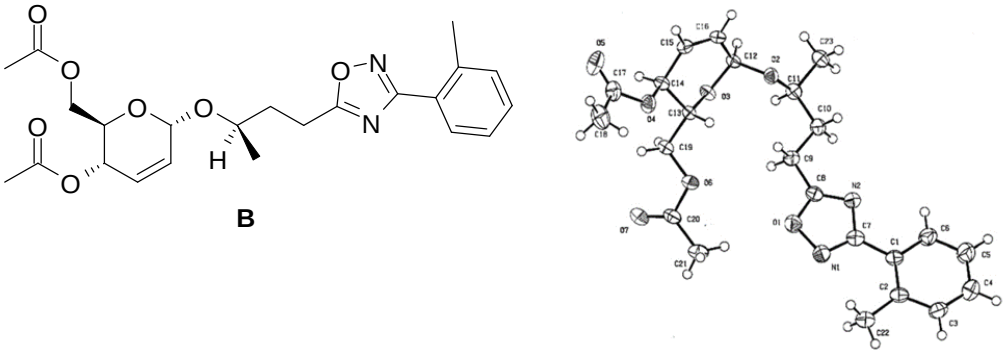
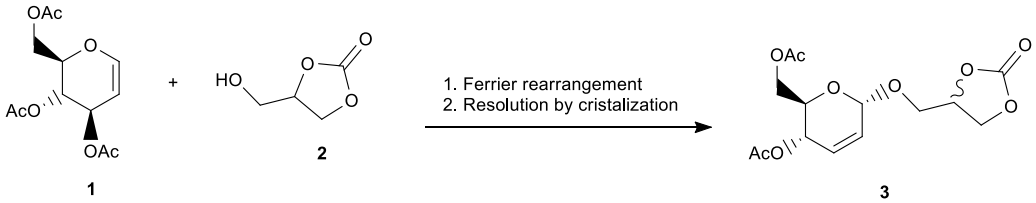


Fig. 11. Molecular and X-ray structure of *C*-glycoside

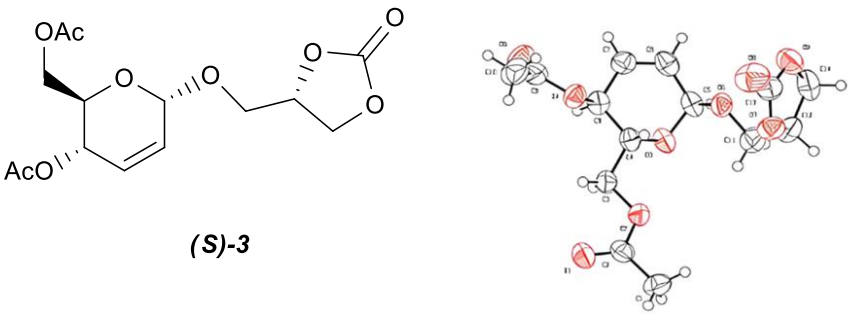


B

Fig. 12. The general structure of glycosides and Ortep Diagram of B



Scheme 1. Synthesis of *O*-glicosyl carbonate



(S)-3

Fig. 13. Diagram of the compound 1-(4,6-di-*O*-acetyl-2,3-dideoxy-*α*-D-erythro-hex-2-enopyranosyl)-(4'*S*)-1,3-dioxalan-2-one

In published work, Costa *et al*. [45] described an efficient synthesis of 2,3-unsaturated *O*-glycosides with glycerol carbonate as the aglycone, using tri-*O*-acetyl-D-glucal **1** and glycerol carbonate **2** as reaction partners via Ferrier rearrangement catalyzed with montmorillonite K-10 doped with iron (III) chloride hexahydrate (Scheme 1).

In controlled conditions, the reaction produced a diastereoisomeric mixture of compound **3** with a yield of 84%, and only the *α*-glucoside was obtained as a mixture of two inseparable diastereoisomers at first, but after spontaneous crystallization, they obtained a single diastereoisomer in 28% yield. The main product was characterized by ^{1}H, ^{13}C, and DEPT NMR

spectroscopy. Additionally, COSY and HSQC were also used to perform a complete assignment and an X-ray crystallographic study was employed to determine the configuration of the diastereoisomer (Fig. 13). The results show that α-glucoside **3** has an (*S*) configuration at C-4 of the carbonate aglycone [45].

4. ADVANTAGES AND DISADVANTAGES OF USING ANALYTICAL METHODS TO DETERMINE ABSOLUTE CONFIGURATION CARBOHYDRATES

The development of practical characterization methodologies with high sensitivity and specificity is one of the pertinent agendas for carbohydrates. Despite the incorporation of different analysis methods, they may not provide conclusive carbohydrate conformations and absolute configuration (AC) confirmation. This challenge is described in the work of Batista et al. [46], in which a reclassification of the configurations of various molecules is carried out. In principle, X-ray diffraction (XRD), nuclear magnetic resonance (NMR), stereocontrolled organic synthesis, or chiroptical spectroscopy, such as optical rotatory dispersion (ORD), electronic circular dichroism (ECD) and circular vibrational dichroism (VCD) and Raman optical activity (ROA) are methods used to obtain AC. Despite the specificity of the methodologies, they all have advantages and disadvantages.

4.1. Nuclear Magnetic Resonance

Carbohydrates have chemical shifts in narrow bands, resulting in a significant overlap of the resonances of each monosaccharide unit present, since their diverse constituents show subtle differences in configuration or in the number and positions of hydroxyl groups [47]. The proton spectrum of glycans can generally be divided into aliphatic (2.7 - 1.0 ppm), carbinolic (4.4 - 3.0 ppm), anomeric (5.6 - 4.4 ppm) and aromatic (8 - 1 ppm) regions. The latter is efficient for glycosides with an aromatic aglycone part [48,49]. One of the challenges associated with the NMR of glycans is determining the spatial arrangement of the group on the higher-numbered asymmetric carbinolic and asymmetric carbon to determine the absolute configuration. A common strategy is to derivatize the hydrolysed glycan with an optically active reagent, the reaction producing mixed products for the monosaccharides.

Non-carbohydrate groups, such as methyl, acetyl, sulphate or phosphate, usually shift the ~ 0.2 - 0.5 ppm field of the proton, and homo- or heteronuclear corrections help to determine the position [50, 51]. In addition, the α-anomer resonates at a lower field than the β-anomer in D-pyranoses in the $^4C_1$ conformation. In ^{13}C spectroscopy, the shift in the standard range varies between 60-110 ppm, and the chemical changes are indicative of the anomeric configuration of the carbohydrates. As they are relatively rigid structures, their couplings are useful for obtaining the anomeric configuration of the sugar, with the steric orientations obtained by the H-H coupling constant and the angular torsions between the bonds in the heteronuclear coupling [52].

In pyranose, the coupling constant between vicinal hydrogens H^1 and H^2 indicates that when both are axial (7 - 8 Hz), they have a higher, equatorial-axial coupling ($J_{1,2}$ ~ 4 Hz) and the others below 2 Hz. This principle is also present in hexopyranose. He points out that the signal from each anomeric proton is split due to coupling with the neighboring proton [52,53]. Thus there are coupling bands that can indicate the relationships between protons: equatorial-axial (1-4 Hz), equatorial-equatorial (0-2 Hz), axial-axial anomeric (7-9 Hz), axial-axial non-anomeric (9-11 Hz), germinal between hydroxymethyl protons (2 Hz) [54].

In the presence of useful techniques for small couplings, such as the Nuclear Overhauser effect (NOE) and the rotating-frame Overhauser effect (ROE), they register the effect of glycosylation or conformational probing, as they are sensitive to interatomic distances. It should be noted that the spectra showing overlapping signals can be worked with two-dimensional homonuclear, which can assign the glycans individually [53]. Monosaccharide residues in the structure of an oligosaccharide present the methylation technique as an analytical tool for identifying the positions of substituted hydroxyl groups. Another strategy is to separate free hydroxyls, using peracetylation of the free hydroxyls with ^{13}C labeled carbonyl. The disadvantages of using chemical derivatization agents in the assignment of protons protected by acetyl [50].

The structural parameters of carbohydrates elucidated by NMR are chain size and type of sugar (aldose or ketose), glycan cycle size, identification of monosaccharides, absolute configuration (D/L), stereo configuration of

anomeric carbon (α/β), location of functions and chains and non-carbon residues in the molecule. This broad characterization is an advantage of the methodology, since only the monomeric composition has its data less easily attributed, when compared to chromatographic and mass spectrometric methods [51].

Finally, the comparative analysis of Nuclear Magnetic Resonance (NMR) data of numerous highly correlated glycans has led to the establishment of empirical rules that associate chemical shift values with carbohydrate structures, and several databases such as GlycoSCIENCES.de, CSDB (Carbohydrate Structure Database) and CASPER (Computer-Assisted Spectral Evaluation of Regular Polysaccharides) have been put forward to consult the data [55]. Thus, the comprehensive analysis of chemical shifts in nuclear magnetic resonance (NMR) offers significant advantages, as it reveals not only the nature of the constituents, but also the way in which they are interconnected. Although it cannot distinguish between enantiomers, it allows the absolute configuration of diastereomeric derivatives to be deduced, thus paving the way for more refined and sophisticated analysis [49].

According to Ben-Tal el al., [56] despite the many advantages of ^{1}H NMR spectroscopy, there are a few limitations that have to be considered when choosing the appropriate NMR techniques. For example, the relatively small chemical shift window or spectral width associated with ^{1}H NMR spectroscopy means that there is a greater likelihood of overlapping peaks. Overlapping peaks lead to greater ambiguity in compound identification and quantification. One way of addressing peak overlap is to perform NMR experiments with stronger magnets and higher magnetic fields.

4.2. X-ray Diffraction

X-ray diffraction analysis of carbohydrates has been for more than long time an most accurate and reliable approach to obtain detailed structural information for this macromolecules. According to information from the literature the technique requires a high-quality crystal, this method provides significant insights into the molecular mechanisms revealing the function of macromolecules, as well as inter/intramolecular interactions forming complex supramolecular assemblies [57-59].

Despite its potential for characterization, the method can have physical limitations such as the polarization factor, the zero of the goniometers, and the generation of double radiation. Another important limitation is that the molecule must contain "heavy" atoms (e.g. bromine) to increase scattering. In addition, crucial signal distortions arise from the influence of the nearest neighbors in any crystal structure and from solvents used during the crystallization process. Finally, the flexibility of the conformers of their oligo- and polysaccharide chains reduces their analysis. Thus, the study of more precise analysis techniques and chemical systems without defined stereoselectivity is one of the guidelines for XRD with glycones.

According to Thakral et al., [60] the XRD is a very powerful technique, because of the ability to interact with atoms in crystal structures. She provides extremely useful information on distances and structures on both the atomic and molecular levels, and it is used for identifying unknown minerals and materials. Still, according to the authors, the high level of structural information that can be obtained is unrivaled by any other technique. The analysis only requires a minimal amount of sample. Several XRD measurement instruments are widely available and data interpretation is relatively straightforward.

However, XRD does have certain limitations. To best identify an unknown material, the sample should be homogeneous. Sample preparation often requires grinding to obtain a powder and typically XRD analysis requires access to standard reference data to make comparisons [60].

The analysis relies a lot on the availability of high-quality crystals. The preparation of suitable crystals, especially for biological macromolecules like proteins, is a well-known challenge. Nevertheless, advances in the aforementioned synchrotron sources can help overcome this limitation, together with the development of X-ray powder diffraction (XRPD) as an alternative to single-crystal X-ray diffraction (SCXD) [61].

In summary, notwithstanding the inherent value of X-ray diffraction (XRD) in elucidating crystalline structures, its less prevalent application in the absolute quantification of carbohydrates within complex matrices stems from the challenges associated with inducing the requisite crystalline state in these compounds.

Methodologies more tailored to carbohydrate analysis, such as chromatography and techniques grounded in chemical reactions, are typically favored for such determinations.

4.3. Chiroptical Spectroscopy

Optical rotation is a crucial tool for studying carbohydrates. It was through the convergence of the deviations of two crystalline forms of D-glucose in water that cyclic glycoside forms were attributed. The results in solutions with disaccharides showed no change in the deviation, contributing to the formation of the intramolecular hemiacetals of the carbohydrates and the α and β forms as hemiacetal enantiomers [62].

Carbohydrates have chiral carbon atoms, giving them optical activity. This activity manifests itself in the ability of carbohydrates to rotate the plane of polarized light, with the resulting angle depending on the nature of the compound, the temperature, the wavelength of the light, and its concentration. Accurate determination of the compound's concentration can be achieved by means of specific optical rotation, provided that all other factors remain constant and the solution does not contain other optically active compounds. This highlights electronic and vibrational circular dichroism (ECD and VCD, respectively) and Raman optical activity (ROA) for chiroptical study [63].

The strong infrared absorption of water (1650 cm^{-1}) prevented analysis of VCD in carbohydrates, and other polar solvents were used, such as dimethylsulfoxide [63]. However, because it is not native to biological processes, Pretovic, Rose and Polavarapu [63] developed films of different carbohydrates, which do not require short-path cells in aqueous solutions. This enabled them to record vibrational circular dichroism (VCD) in the 2000-900 cm^{-1} region for the first time. In this way, the removal of water absorption resulted in higher yields, using cells two orders of magnitude smaller than those required for corresponding VCD measurements in aqueous solutions.

Chiroptical methods on carbohydrates have certain behaviors, in which VCD shows low signals due to the flexibility of the sugars, and when solubilized in water, the strong absorption of water interferes with that of the sugars. In ECD, it is limited due to the few electronic transitions accessible in the ultraviolet/visible region. In ROA, these limitations are not seen, due to the presence of the glycosidic bond in the structure being well defined, with rich and detailed spectra of the stereochemistry [64].

Finish, to determine the absolute configuration according to Li, Ferreira, and Ding [65] since each of the aforementioned methods has limitations, careful selection of an appropriate, or a combination of two or more methods, depending on the nature of the carbohydrate is crucial

5. CONCLUSIONS

This review describes a survey of methods for absolute configuration (AC) determination of carbohydrates, namely, X-ray diffraction analysis (XRD), Mosher NMR analysis, means of chiroptical spectroscopy (ORD, ECD, VCD, and ROA) with their respective advantages and disadvantages.

Although everyone knows the importance of using the methods of X-ray diffraction analysis, Mosher NMR analysis, electronic circular dichroism (ECD or CD), vibrational circular dichroism (VCD), and ROA in AC study, errors can also appear in different situations. To avoid such mistakes, it is important to list reasons for the errors and the methods that can prevent them

The main drawback of NMR-based studies is its relatively intrinsic low sensitivity. Efforts have been made to overcome this problem, such as using higher magnetic fields. About computational methods, molecular docking can be used to predict the structures of carbohydrates. Unlike pyranose, furanose rings can adopt different conformations with little difference in their free energies, resulting in more ring flexibility.

However, every method has its range of applications. This leads, in some cases, to incorrect conclusions by researchers who are not familiar with these methods. This review, provides experimental chemists and researchers with more details about the use of each method and its advantages, especially hope that this experience may help readers be more interested in the topic.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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