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Preparation, Characterization, and Biological Study of a New Inclusion Complex of 1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-[benzyliden(amino)]-*β*-*D*-glucosamine and *β*-Cyclodextrin

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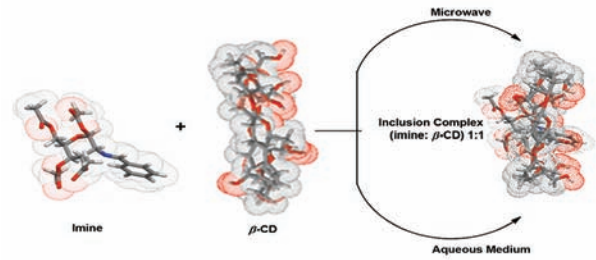
ABSTRACT In this study, an inclusion complex based on *β*-Cyclodextrin (CD) and 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-[benzyliden(amino)]-*β*-*D*-glucosamine (imine) were prepared in order to modify imine's solubility and improved its stability. The inclusion complex was obtained by equimolar quantity 1:1 under microwave irradiation and characterized by X-ray diffraction and melting point. The effect of pH on the formation of the inclusion complex in aqueous medium buffered at 25°C was examined. Spectrophotometric measurements were carried out to visualize the interaction between imine and *β*-CD in the complex in the liquid state. The results obtained showed that the complexation was favored in a neutral medium (pH = 6.9). The stoichiometric ratio 1:1 of the complex was confirmed by the A_L solubility diagram according to the Higuchi and Connors method, and the stability constant is K_s = 583.38 M^{-1} at 25°C, which proves a good capacity of complexation in the neutral medium. The biological activity of free imine and complex was examined against different pH.



KEYWORDS Azomethin-*O*-acetyl-*β*-*D*-glucosamine, *β*-Cyclodextrin, Biological activity, Inclusion complex, Solubility, Stability.

INTRODUCTION

D-Glucosamine is an important class of heterocyclic compounds widely used in various fields of medicine, and is naturally synthesized by the organism from glucose and glutamine.[1] It plays a preponderant role in maintaining the integrity of the cartilage of all joints.[2] It has a free amino group, allows the chemical substitution producing several *D*-glucosamine derivatives with a large spectrum of applications, *D*-glucosamine derivatives have attracted a lot of attention from chemists because of their wide applications in organic chemistry and in therapeutic.[3-5] Among these derivatives, azomethines are obtained by a condensation reaction;[6-11] these derivatives are important scaffold, have

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attracted the interest of chemists because of their wide applications in synthetic chemistry and in biology.[12,13] They also serve as a basis for the synthesis of various heterocyclic compounds.[14,15] The azomethine groups are the most widely used and they play an essential role, especially in the pharmacological field **[Figure 1]**.[16-18]

Azomethines, in general, and azomethine based on *β-D*-glucosamine in particular, is insoluble in water. It hydrolyzes easily,[19] which makes their application, reduced in the various fields;[20,21] for this reason, we have thought about forming an inclusion complex based on *β*- Cyclodextrins (CD) to increase solubility and improve stability in the aqueous medium.

CDs are a cyclic oligosides **[Figure 2]**[22] with the ability to encapsulate entirely or in part, in their hydrophobic cavity, a wide variety of molecules,[23] forming inclusion complexes.[24,25] This integration process leads to changes in the physicochemical properties of the guest, such as solubility, stability, and biological properties, etc.[25,26] These three properties are developed in this work.

The method of preparation of the inclusion complex must be chosen according to the applications envisaged because it influences the physicochemical characteristics and the dissolution of the complex. There are various techniques for preparing inclusion complexes, which can be carried out in the liquid state and/or in the solid state. Although researchers have reported azomethines derived from *β-D*-glucosamine acetyl, have no reports on complexation in CDs and even the study of solubility, stability, and biological activity of imine in the presence of CDs.

In the light of these bibliographic deficiencies, this work was explored by our research group and in the context, we

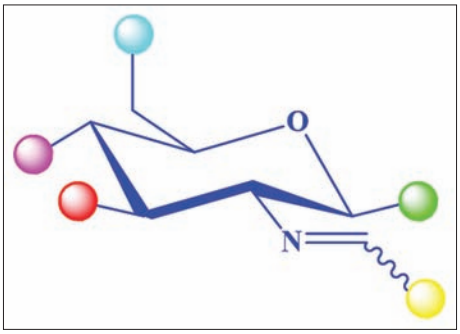


Figure 1: Structure of azomethine based on modified *β-D*-glucosamine

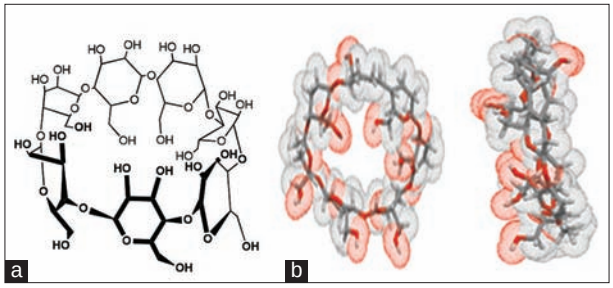


Figure 2: (a) Two-dimensional structure of *β*- cyclodextrins showing the arrangement of glucose monomers and (b) three-dimensional structure created by molinspiration galaxy 3D structure generator v2018.01

represent the improvement of the procedure of the synthesis of imine under the catalysis of sodium hydroxide (NaOH) in tetrahydrofuran (THF), then the preparation of a new inclusion complex of imine and *β*-CD in 1:1 equimolar quantity in the solid state by microwave irradiation. The complex formed was characterized by X-ray diffractions (XRD) and melting points.

We then carried out a study on the influence of pH on the formation of inclusion complexes at room temperature in buffer solutions. From the obtained results, we have studied the phase of the solubility of the imine with different concentrations of *β*-CD at pH = 6.9 at 25°C. Furthermore, we studied the thermodynamic process of formation of the (imine:*β*-CD) complex by the calculation of the free enthalpy ΔG° at 25°C of each concentration of *β*-CD.

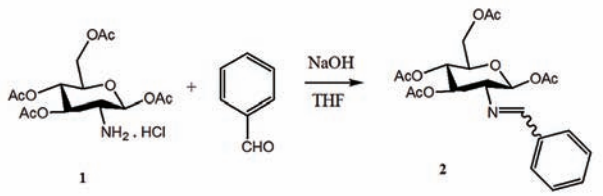
Finally, free imine and complex at different pH were also tested for their antimicrobial activities against the Gram-positive and Gram-negative bacteria.

RESULTS AND DISCUSSION

Synthesis of imine 2

First, we carried out the synthesis of the imine according to the literature procedure[27,28] which involves a condensation reaction of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-amino-*β-D*-glucosamine hydrochloride (**1**) with benzaldehyde at reflux under a nitrogen atmosphere then by the use of sulfuric acid and glacial acetic acid as catalysts. However, the method did not give acceptable results. This led us to attempt the condensation reaction differently. Hence, the strategy that we adopted for the synthesis of imine was to condense equimolar quantities of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-amino-*β-D*-glucosamine hydrochloride (**1**) with benzaldehyde in the presence of NaOH as a catalyst under reflux in THF. Interestingly, this modification gave the desired imine 2 in good yield [**Scheme 1**].

The IR spectrum of imine **2** showed a sharp peak in 1645 cm^{-1} due to C=N bond. Ultraviolet–visible (UV-Vis) absorption spectrum of imine in absolute EtOH (C = 10^{-3} mol. L^{-1}, T = 25°C, ε: [L mol^{-1} cm^{-1}], λ_{max}: [nm]) had a characteristic band at a wavelength between 261 and 292 (λ_{max} = 280 nm of an absorbance of 2.71) and molar extinction coefficient (ε = 2.71.104 L. mol^{-1}. cm^{-1}). This band was attributed to the electron transition (π-π*) of the conjugated system (C=C aromatic cycle, C=N), with a higher intensity.



Scheme 1: Synthesis of imine 2

Analysis of the inclusion complex

Analysis of the inclusion complex of (imine:β-CD) 1:1 formed through MW by XRD

Based on XRD studies, it is concluded that crystallinity and amorphicity are important factors that must be related to the solubility of the compounds.[29] However, they are useful to control the change in the crystallinity of the compounds during the host-guest interaction.[29] **Figure 3** shows the spectrum of XRD of imine, *β*-CD, and the complex formed.

The dynamic recrystallization (DRX) diffractogram of imine shows a crystalline profile with two intense peaks at 8° and 18° and the *β*-CD spectrum shows fine mean peaks, but imine in the presence of *β*-CD does not represent peaks, so the *β*-CD has modified the crystallographic properties of imine and this change can be attributed to the formation of an inclusion complex, this result is in agreement with those of the literature.[30,31]

Analysis of the melting point of the inclusion complexes of (imine:β-CD) 1:1 formed through MW

Comparing the melting point values of free *β*-CD (280–281°C) and free imine (131–133°C) with the inclusion complex (197–198°C), we found that it is totally different. This observation provided support for the possibility of the formation of the inclusion complex.

Analysis of the inclusion complex 1:1 of (imine:β-CD) formed in the liquid state by UV-vis

From the spectrum **[Figure 4]**, the addition of *β*-CD to the solution of imine in the different pH produces an increase of the absorbance and a shift of the absorption maxima significantly, so we can attribute this to the effect of *β*-CD on the stability of imine by the formation of a complex where *β*-CD inhibited the hydrolysis of the double bond (C=N). The azomethine bond is easily hydrolyzable in an aqueous medium, many studies have been devoted to the study of the decomposition of imines by the addition of a molecule of water on the carbon-nitrogen double bond (C=N),[32,33] because of its effective role in certain biological reactions,[34] but so far have no reports on the inhibition of hydrolysis by the effect of complexation by CDs.

The different values of the wavelengths and absorbance obtained from the spectrum of *β*-CD, imine, and complex in the different pH are grouped in **Table 1**.

According to the **Table 1**, the inclusion complex in neutral medium has the highest absorbance compared to other complexes formed in the other two medium, it can be concluded that the neutral medium is very favored for complexation. This study showed that the presence of *β*-CD regardless of the medium used, protected the imine against protonation and decomposition.

Analysis of the complexes formed in the different pH by DRX

Following these results, we studied the influence of *β*-CD on the imine by XRD in a different medium. The XRD diagrams of *β*-CD, imine, and the inclusion complexes obtained are shown in **Figure 5**. The analyses of the XRD show an amorphous profile for imine in the presence of

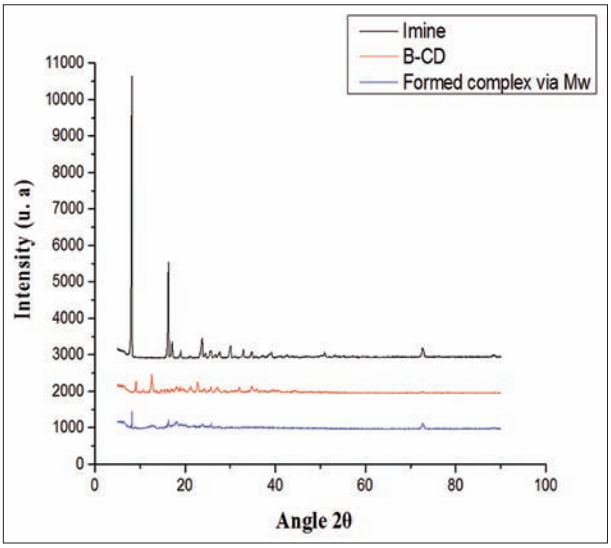


Figure 3: Diffractogram of imine, *β*-cyclodextrins, and complex formed through MW

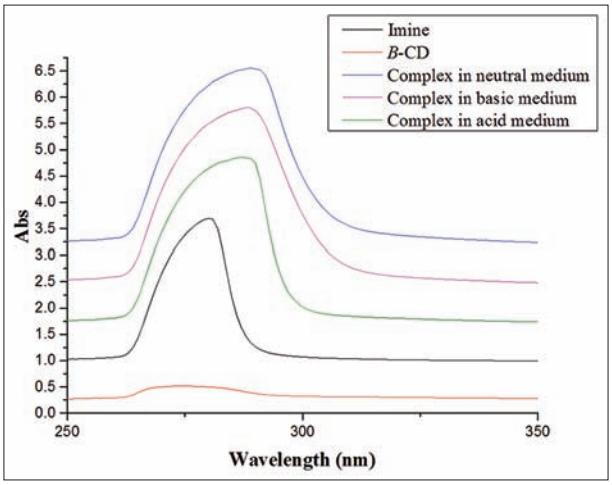


Figure 4: Ultraviolet–visible absorption spectrum of imine, *β*-cyclodextrins, and the inclusion complex formed in the different pH

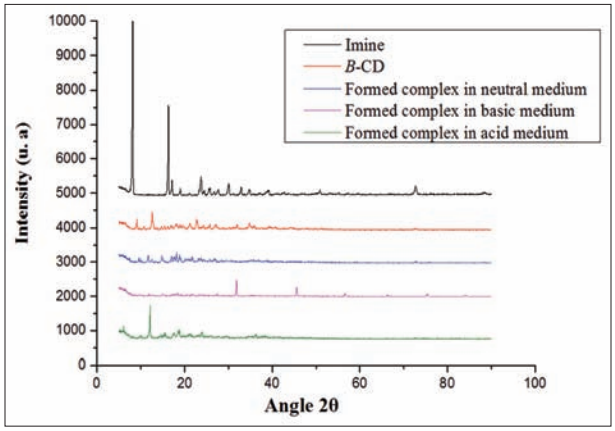


Figure 5: Diffractogram dynamic recrystallization of imine, *β*-cyclodextrins, and complexes at different pH

β-CD in the neutral medium, a semi-crystalline profile for complexes with a basic and acidic medium. We can attribute this change to the presence of β-CD, which modified the crystallographic properties of imine in the three mediums, but it is very remarkable in the neutral medium. Hence, we can conclude that the stability of the imine in this medium can inhibit the hydrolysis of the double bond and we can attribute this to the favored complexation in the neutral medium, which confirms the results obtained by UV-Vis.

Phase solubility study

The results obtained are presented in **Table 2** and **Figure 6,** which summarize (λ_{max}) the absorbance and the concentration of imine solubilized in the presence of aqueous solution of β-CD at different concentrations.

Of the evolution of the absorbance as a function of the β-CD concentration, at pH = 6.9 shown in **Figure 6**, for aqueous mixtures having a constant concentration of imine, we observed that the intensity of the band positioned at 280 nm depends on the concentration of β-CD.

From **Figure 6**, we noticed that intensity of the band's increases by the increase of the concentration of β-CD and we know that β-CD does not absorb in this spectral range and the concentration of imine is kept constant, so this reveals that the complex formation (imine:β-CD) has taken place. These observations also allow us to deduce that the spectral intensity indirectly reflects the proportion of imine included in the study. To verify the variation of the absorbance, we plotted the latter as a function of the β-CD concentration **[Figure 7]**.

We observed an increase in absorbance as a function of the increase in β-CD concentration. Based on these results, the imine solubility curve was plotted as a function of β-CD concentration **[Figure 8]**.

Table 1: Comparison between the imine, the β-CD, and the inclusion complex (imine:β-CD) 1:1 formed in different pH

Compound	λ (nm)	Abs	Molar extinction coefficient ε (L. mol^{-1}. cm^{-1}) 10^3
Free imine	280	2.71	2.71
Complex at pH=4	287	3.21	3.21
Complex at pH=6.9	289	3.42	3.42
Complex at pH=8.5	288	3.37	3.37
Free β-CD	274	0.28	2.80

CD: Cyclodextrins

Table 2: Concentration of imine solubilized in the presence of β-CD

(Imine:β-CD)	Complex (mol/L)	Abs	λ_{max}	$Imine_{solubilized}$ (mol/L)×10^{-3}
1:0	(0.01/0.00)	2.750	280	1.014
1:1	(0.01/0.01)	2.863	281	1.056
1:2	(0.01/0.02)	2.893	283	1.067
1:3	(0.01/0.03)	3.202	287	1.181
1:4	(0.01/0.04)	3.437	289	1.268

CD: Cyclodextrins

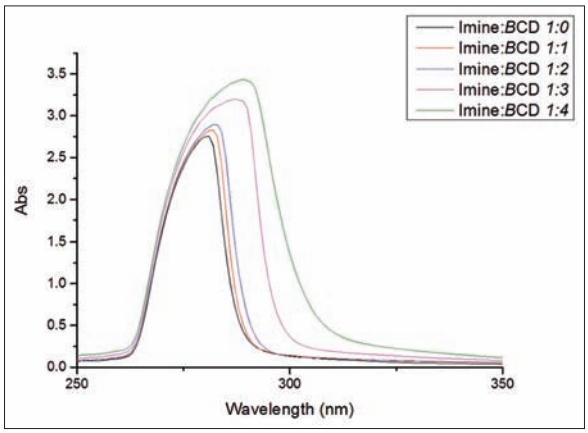


Figure 6: Ultraviolet spectrum of imine in an aqueous solution of β-Cyclodextrins at different concentration

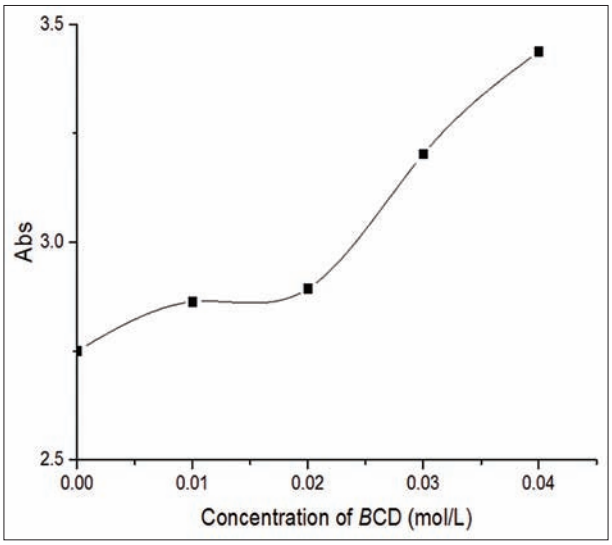


Figure 7: Absorbance spectrum as a function of β-cyclodextrins concentration

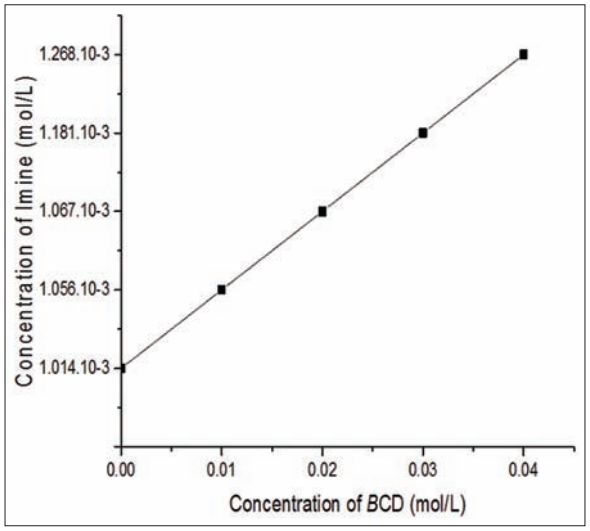


Figure 8: Phase solubility diagram of imine associated with β-cyclodextrins at 25°C

The solubility diagrams of imine with *β*-CD in the neutral medium are a line that does not pass through the origin, this indicates that the concentration in imine increases proportionally with increasing of *β*-CD concentration. According to Higuchi-Connors, it is an A_L type isotherm[35,36] because the solubility of imine increases linearly as a function of the concentration of the *β*-CD. This profile is attributed to the formation of 1:1 type complexes. From the solubility phase diagram of imine in an aqueous solution of *β*-CD at 25°C **[Figure 8]**, we calculated the stability constant $K_{1:1}$, using the equation **(2);** with slope = 5.8338. 10^{-3}; S_0= (0.01mmol/L) at 25°C.

The stability constant ($K_{1:1}$) for the complex at room temperature, at a 1:1 stoichiometry, calculated from the solubility curve was 583.38 M^{-1}, which indicates the formation of the stable complex since Ks is in the range of 200–5000 M^{-1} indicates a good complexing capacity.[37,38] This also suggests that there is an increase in the dissolution profile that would certainly increase the stability of imine. In parallel, we were calculated the free transfer energy (ΔG°) of imine in pure water at different concentrations of *β*-CD using the values of the solubility curve **[Figure 8]** and the application of the equation **(1).**[39,40] The values obtained for ΔG° are presented in **Table 3**.

$$\Delta G^\circ = -2.303RT\log\left(\frac{Ss}{S0}\right) \quad (1);[39]$$

Where $\left(\frac{Ss}{S0}\right)$: The ratio of molar solubility of imine in an aqueous solution of *β*-CD to that of pure water.

From the results shown in **Table 3**, we found that complexing reactions of the imine with *β*-CD are spontaneous because the free enthalpy changes are negative (ΔG°<0). Moreover, these negative free enthalpy variations suggest that the inclusion process is enthalpically favorable which is attributed to the stronger van der Waals interactions between imine and *β*-CD and a more profound penetration of the guest molecule[39,41] into the hydrophobic cavity. **Figure 9** represents the evolution of the free energy ΔG° at 298K of the imine solubilization process as a function of the concentration of *β*-CD in a neutral medium.

It is noted that ΔG° strongly decreases when the concentration of *β*-CD increases this considerable decrease of ΔG° in the presence of *β*-CD can be interpreted by the encapsulation of imine accompanied by the formation of an intramolecular hydrogen bond between the hydroxyl groups of *β*-CD and the four acetyl groups (OAc) of imine.

Table 3: Gibbs free energy (ΔG°) for the solubilization process of imine in an aqueous solution of *β*-CD at 298K

Concentration of *β*-CD (mol/L)	ΔG° (kJ/mol, 298K)
0.01	−17.12
0.02	−18.84
0.03	−19.85
0.04	−20.56

CD: Cyclodextrins

Variations in free enthalpy are similar to experimental data.[42]

In vitro antibacterial activity

The evaluation of the antibacterial activity of free imine and inclusion complex at different pH carried out in solid medium by diffusion by the disk and in liquid medium by microdilution. The first method is more practical and rapid. In this method, the biological activity of free imine and inclusion complexes at different pH was evaluated by the presence or absence of inhibition of bacterial growth. After incubation, the inhibition diameter was measured in millimeters, including disc. Each test was performed three times in three successive experiments. The overall results of this study are summarized in **Table 4** and **Figure 10**.

According to the results obtained, practically no inhibition zone of *Pseudomonas aeruginosa* strain (G^+) was observed for all products; therefore, an absence of antibacterial activity for this bacterium. On the other hand, the inclusion complex at pH = 6.9 has a relatively variable zone of inhibition (22–29 mm), according to *the Staphylococcus aureus* (G^+) and *Escherichia coli* (G^-) strain reflecting a relatively strong antibacterial activity. Comparing the diameter of the inhibitory zones of *E. coli* and *S. aureus*, we find that the sterile zone diameter of *S. aureus* was greater than that of *E. coli*, so the strain of *S. aureus* is more sensitive than *E. coli* toward the inclusion complex at pH = 6.9 **[Figure 11]**. We conclude that the diameter of halos varies according to the effect of *β*-CD on the imine, the nature of the medium and the strains tested. The second method had given the same result.

A very important result, concerning the antibacterial activity of free imine and inclusion complexes at different pH, showed that the presence of *β*-CD inhibited the protonation of imine in the aqueous medium; therefore,

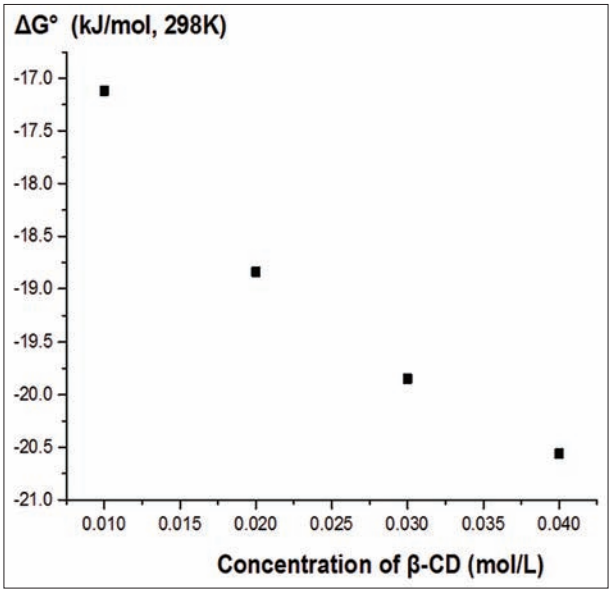


Figure 9: Evolution of ΔG° of the imine solubilization process as a function of the concentration of the *β*-cyclodextrins in a neutral medium at 298 K

Table 4: *In vitro* test results for antimicrobial activity with *S. aureus, E. coli,* and *P. aeruginosa*

Compounds	Diameters of the inhibition zones (mm)		
	S. aureus	*E. coli*	*P. aeruginosa*
Imine: *β*-CD (pH=6.9)	29	22	6
Imine: *β*-CD (pH=8.5)	10	10	6
Imine: *β*-CD (pH=4)	6	6	6
Free imine	8	7	6

CD: Cyclodextrins, *P. aeruginosa: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli*

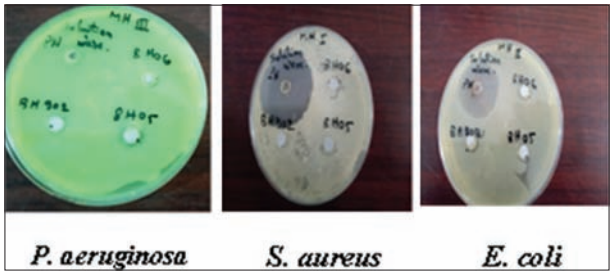


Figure 10: Effect of inclusion complex (complex BH 06 at pH = 4), (free imine BH 5), (complex BH 902 at pH = 8.5) and (complex PN at pH = 6.9) on *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli*

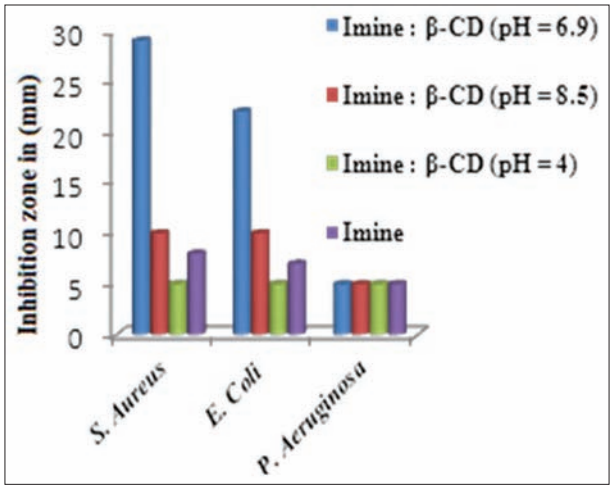


Figure 11: Histogram of free imine inhibition diameters and complexes at different pH

it can be concluded that *β*-CD improved the antibacterial activity of imine in the aqueous medium and in particular in a neutral medium (pH = 6.9).

EXPERIMENTAL SECTION

General

The reagents and solvents were procured mainly from Sigma-Aldrich, 98% NaOH, 37% hydrochloric acid (HCl), petroleum ether, ethyl acetate, dichloromethane, 95% sulfuric acid (**H_2SO_4**), THF, magnesium sulfate ($MgSO_4$), benzaldehyde and distilled water. 1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-amino-*β*-*D*-glucosamine hydrochloride (**1**) was synthesized and characterized previously in our laboratory. The *β*-CD (*β*-CD ≥97% Sigma-Aldrich) provided by Roquette Frères (Lestrem, France) was recrystallized twice in distilled water, filtered, and dried 24 h at 100°C under vacuum before use.

Irradiation for the preparation of the complex in the solid state was carried out by domestic microwave LG marks.

Unless otherwise stated, all reactions were carried out in oven-dried glassware. Reactions were monitored by thin-layer chromatography (TLC) on a Merck silica gel 60 F254 plates. TLC spots were visualized by UV.

Column chromatography was performed on Merck silica Gel 60 (particle size 40–63 µm). Nuclear magnetic resonance (NMR) spectroscopies were recorded with a Bruker (Forward), (300 MHz for ^{1}HNMR and 400 MHz for ^{13}C NMR). Tetramethylsilane (TMS) was used as an internal reference. Chemical shifts for spectra in $CDCl_3$ are given in ppm. High-resolution mass spectra (HRMS) were recorded on a Bruker MicrOTOF-Q spectrometer using electrospray ionization (ESI) and tandem quadrupole coupled with a time-of-flight mass analyzer. Infrared (IR) spectroscopy was recorded on a Nicolet Magna-IR 550 Fourier transform IR-Thermo scientific-IS50-NIR-spectrometer (Madison, Wisconsin, USA), in the range of 500–4000 cm^{-1}. Absorption spectra: UV-Vis spectroscopy (Perkin Elmer UV-Vis Lambda 10) with 1 mm quartz cells was used for all spectroscopic studies. A wavelength absorption profile was obtained in the range of 200–400 nm. The melting points were measured by a B-540 type Büchi apparatus in open capillary tubes. pH was measured using a digital pH meter (BECKMAN), calibrated at 25°C with buffer solutions at acetic acid-sodium acetate 0.05 M (pH = 4), 0.05 M phosphate (pH = 6.9) and/or 0.05 M borate (pH = 8.5) to 50% methanol gradient with an accuracy of 0.01 pH units.

1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-[benzyliden(amino)]-*β*-*D*-glucosamine (2)

Due to the poor results obtained from literature procedure,[27,28] a modified procedure was adopted for the synthesis of imine.

Procedure: In a 100 mL flask surmounted with a condenser containing 100 mg (2.5 mmol) of NaOH in 15 mL of THF, was added 1000 mg (2.610 mmol) of 1,3,4,6-*O*-acetyl-2-deoxy-2-amino-*β*-*D*-glucosamine hydrochloride (**1**), dissolved in THF(5 mL). To the resulting mixture was slowly added a solution of benzaldehyde (2.5 mmol) in THF (5 mL). The mixture, with stirring, was refluxed under a nitrogen atmosphere for 1 h until the total NaOH got dissolved. The crude reaction product was then filtered and washed with THF to give a powder corresponding to the imine. The purity of the compound was checked by TLC, using (CH_2Cl_2:MeOH, 9.5:0.5) as eluent. After evaporation of the filtrate in open air, 900 mg of white brilliant grain was thus recovered. Chemical formula: $C_{21}H_{25}NO_9$; Exact Mass: 435g/mol; Yield 85%; TLC: R_f = 0,6 (CH_2Cl_2:MeOH, 9.5:0.5) ; M.p. 131–133°C (The crude product was recrystallized from ether); ^{1}H NMR ($CDCl_3$) (300 MHz, δ, ppm (*J*, Hz): 1.88 (s, 3H, 2×CH_3), 2.02 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 3.37 (dd, 1H, *J* = 5.3 Hz, *J* = 13.3 Hz, H-6a), 3.41–3.49 (m, 2H, H-2, H-6b), 3.94 (ddd,

1H, *J* = 2.7 Hz, *J* = 5.3 Hz, *J* = 9.9 Hz, H-5), 5.11 (dd, 1H, *J* = 9.7 Hz, *J* = 9.9 Hz, H-4), 5.43 (dd, 1H, *J* = 9.7 Hz, H-3), 5.95 (d, 1H, *J* = 8.4 Hz, H-1), 7.30–7.45 (dd, 5H, *J* = 8.7 Hz, Ar-H), 8.40 (s, 1H, N=CH); ^{13}C NMR ($CDCl_3$), (100 MHz, δ, ppm) : 20.4 ($3\times CH_3$), 20.6 ($2\times OCH_3$), 50.6 (C-6), 69.1 (C-4), 72.7 (C-2), 73.06 (C-3), 73.6 (C-5), 92.9 (C-1), 113.9–130.1 ($6\times C_{arom}$ (Ar)) Six carbon ring), 164.2 (N=CH), 168.6 ($COCH_3$), 169.4 ($COCH_3$), 169.7 ($COCH_3$), 170.0 ($COCH_3$). HRMS (ESI neg) calcd for $C_{21}H_{24}NO_9$ (M-H) 434.2055, found 434.2050. IR (KBr), ν, cm^{-1}: 1630–1675 (CH=N), 1550 ($C=C_{arom}$), 2831–3056 ($C\text{-}H_{Ar}$); 1229 (C-C(O)-C), 1028, 927. 1735 (O-C=O), 1432-1372-1260 ($C\text{-}O\text{-}C_{ester}$), 1143 (C-O-C).

Formation of inclusion complexes

The possibility of forming an inclusion complex between the *β*-CD and the imine to increase its solubility in water and improve its stability, according to two procedures: In the liquid phase and in the solid phase, has been realized.

Formation of inclusion complex of type 1:1 in the solid state by microwave irradiation

Microwave irradiation is a method to quickly reach the temperature necessary for the formation of the inclusion complex while keeping a good uniformity of the properties of the complex. The reaction time is usually very short and the temperature does not exceed 60°C. The complex will subsequently be recovered in solid form. For the formation of the complex, we added a quantity of 200 mg (0.45 mmol) of imine to 520 mg (0.45 mmol) of *β*-CD, 2 mL of ethanol was added and the paste obtained was mixed for 2 min. It was irradiated for about 10 min by microwave in a domestic oven at 350 Watt, until the appearance of the crystals, the crude product was purified by crystallization in the mixture of (AcOEt:PET, 1:1), the mixture was left to dry naturally, until the appearance of a precipitate, then filtered and dried under vacuum, we thus recovered (570 mg, 90% yield) of white powder. TLC: R_f = 0.79 (CH_3Cl:MeOH, 99:1); M.p. 197–198°C.

Formation of inclusion complex type 1:1 in the liquid state

This study aims to highlight the formation of the complex between imine and *β*-CD indirectly, and to determine the effect of the latter on the stability of the complexes in different buffered medium, to confirm and envisage the medium of complexation in the different forms: Protonated, neutral, and/or basic. The concentration of the used solutions of *β*-CD and imine is of the order of 10^{-3} M and equimolar mixture 1:1 in the three buffer solutions: The acidic medium (protonated) (pH = 4), the neutral medium (pH = 6.9), and basic medium (pH = 8.5).

After stirring, for 24 h at room temperature to accelerate the formation of the inclusion complex, the complexation in the different medium was followed by UV-Vis spectroscopy.

Phase solubility study

Phase solubility study was performed according to the method of Higuchi and Connors.[43] To do this, we dissolved a quantity of imine in 250mL Erlenmeyer Flask, with Rubber Stopper, containing a buffered aqueous solution of *β*-CD (neutral medium), to obtain the imine:*β*-CD solutions, at different molar ratios 1:0, 1:1, 1:2, 1:3, and 1:4. The mixtures were stirred for 48 h at room temperature until equilibration of the solutions. After filtration on a 0.45 μm Whatman filter of the samples, diluted solutions were prepared for analysis by the UV-Vis spectrophotometer. The stability constant Ks was calculated from the phase solubility diagrams using the following equation:[44,45]

$$K = \frac{\text{Slope}}{\mathbf{S}_0(1-\text{Slope})} \quad (2)$$

Where the slope was obtained from the plot of imine concentration against *β*-CD concentration and s_0: The intrinsic solubility of imine (in the absence of *β*-CD).

In vitro biological activity study

The free imine and all inclusion complex at different pH were tested for their antibacterial activity against Gram-positive *S. aureus* (ATCC25923) and two Gram-negative (*E. coli* [ATCC25924] and *P. aeruginosa* [ATCC27853]) bacterial strains, using two different methods according to the literature protocol: Disk of sterile Wattman filter paper of 6 mm in diameter tests[46-48] and broth microdilution in the liquid method.[46,49]

CONCLUSION

A new inclusion complex 1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-[benzyliden(amino)]-*β*-*D*-glucosamine (imine 2) with *β*-CD in equimolar quantity 1:1 by irradiation through microwave was successfully prepared and characterized by DRX and melting point. UV-Vis spectroscopy of imine in the presence of *β*-CD in neutral medium showed a significant bathochromic and hyperchromic effect; this change can be attributed to the presence of *β*-CD and to the nature of the complexing medium which favors the neutral medium. The proposed molar ratio 1:1 at pH 6.9 was confirmed by the A_L solubility diagram with an apparent stability constant of 583.38 M^{-1}; this value confirms the formation of the stable complex. The inclusion complex at pH = 6.9 has significant activity with *S. aureus* (G^+) and *E. coli* (G^-) in contrary to imine and the inclusion complexes at pH = 4 and pH = 8.5. Hence, we can say that *β*-CD improved the antibacterial activity of imine in the neutral medium. This proves that the inclusion in *β*-CD in the neutral medium appears as a promising formulation for imine and opens several questions, which we will continue our research to find the right answers and in another side, we will form inclusion complex (host: Imine) by other complexing agents (host molecules) (HP-*β*-CD, calixarene, and crown ether) to study the effect of the nature of the medium (pH) on imine based on modified *β*-*D*-glucosamine.

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