EASY-IMPLEMENTING ANTIMICROBIAL POLYPROPYLENE MATERIALS MODIFIED BY SILVER(I) AND COPPER(II) IONS

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Keywords: Polypropylene; Antimicrobial activity; Chemical activation; Functionalization.

Common polypropylene exhibiting no antimicrobial activity was effectively transformed into novel functional material. A surface of polypropylene materials was preliminarily activated via two chemical methods which are the treatment of the sample with water solution of sodium hydroxide in presence of iron(II) sulfate under boiling temperature and treatment with water solution of hydrogen peroxide in presence of iron(II) sulfate (to activate decomposition of the peroxide). Silver(I) and copper(II) ions were immobilized onto the surface of activated polypropylene, giving a set of four hybrid materials. Resulting functional polymers are of continuous and comprehensive antimicrobial activity. An impact of preliminary chemical activation of the polypropylene surface on the antimicrobial activity of the hybrids was shown. The highest efficiency in terms of antimicrobial properties is manifested by nonwoven polypropylene material activated by 20% water solution of chemically pure sodium hydroxide in the presence of iron(II) sulfate under boiling temperature for 2 hours followed by immobilization of silver ions.

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INTRODUCTION

Polymers are of wide application in almost every sphere of humanity. Polypropylene is one of the most widely used and available commercial commodity polymers produced in tons. In order to impart it more functions, one need to functionalize a surface by some active compounds because polypropylene itself has limited application range due to brittleness and hydrophobic nature. There are three common ways to realize the functionalization, namely: plasma treatment that is expensive, but environmental-friendly;[1,2,3] microwave treatment being controversial;[4,5,6]and relatively dirty but cheap chemical activation.[7,8] Resulting surface acquires activated groups being able to form bonds with different compounds that make polypropylene to be a potential carrier. This approach is used, for example, for catalysis of oil sweetening.[9,10] However, one of the most significant polymers application area is medicine.

Use of biomedical polymer materials modified by curative and antimicrobial compounds becomes increasingly common in recent years.[11,12] Practical medicine concluded advisability of using hybrid medicinal drugs being principally (polymer carrier)+(a biologically active substance (BAS)) combination.[13] Chemical modification of polymer surface by BAS requires the initial materials to be biologically inert, mechanically durable and able to link BAS after preliminary activation. Polypropylene (PP) meets all of these requirements. Materials based on it are widely used in medical practice, which for example, are suture materials for microsurgery, surging clothes, dressing and covering means made of non-woven fabric produced using spunbond technology ("Spunbond"), etc.

There are two common and easily available metals exhibiting antimicrobial activity, namely copper and silver.[14,15,16,17] In order to approach obtaining antimicrobial activity being easy and at the same time effective these very two elements were chosen for the work.

We previously obtained PP films functionalized by indomethacin,[18] PP films and nonwoven materials containing immobilized gentamycin.[19] All compounds exhibited antimicrobial activity. Although microorganisms become resistible towards antibiotics, there is no adaptation to antiviral, antifungal and antiseptic action of silver and copper ions. Therefore, the work provides data on PP material's modification by copper and silver ions to impart them antimicrobial properties. The goal was to develop a cheap, easy-implementing and efficient approach for obtaining antimicrobial materials, which could be performed right on the place of probable application, for example, hospital.

RESULTS AND DISCUSSION

PP is one of the most inert polymers that is to say it contains no active functional groups neither on the surface no in the volume. Therefore, it is impossible to anchor antimicrobial substances on the surface of the initial PP. That is why chemical activation of PP surface being part of the entire modification process is of great importance.

Successful activation of polypropylene surface is proved by spectrophotometric studying resulted in samples. Spectrophotometric studies of chemically activated polymer surface by ATR IR technique indicate the formation of absorption bands in the range of 3650–3100 cm^{-1} correspondings to valent vibrations of H-bonded OH-groups upon alkaline etching at elevated temperature (method 1). This is supported by absorption bands in the range of 1680–1600 cm^{-1} related to stretching vibrations of OH-groups. Moreover, this kind of activation leads to the formation of absorption bands in the range of 1710–1650 cm^{-1}

characterizing asymmetrical vibrations of carbonyl groups of modified polymer surface layer as well as absorption band in the range of 1580–1560 cm^{-1} which are attributed to carboxylate-ions.[20,21]

Similar spectral change is observed upon chemical activation of PP by the solution of hydrogen peroxide in the presence of iron(II) salt (method 2). IR-spectra show formation of primary alcohol groups upon activation according to method 2 that is confirmed by absorption bands in the range of 3000–2800 cm^{-1} (stretching vibrations of H-bonded OH-groups) complemented by absorption bands in the range of 1350–1240 cm^{-1} (deformation vibrations of OH-groups). There is also an increase of absorption band in the range of 1180–1160 cm^{-1} intensity corresponding to vibrations of C-(O)H groups. In addition, signal accumulation is observed in the range of 1750–1730 cm^{-1}, which corresponds to asymmetric valence vibrations of the carbonyl group and is confirmed by the amplification of the absorption band signal in the range of 1380–1360 cm^{-1} (deformation vibrations of the carbonyl group).[20,21,22] ATR IR spectra of PP film before treatment and after are shown in Figure S2. Spunbond exhibits similar spectral behavior.

Summarizing the aforesaid, suchlike chemical activation of polymers leads to the formation of the modified surface having a large number of reactive groups. Herewith, the amorphous phase is predominantly etched and globular structures of the crystal phase are redistributed.[23] Such changes of the activated PP surface were observed by AFM. Since the PP films have low surface stiffness, the AFM intermittent contact mode was applied, which allows to significantly reduce the lateral forces between the probe and the sample. Figure 1 provides 3D micrographs of the PP film's surface obtained by AFM method.

Figure 1 demonstrates the chemical activation being able to change the surface morphology. The surface of unactivated PP films is characterized by the presence of large peaks and depressions, some of the concentration areas are of 10-45 μm high and 0.8-2.0 μm diameter (Figure 1, a). Surface morphology is smoothed resulting from chemical activation that is shown in Figure 1, b, where the height of some concentration areas is 0.3-3.0 μm and diameter is 0.1-0.5 μm. The samples activated according to method 2 exhibit resembling characteristics, the height of the concentration areas is 0.2-1.7 μm and diameter is 0.05-0.18 μm (Figure 1, c). AFM data are supported by SEM images given in Figure S1.

Table 1. Physicomechanical characteristics of the initial and activated polypropylene film.

***t*, min**	**The workload at break (Nm^{-1})**	**Elongation at break, %***
Untreated		
0	9.8 ± 0.4	35 ± 1.7
Activated by 30 % aq. H_2O_2		
90	9.4 ± 0.5	32.5 ± 1.5
120	9.5 ± 0.4	32.2 ± 1.7
Activated by 20 % aq. NaOH		
90	7.2±0.9	28.3±1.7
120	7.1±1.0	28±1.7

*Relative to initial sample length equal to 60 mm working speed at break is 100 mm s^{-1} (constant for all samples); error in measuring the working load and elongation at break is not greater than 5 %.

Obtained data suggest a less essential change of the surface in case of chemical activation according to method 2 compared to that one according to method 1. This fact indicates less harsh modification of supramolecular polymer surface, that is in agreement with results of a study of the strength characteristics of activated PP samples given in Table 1 showing treated materials to lose mechanical durability insignificantly.

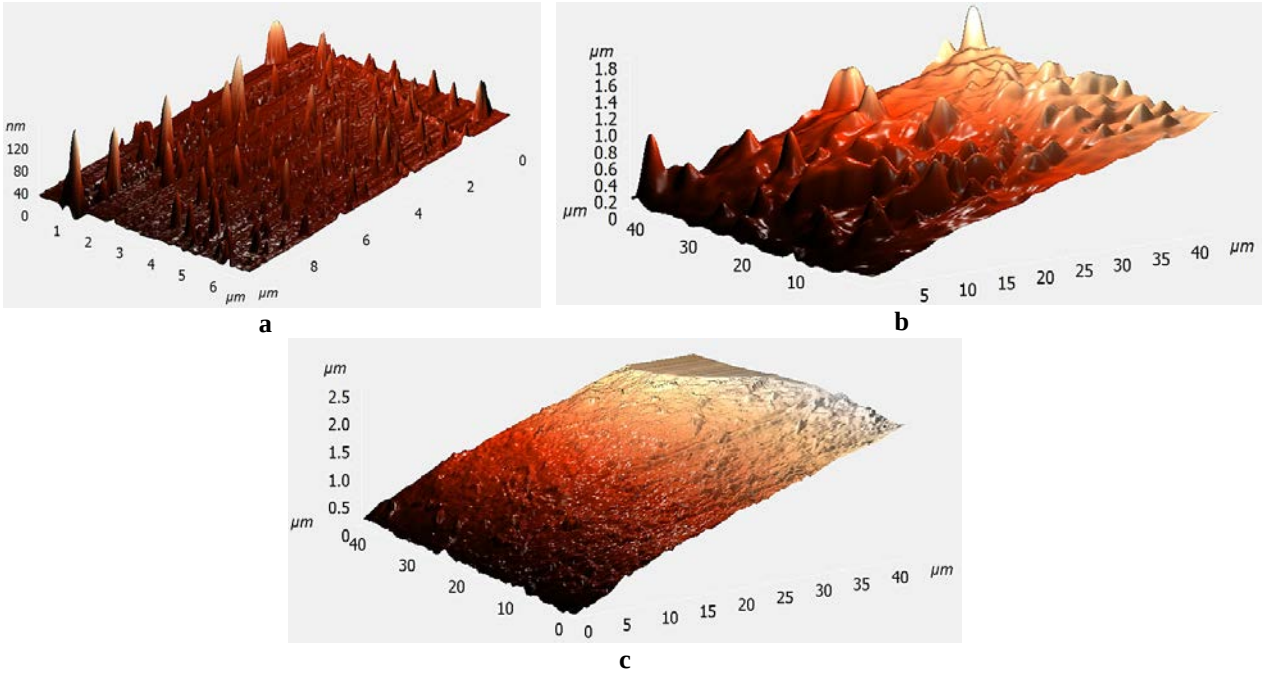

Figure 1. 3D micrographs of the surface according to the size's distribution (AFM data): **a** – initial PP film; **b** – PP film activated according to method 1; **c** – PP film activated according to method 2.

This indirectly suggests smooth morphology's modification without inner structure changes. Chemically activated samples were treated by water solutions of silver(I) and copper(II) nitrates in order to obtain PP materials (film, "Spunbond") being antimicrobial. Since the chemically activated surface of PP materials has active oxygen-containing functional groups and immobilized substances are dissociating, the way of anchoring metal ions onto the surface of polymer-carrier proceeds through the formation of chelate bonds in case of Cu, while Ag is bonded through weak coordination interaction. Proposed mechanism for the process is presented in Figure 2.

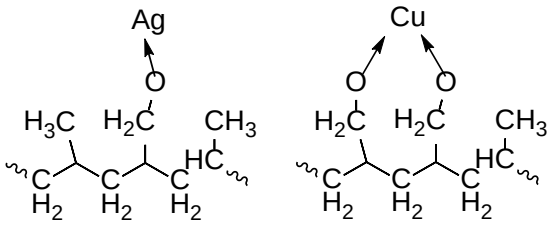

Figure 2. The proposed binding mechanism for Ag and Cu with polypropylene.

Obtained modified PP materials were tested toward antimicrobial activity. Experimental data are presented in Figure 3, Table 2. Conditions A and B used upon antimicrobial experiment were applied in order to find out the antimicrobial effect of weakly-bonded metal ions.

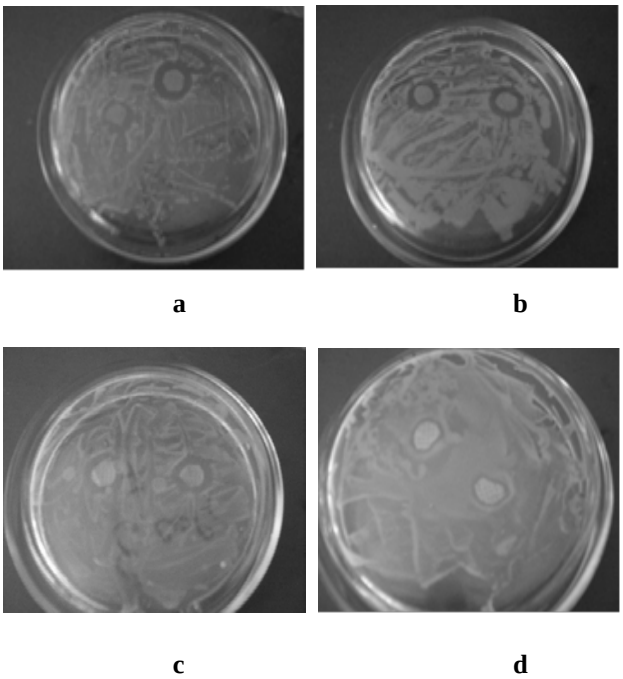

Figure 3. Images of modified "Spunbond" material (activation according to M1 with silver) toward *Staphylococcus aureus*(a), *Bacillus subtilis*(b), *Escherichia coli*(c) and *Pseudomonas aeruginosa* (d).

Table 2 shows unmodified materials to have no antimicrobial properties. The samples containing silver ions are more active than copper-containing ones as a rule that is explained by the difference in action mechanisms of silver and copper ions toward microbial cells.

Nonwoven PP material (Spunbond) is known to have more developed specific surface compared to PP film and thus greater possibility to bind metal ions.[23] This fact is indirectly supported by more pronounced antimicrobial effect observed in the case of Spunbond compared to the film. Storing for 7 days of samples in the medium of infected agar decreases antimicrobial activity but not more than two times. Films activated according to method 2 and modified by copper ions are of exception – they have completely lost their activity on the fifth day. Loss of activity is more pronounced in the case of Spunbond usage. At the same time, some of the films modified by silver ions have not changed antimicrobial activity even after 7-days soaking.

Based on obtained results one can propose the duration of antimicrobial activity of obtained polymer materials is related to the mechanism of metal ions binding by chemically activated PP carrier, the amount of immobilized substance exhibiting antimicrobial properties and the sterilization method of such materials.

EXPERIMENTAL

Following materials,being samples of 25×60 mm size, were used in present work: biaxially oriented commercial film of isotactic polypropylene (PP film) TU RB 00204079.164-97 (RF, Moscow, JSC "Moscow Oil Refinery") (molecular mass 400-700 thousand, thickness 20 μm);non-woven polypropylene material "Spunbond" (Spunbond) grade N-55/2100 (Republic of Belarus, Mogilev, Mogilev Kkhimvolokno OJSC) with specific density of 40 g/m^2, molecular weight 400-700 thousand, thickness 40 μm).

Method 1 (M1). PP material sample of 20×65 mm is treated by wt.20% water solution of sodium hydroxide in the presence of iron(II) sulfate under boiling temperature for 2 hours.

Method 2 (M2). The similar sample is treated by wt.37% water solution of hydrogen peroxide in the presence of iron(II) sulfate (to activate decomposition of the peroxide) under the temperature of 60ºC for 2 hours.

After activation, the samples were thoroughly washed by distilled water (till pH ≅ 5.5 for method 1; negative reaction toward hydrogen peroxide according to iodine-starch paper for method 2). The change in the chemical composition of the surface layer of polypropylene was studied by multiply attenuated total reflection infrared spectroscopy (ATR IR). The measurements were carried out on an "Avatar 360" FT-IR ESP spectrophotometer in the wave-number range of 400–4000 cm^{-1}. The ATR prism of the crystalline zinc selenide was used. The angle of incidence of the beam at the media interface was 45°, the number of reflections was 12. The spectra were recorded with an accumulation of the signal based on the results of 32 scans. The surface structure of modified films was investigated by AFM method (in a semi-contact mode using cantilevers NSG 11) on an atomic force microscope Solver P-47 Pro.

Table 2. Antimicrobial properties of polypropylene materials modified by metal ions (Me).

№	PP material	Me	Experiment condition*	Inhibition level** of test culture growth around the sample, mm			
				St.au.	*E.c.*	*Bac.s.*	*Ps.ae.*
			Initial material				
	Film	-	-	Growth under the sample, g s^{-1}			
	Spunbond	-	-	Growth under the sample			
			Activation according to method 1 followed by Me immobilization				
1	Film	Ag	A	11	13	12	12
2			B	10	10	10	10
3	Spunbond	Ag	A	20	21	19	17
4			B	10	13	10	12
5	Film	Cu	A	10	12	12	11
6			B	g/s	10	10	10
7	Spunbond	Cu	A	12	14	14	13
8			B	10	10	10	10
			Activation according to method 2 followed by Me immobilization				
9	Film	Ag	A	10	10	10	12
10			B	10	10	10	10
11	Spunbond	Ag	A	20	18	15	12
12			B	10	10	10	12
13	Film	Cu	A	10	10	11	12
14			B	g/s	g/s	10	11
15	Spunbond	Cu	A	10	14	10	11
16			B	10	10	10	11

Mechanical tests were carried out on the breaker PM-30-1 according to the known method.[24] The speed of the extension of the clamps of the testing machine is 100 mm min^{-1}, the studies were carried out for a set of 10 samples.

Activated PP materials were treated by water solutions of silver or copper nitrates to impart antimicrobial activity. Immobilization of Ag^{+} and Cu^{2+} ions was performed by means of 2% water solutions of corresponding salts under the temperature of 17 ± 2 ºC for 12 hours. Antimicrobial activity tests of the samples were performed right after immobilization of the ions and washing by distilled water. Improved “agar plate" method on base of meat-peptone agar (MPA) was applied in order to study the antibacterial activity of modified PP materials. The following test cultures being primary causative agents of wound infection were used: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus subtilis* (ATCC 6633). The microbial load was 10^7 cells ml^{-1}. Each Petri dish of 15 cm diameter was loaded with 20 ml of contaminated with a microorganism environment. Then, test samples of the material having ~10mm diameter were placed onto not hardened MPA under the temperature of +45ºC in the way that the sample was in contact with the substratum.

The dished were maintained under room temperature for 1-2 hours to eliminate time variation upon the sample's loading. After this, the dishes were incubated under the temperature of (36 ± 1) ºC for 24-48 hours. Uneven inhibition zones were observed around test samples of the material after incubation. Diameters of inhibition zones were measured in narrowest place.[25]

Conclusion

In this way, by means of various methods, we found the chemical treatment of polymer to change the morphology of the PP supramolecular surface becoming rough. Based on IR spectra, one can conclude successful activation of the surface via the formation of oxygen-containing groups

binding copper and silver ions. Absence of dramatical loss of physicochemical characteristics (within 5%) suppose studied methods to be applied for obtaining antimicrobial polypropylene materials of biomedical purposes.

The highest efficiency in terms of antimicrobial properties is manifested by Spunbond material activated by 20% water solution of sodium hydroxide in the presence of iron(II) sulfate under boiling temperature for 2 hours followed by immobilization of silver ions.

Hereby, obtained PP materials provide one of the possible ways of biologically active polymers creation that is of great importance and could be recommended for medicine, veterinary or agriculture purposes (disposable medical clothing, suture, plugging, dressing and covering materials, etc.). The synergy of inert carrier and the active substance is quite a simple formula giving outstanding results. Novel materials combine properties of starting ones in order to perform tasks they were made for.

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HPLC METHOD DEVELOPMENT FOR DETERMINATION OF PYRAZINAMIDE AND RELATED SUBSTANCE BY USING QUALITY BY DESIGN (QBD) APPROACH

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Keywords: Quality by Design (QbD); HPLC; Pyrazinamide; Box-Behnken design

A robust and simplified high-performance liquid chromatography (HPLC) method was developed for the estimation of Pyrazinamide and its related substance. A systematic approach, one of the parts of QbD (quality by design) was used in suitable analytical method development. The HPLC segregation method was carried out with C-18 Column (3.9x300 mm, I. D. 10 µm), a mobile phase of phosphate buffer: acetonitrile (pH 3.0) 90:10 v/v, detected at 270 nm. Optimization to this method was done by response surface methodology by applying a three-level Box Behnken design with three center points.

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Introduction

Analytical methods contribute to the design process, manufacturing of high-quality drug products and its development pattern. Hence analytical method should be accurate and specific as well as robust. Now a day's QbD (quality by design) is applied to analytical techniques to get a reliable method to analyze the quality of the product. It also provides better regulatory compliance.[1-4] There was no method reported for the HPLC development method in a QbD environment for Pyrazinamide (PZA) drug. Hence, in this systematic HPLC method using QbD principles was outstanding in ensuring the quality of the method throughout the product lifecycle. This issue has presented a new challenge to the analytical chemistry field. A precise and applicable component of the QbD is the understanding parameters and their interaction results by a desired set of experiments.

This current study involves the development of overall science and risk-based HPLC method and subsequent validation in the analysis of active pharmaceutical constituent. QbD is a systemic process for building into a product from the final output process. QbD process means a complete understanding of the product with its related process of manufacturing, overall involvement of an investment in time and resources upfront in the discovery and development of the product. For QbD the product and process knowledge base must include an understanding of the variability of raw material, the relationship between a process and product critical quality attributes (CQA) and the association between a CQA and products clinical properties. QbD method of work deals with systematic science and depends upon product-based development and its risk factors affecting, its designing factors and techniques which should be according to ICH guidelines such as ICH Q8, Q9, and Q10. PZA is a first-line anti-tubercular agent. PZA tablets are used to treat active tuberculosis. WHO has listed this drug in the essential medicines category. PZA is applicable with a combination of other medications such as Rifampicin, Isoniazid, Streptomycin and Ethambutol. It is used in the first two months of the treatment to reduce the duration of treatment required. PZA is a potent antiuricosuric drug. It is used in hypouricemia and hyperuricosuria. It is safe in pregnancy. It is soluble in water, methanol and phosphate buffer. It possesses excellent oral absorption, metabolizing by the liver and is mainly excreted in the urine.[5] Pyrazinamide undergoes diffusion process through the mycolic acid present within this bacteria and pyrazinamidase enzyme converts to active pyrazinoic acid from pyrazinamide and binds to S1 protein attached to the ribosome and hence it shows inhibition effect for the killing of *mycobacterium tuberculosi.* In synthetic pathway[6] (Figure 1), Deamidation of PZA yields pyrazine-2-carboxylic acid. Ring oxidation is another major pathway, leading to 5-hydroxypyrazinamide, which in turn hydrolyzed to 5-hydroxypyrazinoic acid.

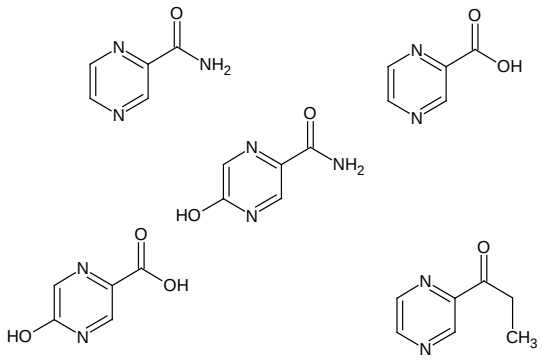

Figure 1. Pyrazinamide and its related impurities.

MATERIALS AND METHODS

Reference active pharmaceutical ingredient of Pyrazinamide was procured from Lupin Limited, Aurangabad. HPLC grade acetonitrile, phosphoric acid of Merck was used. All liquid solutions were prepared with HPLC grade ready water obtained in-house, through Milli-Q water purification system (Millipore, USA).

Instrumentation

HPLC analysis was done by using a Shimadzu HPLC SILAD vp model chromatograph equipped with an LC20 AT isocratic delivery system (pump), SPD-10Avp detector, and the analytical column was C-18 column (3.9 × 300 mm), 10 µm particle size). Data acquisition and processing were performed using Class Vp 5.13 software. Deionized water was prepared from a Milli-Q water purification system (Millipore, USA). The UV detection was done using SHIMADZU UV visible spectrophotometer (double beam), and the wavelength range of 200 to 400 nm.

Mobile phase system

The phosphate buffer solution was prepared by dissolving 6.8 g of potassium dihydrogen phosphate and 1.844 g of sodium hydroxide into a 1000 mL volumetric flask and dilute with water to produce final 1000 ml volume. Adjust the pH 3.0 with phosphoric acid. The buffer solution was degassed and sonicated and filtered prior to use for HPLC analysis. Dissolve 90 % of phosphate buffer solution in 10% of acetonitrile to produce mobile phase of the buffer: acetonitrile having concentration 90:10 v/v.

Pyrazinamide sample preparation

Pyrazinamide stock solution for optimization of experiments was prepared by accurately weighing 40 mg of Pyrazinamide and dissolving in 100 ml phosphate buffer to yield a final concentration of 400 µg mL Pyrazinamide. Transfer 10 ml standard stock into a 100 mL volumetric flask, dissolve and make up the volume with phosphate buffer. From the above stock solution, 4 µg mL samples were prepared for analysis

Wavelength selection for analysis

Appropriate dilutions of Pyrazinamide were prepared and samples were scanned using UV spectrometer in the range of 200 nm to 400 nm. An absorbance maximum was obtained at 270 nm.

Analytical target profile

A systematic approach for the development of product and its process design is termed as QbD[2] and hence determination of systematic goal in product development is necessary for a better understanding of the process and product development.[7,8] Here method intent was to develop HPLC method of Pyrazinamide which is robust, accurate, precise and USP resolution more than 2, a number of theoretical plates as per requirement and short analysis time, i.e., less than 10 min as per QbD norms a robust method should be developed with the help of visualized a design space.

Risk assessment

It is commonly understood that risk is defined as the combination of the probability of occurrence of harm and severity of that harm. The quality of process and method used in QbD is a prime part in finding out the risk assessment system and it also determines the efforts taken during variable input and its process. Critical attributes can be found from the risk assessment system, which can affect the product quality at the final stage. In the effective communication between Industries, FDA, R&D, Mfg plant risk assessment system plays a vital role.

Various tools for risk assessment are as follows: Ishikawa or fishbone diagram, failure mode effect analysis (FMEA).

Initial chromatographic conditions

Chromatographic separation was carried out with C18 column, different mobile phases were tried starting with methanol and phosphate buffer, The separation was carried out by C-18 column (3.9×300 mm, 10-µm particle size) with mobile phase of buffer (pH-3.0): acetonitrile (90:10 v/v) degassed in a sonicator for 10 min and filtered through 0.2µ membrane filter before use. Peak was obtained with a retention time of 5.20 min and by the flow rate of 1 mL min^{-1} and a column temperature of 30 °C. Before the process of the injection of drug solution, the column was balanced with mobile phase flowing through the system. Detection was done using UV detector at 270 nm. Further changes were done according to the optimization model. pH was changed by using phosphoric acid.

Table 1. Chromatographic factors and response variables for Plackett-Burman experimental design

Chromatographic condition	Level used		
	Low	Centre	High
Flow rate, mL min^{-1}	0.5	1	1.5
Injection volume, µL	7	10	13
Column oven temp., °C	28	30	32
MeCN concn., %	2	10	18
Wavelength, nm	260	265	270

Method design

The screening was done by Plackett-Burman design using Design Expert 9 software.

Five factors were selected as follows: flow rate, injection volume, column oven temperature, acetonitrile concentration, detection wavelength. The total runs obtained were 12 in number; the response for the design was a resolution between the drug and the related substance. The results were then put in the design to optimize the method further. (Table 1 and 2).

Table 2. Plackett-Burman experimental design pyrazinamide

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response
	A: Flow rate mL min^{-1}	C: ACN concn., %	D: Column oven temperature, °C	B: Injection volume, µL	E: Detection wavelength, nm	Resolution
1	1.5	2	32	13	270	11.13
2	0.5	18	32	7	270	3.31
3	1.5	2	32	13	260	9.21
4	1.5	18	28	13	270	2.43
5	0.5	2	32	7	270	14.12
6	1.5	18	28	7	260	2.29
7	1.5	2	28	7	270	8.74
8	0.5	2	28	13	260	14.15
9	0.5	18	28	13	270	3.41
10	1.5	18	32	7	260	2.09
11	0.5	18	32	13	260	2.70
12	0.5	2	28	7	260	15.16

Optimization was done by response surface methodology, applying a three-level Box Behnken design with three-center points **(**Table 3**)**. Three factors selected were flow rate, acetonitrile concentration and column oven temperature in the mobile phase. Evaluation of the main factor, their interaction and quadric effect on peak USP resolution factor were done. An injection volume of 10 µL, detection wavelength of 270 nm was kept constant as their effect on the resolution was less significant.

Table 3. Chromatographic factors and response variables for box-Behnken experimental design

Chromatographic conditions	Level used		
	Low	Centre	High
Flow rate (X_1)	0.8	1	1.2
Acetonitrile concentration, X_2	8	10	12
Column oven temperature (X_3)	28	30	32

Experiments were conducted by making triplicate injections (total 51 runs) of standard Pyrazinamide solution and the average of USP Resolution was analyzed using Design Expert 9 Software **(**Table 4**)**. Application of multivariate regression analysis resulted in a fitted full quadrate model for the average responses for peak USP resolution given by the equation:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$$

where

Y represent as a response,

β_0 represents an arithmetic mean response,

β_1 β_2 and β_3 are regression coefficients of the factor X_1, X_2 and X_3 respectively.

β_{11}, β_{22} β_{33} are squared coefficients β_{12}, $\beta_{13}$and β_{23} are interaction coefficients.[9,10]

Table 4. Box-Behnken method sued for Pyranizamide determination optimization

Coded (X_1, X_2, X_3)	Flow Rate, mL min^{-1}	ACN concn., %	Column oven temperature, ◦C
000	1	10	30
--0	0.8	8	30
000	1	10	30
+0+	1.2	10	32
+-0	1.2	8	30
000	1	10	30
-0-	0.8	10	28
-0+	0.8	10	32
+0-	1.2	10	28
000	1	10	30
++0	1.2	12	30
000	1	10	30
0++	1	12	32
0--	1	8	28
0+-	1	12	28
-+0	0.8	12	30
0-+	1	8	32

Critical quality attributes

From the software-generated result, the critical factors which affect the resolution and capacity factor were determined. Factor such as flow rate, wavelength and ACN concentration in the mobile phase found to be critical. Selection of the stationary phase was also a critical parameter. The nature of the drug is more retentive on C-18 than C-8.

Method validation

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) Q_2 $(R_1)^{2-4}$ guidelines for linearity, range, precision and robustness. For system suitability, a standard solution of

10 µg mL of Pyrazinamide was prepared by diluting and mixing the drug with methanol. Six replicate injection of the system standard solution were analyzed before sample analysis. The acceptance criteria for Pyrazinamide were less than 2 % relative standard deviation (RSD) for peak area, retention time, symmetry USP resolution factor more than 2 and number of theoretical plates greater than 2000 for all peaks.

Linearity

As per ICH guidelines, the linearity is defined as an analytical procedure which has its ability (within in a given range) to obtain test results and which are directly proportional with the concentration (amount) of analyte in the sample. Standard calibration curves were prepared by taking five different concentrations and then by making serial volume to volume dilution of stock solution with methanol, over the range of 10, 20, 30, 40 and 50 µg mL^{-1}. Three replicate injections of each concentration were made to determine the linearity of Pyrazinamide over the concentration range. Linear concentration curves of peak area versus drug concentration were plotted by using a linear least squares regression and then evaluated for linearity.

Precision

According to ICH, Q2 guidelines, accuracy is defined as the percent relative standard concentration standard deviation of a set of responses. The precision of the method was evaluated for Pyrazinamide drug substance by analyzing standard samples prepared daily from the stock solution. Three replicates of each low (10 µg mL^{-1}), intermediate (20 µg mL^{-1}), high (30 µg mL^{-1}) standard was analyzed daily over three days as a part of validation and quality control. Precision was determined by analyzing the mean, standard deviation and relative standard deviation for peak areas and their resultant concentrations.

An acceptance criterion for precision is that the RSD of the standards should not be more than 2.

Robustness

The robustness of an analytical procedure is defined as a measurement of its capacity to remain unaffected by the small but deliberate change in method parameter and provide an indication of its reliability during normal usage.[3-6] There should be the reliability of an analysis with respect to deliberate variation in method parameters such as flow rate (±0.1mL min^{-1}), pH (±0.1 units), and mobile phase proportion.

RESULTS AND DISCUSSIONS

Pyrazinamide is used as an anti-tubercular agent. Different mobile phases were tried starting with methanol and water, the separation was carried on C-18 column (3.9 × 300 mm, 10 µm particle size) with mobile phase of disodium hydrogen phosphate buffer (pH 3.0): acetonitrile (90:10 v/v) Peak was obtained at retention time of 5.20 min, with flow rate of 1mL min^{-1}, column temperature of 30 °C, at 270 nm wavelength. Further screening was done using Plackett-Burman design and Optimization was done by carrying runs as by Box-Behnken design.

Method design

Plackett-Burman design

The screening was done by using Plackett-Burman design, which gives Pareto chart (Figure 2) and Probability values (*p*-values) for flow rate, acetonitrile concentration, column temperature, and injection volume and detection wavelength.

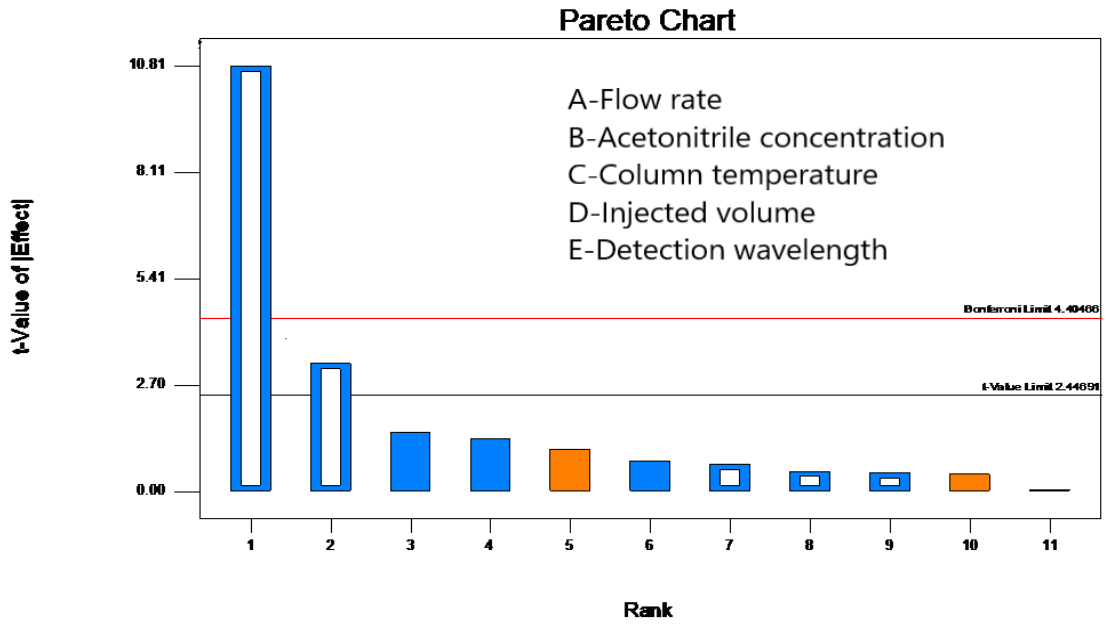

Figure 2. Pareto chart

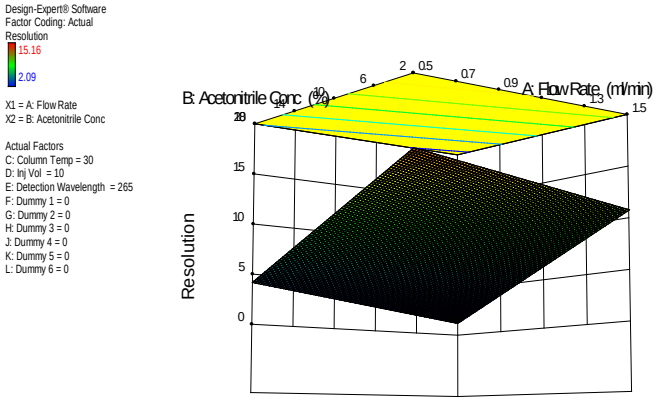

Figure 3. Response surface (3D) and contour plot showing the effects of acetonitrile concentration and flow Rate on USP resolution factor of Pyrazinamide (Plackett-Burman design)

Box Behnken design

Multivariate regression analysis was implemented and then fitted with a full quadratic model which was obtained for the USP Resolution factor of the peak. Factors considered here are column temperature, acetonitrile concentration and wavelength. Regression coefficient and p-values obtained from the software-generated report are given in **(**Table 5).

Table 5. Regression coefficients and associated probability values (*p*-values) for USP resolution of pyranizamide

Term	Regression coefficient	*p*-value
Intercept	3.59	0.0000157
Flow rate	-0.11	0.068765
Acetonitrile concentration	-0.45	0.00000179
Column temperature	-0.017	0.757273
Lack of fit		0.607521

Analysis of variance (ANOVA) was processed to analyze the significance of the factors and interaction terms on the response, i.e., USP resolution of the peak, *p*-value provide the cut-off beyond which we assert that the findings are 'statistically significant' by convention, it is $p<0.05$.[11-17]

A value of probe >*F* was found to be less than 0.05, hence model was found to be significant for prediction of response. The entire model was fitted well for optimization. A lack fit was not significant. Significant factors were found i.e. flow rate (*p*-value 0.068765), acetonitrile concentration (*p*-value 0.00000179), and column temperature (*p*-value 0.757273). Acetonitrile concentration was found to be the most significant.

Two of the factors were found to affect the resolution from their respective coefficients. ACN concentration, the flow rate is showing an inverse relationship with resolution.

Response surface and contour plot were studied to visualize the effect of factor to develop design space for robust method 3D graph are given below in Figure 4. From the graph, some facts about the impact of the factors and their interaction on the response can be found. Curvatures in the contour plot show a linear relationship between factors.

From Figure 4 showing the effect of ACN concentration and flow rate where the wavelength is constant at 265 nm. ACN concentration should be between 8-12 % the Resolution was in limit and above and below this limit Resolution factor get increased. If Column Temperature and ACN concentration receives an increase, then the resolution gets affected. To obtain an optimum set of condition to achieve desired goal composite desirability parameters were applied. The response was set to maximum resolution between Pyrazinamide and the related substance above the target value of 2.

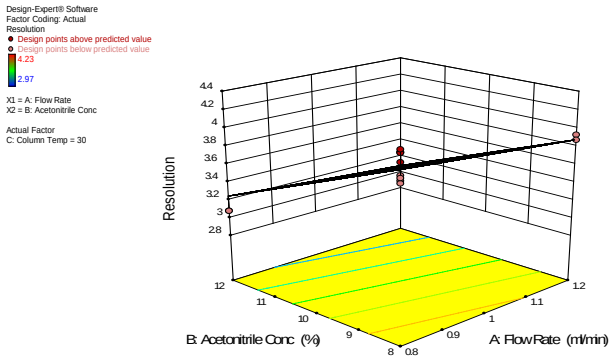

Figure 4. Response surface (3D) and contour plot showing the effects of acetonitrile concentration and flow rate on USP resolution factor of Pyrazinamide (Box-Behnken design)

The optimum condition having desirability was chosen from obtained runs, i.e., column temperature of 30 °C, ACN concentration of 8 % and Flow Rate 0.8 mL min^{-1}. Set of conditions were analyzed to compare predicted response with the actual response.

The chromatogram for optimised condition is shown in Figure 5.

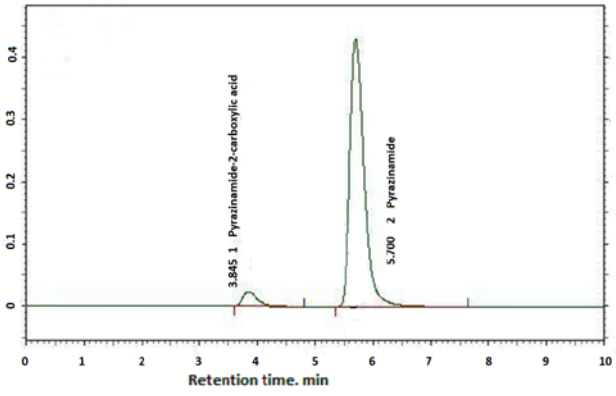

Figure 5. The chromatogram recorded at optimized conditions

Method validation

Method validation was done according to the ICH guideline Q2.[2-5] Results were within the specified limit. This method was found to be precisely accurate and robust and withstand according to ICH guidelines Validation results are given below in (Table 6).

Table 6. Validation of method in term of linearity and precision of Pyranizamide

Validation parameter	Result	Acceptance criteria
Linearity, 10 - 50 µg mL^{-1}	Coefficient of correlation-0.9987	Coefficient of Correlation >0.999
Precision:		
Repeatability	RSD: 1.7320 %	RSD less than 2%
Intraday	0.75%	
Inter day	0.09%	

Linearity

A set of five solutions of Pyrazinamide at a concentration ranging from 10-50 µg mL^{-1} were prepared. Each sample was analyzed in triplicate; calibration curve was constructed by plotting the peak area versus the concentration using linear regression analysis. The correlation coefficient was found to be 0.998 (Table 7).

Table 7. Linearity of Pyranizamide

Standard concentration, µg mL^{-1}	Peak area of Pyrazinamide
10	1083140
20	2137374
30	3178265
40	4043446
50	5174387
Regression Equation	Y=100886x + 96753
Regression coefficient	0.9987

Repeatability

Repeatability was determined by running six replicates of samples and evaluating the average and %RSD for a sample by comparing the peak area (Table 8).

Table 8. Repeatability of Pyranizamide

Sr. No	Concentration, µg mL^{-1}	Peak area
1.	10	1082245
2.	10	1082257
3.	10	1082224
4.	10	1082274
5.	10	1082289
6.	10	1082237
Average		1082254.333
%RSD		1.7320

CONCLUSION

The quality by design approach has been successfully implemented for method development of Pyrazinamide API by using HPLC. All essential parameters of QbD were processed and then implemented in said study. A systematic approach was utilized for developing an efficient and robust method which involves beginning with the determination of target profile characteristics, instrument qualification, risk assessment, and design of the experiment. Interactions and quadratic effects of factors were studied with the least possible runs by using Box-Behnken model in conjunction with response surface methodology. Response surface diagrams and contour plots were studied for coming to a conclusion which factor are affecting response and their limits were recorded. Optimum conditions were obtained; the one with higher desirability was selected and a desirable function which was applied to determine the optimum conditions under a QbD approach.

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CONDENSATION REACTION BETWEEN 3-ACETYLCOUMARINE AND ARYL ALDEHYDES IN THE PRESENCE OF *L*-PROLINE

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Keywords: 3-Acetylcoumarine, salicylaldehyde, ethyl amine, *L*-proline, chalcone.

3-Acetylcoumarine and a series a coumarin-chalcone hybrid compounds have been synthesized in good- to-excellent yields using a simple and efficient method. This method involved the one-pot reaction of salicylaldehyde, an α-ketoester to afford 3-acetylcoumarine and the product has been treated with an aromatic aldehyde using *L*-proline as a bioorganic catalyst to yield 1-(3'-coumarinyl)-3-aryl-2-propen-1-one (3-cinnamoylcoumarins).

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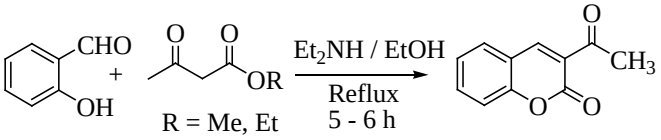
INTRODUCTION

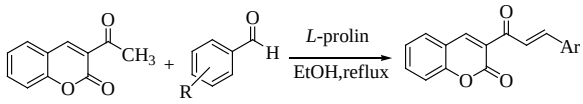
Recently, *L*-proline has gained importance as a versatile catalyst for effecting various organic transformations such as the synthesis of coumarone in ionic liquid[1] density functional study of the *L*-proline-catalyzed α-aminoxylation of aldehydes[2] unsymmetrical dihydro-1H-indeno[1,2-*b*]pyridines[3] and 2-amino-4H-benzochromenes.[4] Also, *L*-proline and *L*-proline derivatives were successfully used as organocatalysts in asymmetric aldol and Michael addition reactions.[5] To the best of our knowledge, there was no attempt to use *L*-proline as a catalyst for the synthesis of 1-(3'-coumarinyl)-3-aryl-2-propen-1-one.

Diethylamine has recently been demonstrated to be an efficient organocatalyst in the various organic transformations such as synthesis of chromenes,[6] Knoevenagel condensation,[7] and aldol condensation and thia-Michael addition process.[8] Also chromenes are an important class of compounds, widely present in plants, including edible vegetables and fruits.[9] Numerous bioactive natural products have been identified, and the presence of the chromene-based structure has been associated with the capacity to prevent disease.[10] In this study, diethylamine was used for the synthesis of 3-acetylcoumarine using alkyl acetoacetates and 2-hydroxy benzaldehyde in ethanol under reflux condition (Scheme 1).



Scheme 1. Synthesis of 3-acetylcoumarine using diethylamine.

Further, 3-acetylcoumarine and aromatic aldehydes, in the presence of *L*-proline, react under reflux condition in ethanol to yield 1-(3'-coumarinyl)-3-aryl-2-propen-1-one in good-to-excellent yields. The protocol represents a simple and efficient route, involving less hazardous solvent and easy separation (Scheme 2).



Scheme 2. Synthesis of 1-(3'-coumarinyl)-3-aryl-2-propen-1-one using *L*-proline.

EXPERIMENTAL

Melting points were measured by using the capillary tube method with an electro thermal 9200 apparatus. IR spectra were recorded on Perkin Elmer FT-IR spectrometer scanning between 4000–400 cm^{-1}. ^{1}HNMR spectra were obtained on Bruker DRX- 300 MHZ NMR instrument.

Synthesis of 3-acetylcoumarin

A mixture of salicylaldehyde (20 mmol.), methyl acetoacetate or ethyl acetoacetate (30 mmol), ethanol (10 mL) and diethylamine (1 mL) were stirred under reflux condition at 4-5 h. After completion of the reaction, the mixture was cooled to afford yellow solid (92 %). The product was filtrated and then was washed with excess water and dried in air. M.p. 119–121 °C. ^{1}HNMR (300 MHz, $CDCl_3$) δ = 8.34 (s, 1H), 7.54–7.42 (m, 2H), 7.26–7.12 (m, 2H), 2.56 (s, 3H).

General procedure for the synthesis of 3-cinnamoyl coumarines using *L*-proline

A mixture of 3-acetylcoumarin (1 mmol.) and the corresponding aldehyde (1 mmol.) were mixed in the presence of L-proline (25 mol %) in ethanol (12 mL). The reaction mixture was stirred under reflux condition for a specified time.

The progress of the reaction was monitored by TLC (4:1 n-hexane:ethyl acetate). After completion of the reaction, the mixture was cooled and the product precipitated slowly after 8 h. The solid material was filtered off, washed with water, dried, and recrystallized from ethanol.

Recycling of the catalyst

The reusability of the catalyst was also investigated on the model reaction. After filtrating of the product, the liquor was evaporated under low pressure and the *L*-proline was washed with dichloromethane (15 mL), dried at 50 °C to provide an opportunity for recycling experiments. The separated catalyst was reused in the mentioned reaction for the synthesis of four times without considerable loss of its catalytic activity (entry 1, table 3; 90, 87, 83 and 78 %).

Spectra data for synthesized compounds

3-((E)-3-Phenylacryloyl)-2*H*-chromen-2-one (entry 1, table 3)

Brown solid. FTIR (KBr): 2978 (alkane,C-H str), 3037(Ar, C-H str), 1715(C=O), 1666 (C=O), 1607 (C=C) cm^{-1}. ^{1}HNMR (300MHz, $CDCl_3$) δ = 8.61(s,1H, C-H), 7.90 (d, 1H, =C-H), 7.71-7.45 (m, 4H, Cumarin), 7.419-7.266 (m, 5H, Ar-H), 6.87 (d, 1H, =C-H).

3-((*E*)-3-(4-Chlorophenyl)acryloyl)-2*H*-chromen-2-one (entry 2, table 3)

Brown solid. FTIR (KBr): 3030(Ar, C-H, str), 1717 (C=O), 1666 (C=O), 1610 (C=C) cm^{-1}. ^{1}HNMR (300MHz, $CDCl_3$) δ = 8.61 (1H, s, C-H), 7.92 (d, 1H, =C-H, J=15.72), 7.84 (d, 1H, =C-H, J=15.75), 7.60-7.70(m, 4H, Ar-H), 7.27-7.43(m, 4H, Ar-H).

3-((*E*)-3-(4-Nitrophenyl)acryloyl)-2*H*-chromen-2-one (entry 3, table 3)

Brown solid. FTIR (KBr): 3042 (Ar, C-H), 1722(C=O), 1666 (C=O), 1610 (C=C) cm^{-1}. ^{1}HNMR (300MHz, $CDCl_3$) δ = 8.65 (s, 1H, C-H), 8.23-6.73 (m, 10H, Ar, CH=CH).

3-((*E*)-3-(3-Nitrophenyl)acryloyl)-2*H*-chromen-2-one (entry 4, table 3)

Brown solid, FTIR (KBr): 3078 (Ar, C-H str), 1728 (C=O), 1687 (C=O), 1608(C=C) cm^{-1}. ^{1}HNMR (300MHz, $CDCl_3$) δ = 8.69 (s, 1H, C-H), 8.49 (s, 1H, H-Ar), 8.21 (d, 1H, J =7.63, H-Ar), 7.78-7.26 (m, 8H, Ar, CH=CH).

3-((*E*)-3-(2-Furyl)acryloyl)-2*H*-chromen-2-one (entry 5, table 3)

Dark brown solid. FTIR (KBr): 3125(Ar, C-H), 1739(C=O), 1680(C=O), 1607 (C=C) cm^{-1}. ^{1}HNMR (300MHz, $CDCl_3$) δ = 8.58 (d, 1H, =C-H), 7.83 (d, 1H, =C-H), 7.70-7.60 (m, 4H, coumarin), 7.45-7.34 (m, 3H, furyl), 6.85 (d, 1H, =C-H).

3-((*E*)-3-(4-Bromophenyl)acryloyl)-2*H*-chromen-2-one (entry 6, table 3)

Brown, solid. FTIR (KBr): 3050 (Ar, C-H), 1707 (C=O), 1741(C=O), 1607 (C=C) cm^{-1}. ^{1}HNMR (300MHz, $CDCl_3$) δ = 8.57 (s, 1H, C-H), 7.90 (d, 1H, =C-H), 7.71-7.50 (m, 4H, coumarin), 7.40-7.16 (m, 4H, Ar-H), 6.59 (d, 1H, =C-H), 7.71-7.50 (m, 4H, Coumarin), 7.40-7.16 (m, 4H, Ar-H), 6.59 (d, 1H, =C-H).

RESULTS AND DISCUSSION

Initially, 3-acetylcoumarine was synthesized from the reaction of salicylaldehyde, an α-ketoester (methyl acetoacetate or ethyl acetoacetate) in ethanol with the addition of little amount of ethyl amine under reflux condition. On the base of our knowledge, the synthesis of 3-acetylcoumarine in the presence of diethyl amine have not been reported previously (Scheme 1).

Table 1. Optimization of the catalyst amount for the synthesis of 1-(3′-coumarinyl)-3-(4″-chlorophenyl)-2-propen-1-one.

Entry	Catalyst, mol %	Yield, %
1	0	0
2	10	40
3	15	47
4	20	75
5	25	90
6	30	90

Reaction conditions: Benzaldehyde (1 mmol), 3-acetylcumarin (1 mmol), solvent: ethanol (12 mL) under reflux

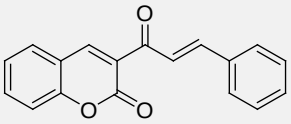
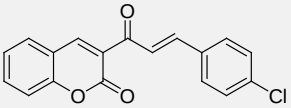
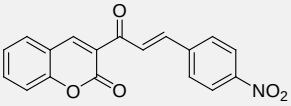
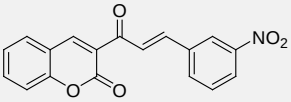
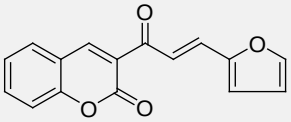
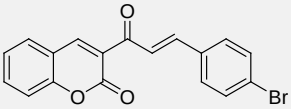
Thus synthesized 3-acetylcoumarine was used for the preparation of 3-cinnamoyl coumarins. In this case, initially, the optimization of the reaction conditions was studied for the synthesis of 3-((E)-3-phenylacryloyl)-2*H*-chromen-2-one. No product was obtained from the reaction of 3-acetyl coumarine and benzaldehyde in the absence of the catalyst under reflux condition in ethanol (Table 1, entry1). The reaction was then performed in the presence of various amounts of *L*-proline to examine the catalytic activity (Table 1, entries 2-6). Among these amounts (25 mol %) of *L*-proline was found to be the most effective catalyst and afforded the desired product in 90 % yield (Table 1, entry 5).

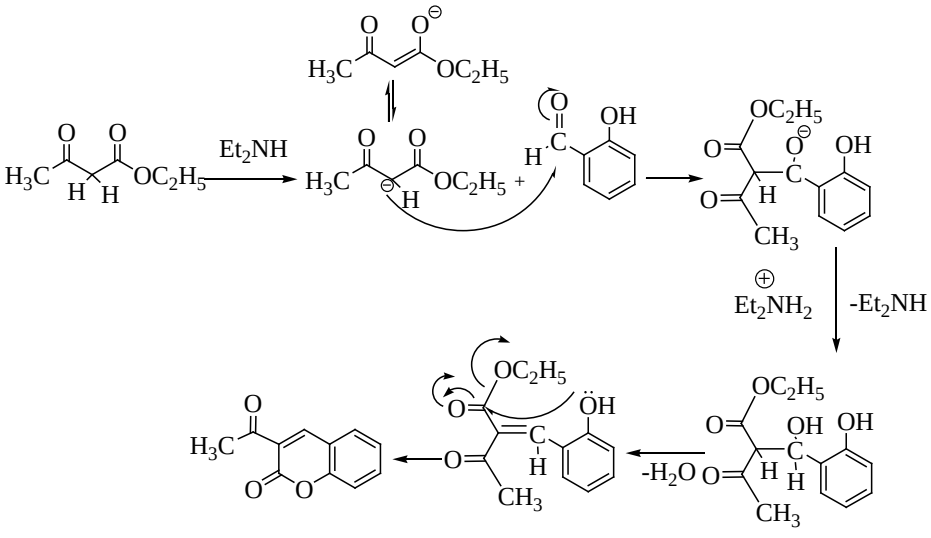
Also, different solvents were evaluated in the benchmark reaction. The results were revealed, that the catalyst more efficiently worked in ethanol under reflux conditions (Table 2).

Table 2. The effect of solvent at different temperatures on the yield of the synthesis 1-(3′-coumarinyl)-3-(4-chlorophenyl)-2-propen-1-one.

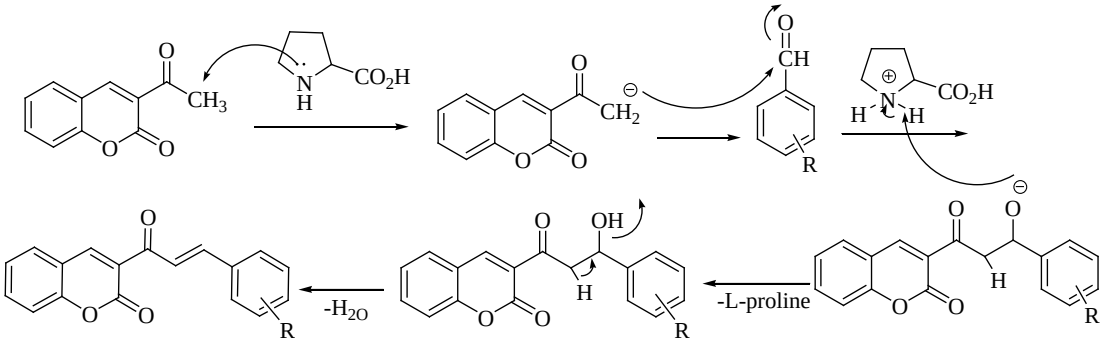
Entry	Solvent	Temp., °C	Time, h	Yield, %
1	EtOH	25	48	60
2	H_2O	25	48	-
3	EtOH	Reflux	17	90
4	H_2O	Reflux	48	-
5	None	100	10	25

Table 3. The synthesis of 1-(3'-coumarinyl)-3-aryl-2-propen-1-one using *L*- proline as a catalyst.

Entry	Aldehyde	Products	Time, h	Yield, %	M.P., °C Found/Reported(ref.)
1	Benzaldehyde		17	88	180-178/180-182[11]
2	4-Chloro-benzaldehyde		17	90	188-190/188-190[11]
3	4-Nitro-benzaldehyde		15	88	152-154/154-156[11]
4	3-Nitro-benzaldehyde		15	87	205-207/205-207[11]
5	Furan-2-carbaldehyde		20	78	93-95/101-103[11]
6	4-Bromo-benzaldehyde		17	88	179-182/189-191[11]
7	2-Hydroxy-benzaldehyde	No reaction	48	-	-
8	4-Dimethylamino-benzaldehyde	No reaction	48	-	-



Scheme 3. Proposed mechanism for the synthesis of 3-acetylcoumarine.



Scheme 4. Proposed mechanism for the synthesis of 1-(3'-coumarinyl)-3-aryl-2-propen-1-ones.

To generalize optimized model reaction, several aromatic aldehydes and 3-acetylcoumarines in ethanol under the same conditions were used for the synthesis of 1-(3'-coumarinyl)-3-(4-chlorophenyl)-2-propen-1-one. All aldehydes possessing electron withdrawing and electron releasing groups reacted in good–to–excellent yields under mild condition. An important feature of this procedure is the survival of variety of functional groups such as nitro groups, halides and heterocyclic aldehyde under the reaction conditions (Table 3).

Proposed mechanism for the synthesis of the preparation of 3-acetyl coumarine and 3-cinnamoyl coumarins have been shown in schemes 3 and 4.

CONCLUSION

The synthesis of potential bioactive hybrid molecules obtained from naturally occurring coumarin-chalcone moieties using *L*-proline as a highly efficient catalyst has been achieved. This method has the advantages of low cost, short reaction time, high atom economy, convenience and efficiency. It also offers a selective approach to the synthesis of coumarins, a structural motif found in a large number of natural products, pharmaceuticals and functionalized materials. Such a selective procedure offers energy saving advantages and reduces the expense of the purification process.

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WATER MEDIATED ONE-POT, FOUR COMPONENT SYNTHESIS OF 3,3-DIMETHYL-13-ARYL-3,4-DIHYDRO-1*H*-INDAZOLO[1,2-*b*]PHTHALAZINE-1,6,11(2*H*,13*H*)-TRIONES

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Keywords: Dimethyl phthalate, one-pot synthesis, green synthesis and water; 2H-indazolo[2,1-*b*]phthalazine-trione derivatives

Water mediated one-pot, four-component synthesis of substituted 2H-indazolo[2,1-*b*]phthalazine-triones from dimethyl phthalate, hydrazine, dimedone and aromatic aldehydes have been reported in the presence of p-toluenesulfonic acid (PTSA) as catalyst at 100 °C for 1.5-2.0 h. This methodology offers several advantages such as good yields, short reaction time, simple procedure, mild condition and environmentally begins.

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INTRODUCTION

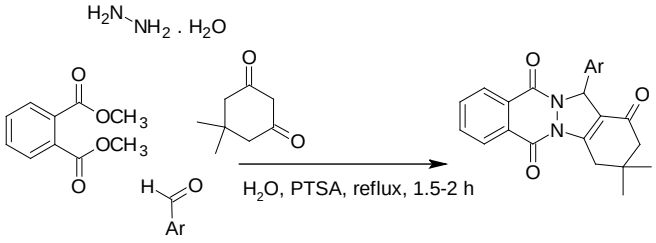
Phthalazines are important heterocycles that are known to possess multiple biological activities such as antimicrobial, anticonvulsant, antifungal, anticancer and anti-inflammatory activities.[1-3] Therefore, a number of methods have been reported for the synthesis of phthalazine derivatives. Nevertheless, the development of new synthetic methods for the efficient preparation of heterocycles containing a phthalazine ring fragment is an interesting challenge. Recent protocols have been employed for the synthesis of title compounds either by one-pot, three-component condensation of phthalhydrazide, dimedone and aromatic aldehydes using Me_3SiCl[4], silica sulfuric acid[5], H_2SO_4[6]. $Mg(HSO_4)_2$,[7] and [bimm]Br under ultra-sonication[8] as catalysts. One-pot, four-component synthesis of phthalic anhydride, hydrazine, dimedone and aromatic aldehydes using various catalytic systems such as starch sulfate,[9] $CeSO_4$-$4H_2O$[10] and PEG-SO_3H[11] have also been performed.

However, many of these methodologies are associated with one or more disadvantages such as the use of expensive catalyst or toxic organic solvents,[12] strong acidic conditions,[6] and harsh reaction conditions,[6,7] the formation of by-products and tedious work-up procedures. According to the principle of safe chemistry, synthetic methods should be designed to use substances that exhibit little or no toxicity to human health and the environment.[13] Nonetheless the development of new synthetic methods for the proficient preparation of heterocycles containing phthalazine ring fragment is an interesting challenge.

Keeping these results in mind, we now aim to report the one-pot, four-component synthesis of 2H-indazolo[2,1-*b*]phthalazine-triones from dimethyl phthalate, hydrazine, dimedone and aromatic aldehydes in water as a solvent in the presence of p-toluenesulfonic acid (PTSA) as a catalyst at 100 ˚C for 1.5-2.0 h.

RESULTS AND DISCUSSION

To find out an appropriate reaction conditions for synthesis of the title compounds by one-pot, four-component reaction among dimethyl phthalate **1** (1 mmol), hydrazine hydrate **2** (1 mmol), dimedone **3** (1 mmol) and benzaldehyde **4** (1 mmol) was selected as a model **(**Scheme 1) and examined with different catalysts in several solvents at different temperatures. The obtained results are summarized in Table 1 and 2.



Ar=Ph, 2-ClC_6H_4, 4-BrC_6H_4, 2-$O_2NC_6H_4$, 3-$O_2NC_6H_4$, 4-ClC_6H_4, 4-$CH_3C_6H_4$, 2-$CH_3C_6H_4$

Scheme 1. Synthesis of 3,3-dimethyl-13-aryl-3,4-dihydro-1*H*-indazolo[1,2-*b*]phthalazine-1,6,11(2*H*,13*H*)-triones

The best results were obtained using water as solvent at 100 ºC for 1.5 hr in the presence of PTSA (1 mmol/10 mmol substrate) as catalyst compares to other solvents like DMF, glycerol, ethylene glycol and PEG-400 at different temperature. It is clear that, water and PTSA are playing very important role in the synthesis of title compound by this method.

Table 1. Effect of acid catalysts on one-pot reaction for yielding 3,3-dimethyl-13-phenyl-3,4-dihydro-1*H*-indazolo[1,2-*b*]phthalazine-1,6,11(2*H*,13*H*)-trione (**5a**) in water at 100 ºC

Entry	Catalyst	mmol/10 mmol substrate	Time, h	5a, %
1	PTSA	0.5	3.0	84
2	PTSA	1.0	1.5	88
3	PTSA	1.5	1.5	80
4	Boric acid	0.5	3.5	82
5	Boric acid	1.0	4.5	80
6	Boric acid	1.5	4.0	81
7	$AlCl_3$	0.5	3.5	80
8	$AlCl_3$	1.0	2.5	78
9	$AlCl_3$	1.5	2.5	74

Table 2. Effect of solvent on the one-pot reaction for yielding 3,3-dimethyl-13-phenyl-3,4-dihydro-1*H*-indazolo[1,2-*b*]phthala-zine-1,6,11(2*H*,13*H*)-trione (**5a**) in the presence of 1 mmol/10 mmol substrate PTSA as catalyst.

Entry	Solvent	Temp., °C	Time, h	5a, %
1	Glycerol	50	10	80
2	Glycerol	100	4	82
3	Glycerol	120	2.5	80
4	H_2O	50	8	80
5	H_2O	100	1.5	88
6	PEG-600	50	12	76
7	PEG-600	100	4.5	78
8	Ethylene glycol	50	4.0	75
9	Ethylene glycol	100	3.0	78
10	DMF	50	8.0	68
11	DMF	100	2.5	65

In the next step, the scope and efficiency of the process were explored under the optimized conditions for the synthesis of title compounds. For this purpose, a broad range of structurally diverse dimethyl phthalate (**1**) were condensed with hydrazinium hydroxide (**2**), aromatic aldehydes (**4a-4h**) and dimedone (**3**) in the presence of water at 100 ˚C temperature, and the results are shown in Table 3.

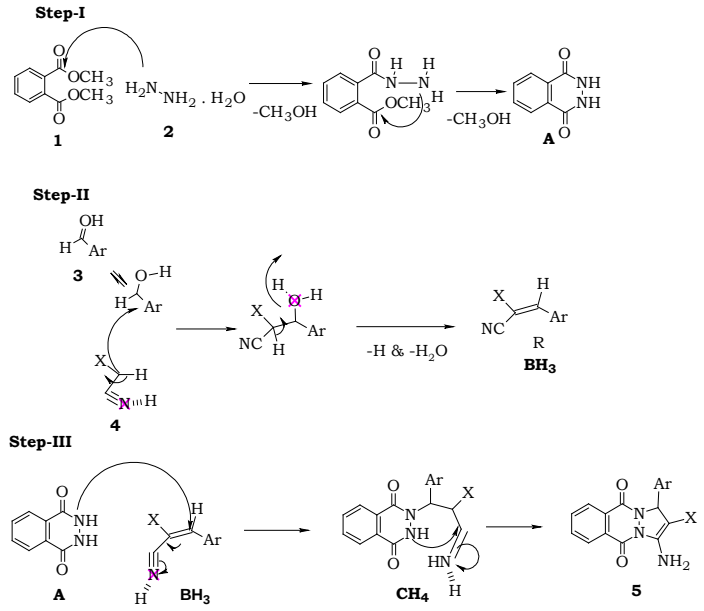
Table 3. Characterization data, reaction time and yields of compounds **5a-5h** obtained from **1**, **2**, **3** and **4a-4h** via one-pot, four component synthesis

Starting materials				Product	Yield, %≠	Melting point, °C
1	**2**	**3a**	**4a**	**5a**	88	203-205
1	**2**	**3b**	**4b**	**5b**	83	268-270
1	**2**	**3c**	**4c**	**5c**	82	262-264
1	**2**	**3d**	**4d**	**5d**	86	239-240
1	**2**	**3e**	**4e**	**5e**	85	267-269
1	**2**	**3f**	**4f**	**5f**	83	258-259
1	**2**	**3g**	**4g**	**5g**	84	226-268
1	**2**	**3h**	**4h**	**5h**	85	243-245

≠ Refers to yields of crude products only.

The yields obtained were good to excellent without the formation of any side-products and all reactions proceed rapidly in short times. The structures of the products were established from their spectral properties (^{1}H NMR & ^{13}C NMR).

Our proposed mechanism contains two steps. The initial formation of phthalhydrazide by nucleophilic addition of hydrazinium hydroxide **2** to dimethyl phthalate **1**. The second step involves the initial formation of heterodiene **(X)** by standard knoevenagel condensation of dimedone **3** and aromatic aldehydes **4**. Then, subsequent Michael-type addition of the phthalhydrazide followed by cyclization affords the corresponding product **5** (Scheme 2).



Scheme 2. Proposed mechanism

EXPERIMENTAL

Melting points are uncorrected and were determined in open capillary tubes in a sulphuric acid bath. TLC was run on silica gel–G and visualization were done using iodine or UV light. IR spectra were recorded using a Perkin – Elmer 1000 instrument in KBr pellets. ^{1}H NMR spectra were recorded in DMSO–d_6 using TMS as an internal standard using a 400 MHz spectrometer. Mass spectra were recorded on an Agilent-LCMS instrument under CI conditions and given by Q+1 values only. Starting materials **1, 2, 3** and **4** were obtained from commercial sources and used as such.

General procedure for preparation of 5a-5h

Dimethyl phthalate **(1)** (10 mmol) and hydrazine hydrate **(2)** (10 mmol) was refluxed at 100 ºC in water for 10-15 min to form phthalhydrazide as intermediate in the presence of PTSA (1 mmol). Then, dimedone **(3)** (10 mmol) and substituted benzaldehydes **(4a-4h)** (10 mmol) were added and the mixture refluxed again for 1.5-2.0 h. After completion of the reaction, ice-cold water (50 mL) was added to the reaction mixture and neutralized (pH: 6.5 to 7.0) with 20 % sodium bicarbonate solution; the solid that separated out was filtered, washed with water (20 mL) two times and dried. The product was recrystallized from ethanol to obtain final compounds.

3,3-Dimethyl-13-phenyl-3,4-dihydro-1H-indazolo[1,2-*b*]phthalazine-1,6,11(2H,13H)-trione (5a)

IR (KBr) in cm^{-1}: 1661, 1625 , 1601(-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.89 (s, 3H, -CH_3), 1.03 (s, 3H, -CH_3), 2.05 (AB system, 2H, -CH_2), 2.54 (AB system, 2H, -CH_2), 4.54 (s, 1H, -CH), 7.09 (m, 1H, Ar-H), 7.11 (m, 4H, Ar-H), 7.87 (d, 2H, Ar-H), 8.07 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 26.3, 28.7, 31.2, 31.8, 50.0, 114.4, 125.1, 126.1, 127.8, 128.0, 132.5, 144.2, 154.6, 162.8, 196.0; HRMS calcd for $C_{23}H_{20}N_2O_3$ $[M+H]^+$: 373.1268. Found: 373. 1238.

13-(2-Chlorophenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-*b*]phthalazine-1,6,11(2H,13H)-trione (5b)

IR (KBr) in cm^{-1}: 1670, 1655, 1631(-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.99 (s, 3H, -CH_3), 1.04 (s, 3H, -CH_3), 2.15 (AB system, 2H, -CH_2), 2.54 (AB system, 2H, -CH_2), 4.53 (s, 1H, -CH), 7.03 (m, 1H, Ar-H), 7.21 (m, 3H, Ar-H), 7.86 (d, 2H, Ar-H), 8.12 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 24.4, 28.6, 30.1, 32.5, 50.3, 114.3, 124.2, 126.5, 127.4, 128.1, 131.4, 143.1, 153.3, 162.5, 196.1; HRMS calcd for $C_{23}H_{19}ClN_2O_3$ $[M+H]^+$: 408.1453. Found: 408.1483.

13-(4-Bromophenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-*b*]phthalazine-1,6,11(2H,13H)-trione (5c)

IR (KBr) in cm^{-1}: 1672, 1642 , 1622 (-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.91 (s, 3H, -CH_3), 1.34 (s, 3H, -CH_3), 2.12 (AB system, 2H, -CH_2), 2.53 (AB system, 2H, -CH_2), δ 4.50 (s, 1H, -CH), 7.08 (m, 1H, Ar-H), 7.23 (m, 3H, Ar-H), 7.86 (d, 2H, Ar-H), 8.12 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 24.2, 28.1, 30.2, 32.2, 50.2, 114.2, 123.2, 126.2, 127.3, 128.2, 131.0, 143.2, 153.2, 162.4, 196.1; HRMS calcd for $C_{23}H_{19}BrN_2O_3$ $[M+H]^+$: 451.2372. Found: 451.2343.

13-(2-Nitrophenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-*b*]phthalazine-1,6,11(2H,13H)-trione (5d)

IR (KBr) in cm^{-1}: 1674, 1652, 1632 (-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.92 (s, 3H, -CH_3), 1.24 (s, 3H, -CH_3), 2.13 (AB system, 2H, -CH_2), 2.52 (AB system, 2H, -CH_2), 4.52 (s, 1H, -CH), 7.12 (m, 1H, Ar-H), 7.23 (m, 3H, Ar-H), 7.86 (d, 2H, Ar-H), 8.24 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 24.3, 28.2, 30.2, 32.3, 50.2, 114.1, 123.2, 126.3, 127.3, 128.3, 131.1, 143.2, 153.2, 162.5, 196.2; HRMS calcd for $C_{23}H_{19}N_3O_4$ $[M+H]^+$: 418.1330. Found: 418.1360.

13-(3-Nitrophenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-*b*]phthalazine-1,6,11(2H,13H)-trione (5e)

IR (KBr) in cm^{-1}: 1672, 1662, 1651 (-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.92 (s, 3H, -CH_3), 1.23 (s, 3H, -CH_3), 2.13 (AB system, 2H, -CH_2), 2.54 (AB system, 2H, -CH_2), 4.49 (s, 1H, -CH), 7.23 (m, 1H, Ar-H), 7.45 (m, 3H, Ar-H), 7.67 (d, 2H, Ar-H), 8.17 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 24.2, 28.0, 30.1, 32.3, 50.1, 114.1, 123.3, 126.4, 127.3, 128.2, 131.0, 143.1, 153.1, 162.4, 195.9; HRMS calcd for $C_{23}H_{19}N_3O_4$ $[M+H]^+$: 418.1330. Found: 418.1372.

13-(4-Chlorophenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-*b*]phthalazine-1,6,11(2H,13H)-trione (5f)

IR (KBr) in cm^{-1}: 1670, 1660, 1653 (-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.99 (s, 3H, -CH_3), 1.24 (s, 3H, -CH_3), 2.03 (AB system, 2H, -CH_2), 2.53 (AB system, 2H, -CH_2), 4.44 (s, 1H, -CH), 7.34 (m, 1H, Ar-H), 7.41 (m, 3H, Ar-H), 7.85 (d, 2H, Ar-H), 8.03 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 24.3, 28.5, 30.1, 32.3, 50.2, 114.3, 123.3, 126.2, 127.3, 128.2, 131.0, 143.3, 153.1, 162.4, 195.8; HRMS calcd for $C_{23}H_{19}ClN_2O_3$ $[M+H]^+$: 408.1453. Found: 408.1492.

13-(4-Methylphenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-b]phthalazine-1,6,11(2H,13H)-trione (5g)

IR (KBr) in cm^{-1}: 1673, 1665, 1651 (-C=O); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 1.2 (s, 3H, -CH_3), 1.26 (s, 3H, -CH_3), 2.7 (s, 3H, -CH_3), 2.13 (AB system, 2H, -CH_2), 2.58 (AB system, 2H, -CH_2), 4.46 (s, 1H, -CH), 7.44 (m, 1H, Ar-H), 7.51 (m, 3H, Ar-H), 7.89 (d, 2H, Ar-H), 8.13 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 24.1, 28.3, 29.1, 30.3, 32.7, 50.2, 114.1, 123.0, 126.4, 127.2, 128.1, 131.1, 143.3, 153.5, 162.3, 195.1; HRMS calcd for $C_{24}H_{22}N_2O_3$ $[M+H]^+$: 386.1251. Found: 386.1223.

13-(2-Methylphenyl)-3,3-dimethyl-3,4-dihydro-1H-indazolo[1,2-b]phthalazine-1,6,11(2H,13H)-trione (5h)

IR (KBr) in cm^{-1}: 1671, 1658, 1656 (-C=O); ^{1}H-NMR (DMSO-d $_6$, 400 MHz): δ 0.98 (s, 3H, -CH_3), 1.22 (s, 3H, -CH_3), 2.70 (s, 3H, -CH_3), 2.13 (AB system, 2H, -CH_2), 2.48 (AB system, 2H, -CH_2), 4.42 (s, 1H, -CH), 7.31 (m, 1H, Ar-H), 7.44 (m, 3H, Ar-H), 7.84 (d, 2H, Ar-H), 8.13 (d, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 20.2, 28.2, 30.2, 31.2, 32.4, 51.3, 113.2, 123.1, 125.1, 127.2, 128.1, 131.3, 143.2, 153.0, 162.1, 195.2; HRMS calcd for $C_{24}H_{22}N_2O_3$ $[M+H]^+$: 386.1251. Found: 386.1223.

CONCLUSION

In summary, water-mediated one-pot, four-component synthesis of substituted 2H-indazolo[2,1-*b*]phthalazine-triones have been reported with several advantages such as good yields, short reaction time, simple procedure, mild condition and environmentally begins.

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GREEN SYNTHESIS OF 4-((UN)SUBSTITUTED BENZYLIDENE)-N-ARYLAMINO-2-((UN)SUBSTITUTED STYRYL)-1*H*-IMIDAZOLE-5(4*H*)-ONE DERIVATIVES

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Keywords: Green chemistry, phenylhydrazine, DBU, Schiff bases.

We have achieved an efficient and green synthesis of 4-(benzylidene/substituted benzylidene)-N-aryl amino-2-(styryl/substituted styryl)-1*H*-imidazole-5(4*H*)-one derivatives in good yields by using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a catalyst.

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INTRODUCTION

Heterocyclic compounds containing imidazole moiety have many pharmacological properties and play an important role in biochemical processes. Highly substituted imidazoles are the key intermediates in the synthesis of various therapeutic agents and act as a subunit in drugs such as Olmesartan, Losartan, Eprosartan (angiotensin II receptor antagonist), Metronidazole (antibiotic), Trifenagrel (platelet aggregation inhibitor), Dacarbazine (antineoplastic), Cimetidine (H2-receptor antagonist), methimazole (antithyroid), Pilocarpine (muscarinic receptor agonist), Etomidate (intravenous anesthetic) as well as plant growth regulators,[1] fluorescence labeling agents, biological imaging[2] and chromophores for non-linear optic systems. These moieties have been reported as antibacterial, anti-inflammatory, antihypertensive, antithrombotic, fungicidal,[3] anti-allergic, antiviral[4] and herbicidal properties. On the other hand, an ionic liquid catalyzed reaction have gained considerable attention because of their interesting properties like high thermal stability, non volatility, eco-friendly benign nature and reusability leading to proceed the reaction effectively with high yields in shorter reaction times.

In view of the diverse pharmacological properties of these potent compounds, many methodologies have been developed using various catalytic systems such as InF_3,[5] $InCl_3.3H_2O$,[6] $BF_3.SiO_2$,[7] $Zr(acac)_4$,[8] I_2,[9] n-tetrabutylammonium bromide (TBAB),[10] CAN,[11] DABCO,[12] $Yb(OTf)_3$,[13] *L*-proline,[14] zirconium(IV)-modified silica gel,[15] p-toluenesulfonic acid (p-TSA),[16] Recently polyethylene glycol,[17] and molecular iodine[18] were also used efficiently for this reactions. However, many of these reported methods suffer from one or several drawbacks such as low yields, prolonged reaction times, use of toxic, costly, moisture sensitive, excess quantity of reagents, harsh reaction circumstances, special apparatus, difficult workup procedure and difficulty in recovery and reusability of the catalysts. Therefore, there is still a need to build up an efficient, eco-friendly and easy method for the synthesis of imidazole derivatives

Based on the literature, this prompted us to synthesize 4-(benzylidene/substituted benzylidene)-N-arylamino-2-(styryl/substituted styryl)-1*H*-imidazole-5(4*H*)-one derivatives from Schiff bases by making use of DBU as a catalyst.

EXPERIMENTAL

Melting points are uncorrected and taken in open capillary tubes in sulphuric acid bath. TLC was run on silica gel-G and visualization was done using UV light. IR spectra were recorded using Perkin-Elmer 1000 instrument in KBr pellets. ^{1}HNMR spectra were recorded in DMSO-d_6 using TMS as internal standard with 400 MHz spectrometer. ^{13}C NMR spectra were recorded in DMSO-d_6 using TMS as internal standard with 100 MHz spectrometer. Mass spectra were recorded on Agilent-LCMS instrument under CI conditions and given by Q+1 value only.

Preparation of (*Z*)-2-acetamido-N-phenyl-3-(phenyl/substituted phenyl)prop-2-enamides (2a, 2b)

A mixture of 4-(benzylidene/substituted benzylidene)-2-methyl oxazolin-5-ones (**1a, 1b**) (10 mmol) and phenylhydrazine (10 mmol) was dissolved in ethanol and refluxed for 5 h at 80 ºC. The completion of the reaction was monitored by TLC (1:3 of EtOAc:hexane). Then this reaction mixture was cooled to room temperature and poured into ice-cold water (50 mL), separated solid product was collected, washed with water (10 mL) and dried. The product was recrystallised from ethanol to obtain (*Z*)-2-acetamido-N-phenyl-3-(phenyl/substituted phenyl)prop-2-enamides (**2a, 2b**). The formation of **2a** and **2b** from oxazolin-5-one derivatives (**1a, 1b)** has been confirmed from the spectral data.

The IR spectrum of the enamides showed peeks for NH group and C=O and absence of the peak for lactone ring. The ^{1}H NMR spectra of the enamides showed signals for

NHPH and NHCO groups. The mass spectra of the compounds exhibited molecular ion peaks (M^+) corresponding to their molecular weights.

Preparation of 4-(benzylidene/substituted benzylidene)-N-aryl amino-2-(styryl/substituted styryl)-1*H*-Imidazole-5(4*H*)-one derivatives (4 a-l)

Equimolar quantities of (Z)-2-acetamido-N-phenyl-3-(phenyl/substituted phenyl)prop-2-enamides (**2a-2b**) (10 mmol) and Schiff bases **3a** and **3b** (10 mmol) were mixed together in 20 mL of ethanol in the presence of DBU(1 mmol) as catalyst. The mixture was refluxed for 2 h. The completion of the reaction was checked by TLC (1:3 of EtOAc:hexane), then this reaction mixture was cooled to room temperature and poured into ice-cold water (50 mL). The separated solid product was collected, washed with water (10 mL) and dried. The product was recrystallized from ethanol to obtain 4-(benzylidene/substituted benzylidene)-N-aryl amino-2-(styryl/substituted styryl)-1*H*-Imidazole-5(4*H*)-one derivatives (**4a-4l**). The physical (Table 1) and spectral analysis of the compounds is given below.

Table 1. Physical data of the products **4a-4l**.

No.	Starting materials	Mol. formula of product	Yield* , %	M.P., ˚C
1	**2a**, **3a**	$C_{24}H_{19}N_3O$	68	165-166
2	**2a**, **3b**	$C_{25}H_{22}N_3O_2$	65	168-169
3	**2a**, **3c**	$C_{24}H_{18}N_3OF$	66	161-162
4	**2a**, **3d**	$C_{24}H_{18}N_4O_3$	61	168-171
5	**2a**, **3e**	$C_{24}H_{18}N_3OCl$	61	154-156
6	**2a**, **3f**	$C_{24}H_{18}N_3OCl$	65	219-221
7	**2b**, **3a**	$C_{24}H_{18}N_3OCl$	68	154-156
8	**2b**, **3b**	$C_{25}H_{21}N_3O_2Cl$	68	171-172
9	**2b**, **3c**	$C_{24}H_{18}N_3OFCl$	68	211-213
10	**2b**, **3d**	$C_{24}H_{18}N_4O_3C1$	63	211-212
11	**2b**, **3e**	$C_{24}H_{18}N_3OC1_2$	64	206-208
12	**2b**, **3f**	$C_{24}H_{18}N_3OC1_2$	69	210-212

***** Refers to yields of crude products only.

Spectral analysis of (4Z)-4-benzylidene-1-methyl-2-styryl-1*H*-imidazol-5(4*H*)-one derivatives 4a-4l)

4a: IR (KBr): 3444 (-NH), 1668 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 17H, Ar-H**,** =CH-Ar and NH**),** 8.0-8.4 (d, 2H, -CH=CH). ^{13}CNMR δ = 109.5, 109.8, 116.1, 119.3, 123.3, 123.4, 123.4, 127.3, 128.0, 128.8, 130.0, 130.3, 137.1, 137.1, 138.1, 138.6, 141.1, 141.3, 167.1. MS: M+H = 366.

4b: IR (KBr): 3436 (-NH), 1674 (-C=O) cm^{-1}. ^{1}HNMR δ = 4.2 (s, 3H, -CH_3), 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 109.4, 109.5, 115.3, 114.2, 123.3, 123.4, 123.5, 126.2, 127.0, 128.4, 130.2, 130.4, 137.2, 137.4, 138.2, 138.5, 141.0, 141.3, 165.2. MS: M+H = 396.

4c: IR (KBr): 3432 (-NH), 1664 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH**),** 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 110.3, 110.8, 114.3, 118.2, 122.2, 123.2, 123.5, 125.2, 127.2, 127.2, 130.4, 130.9, 137.2, 137.6, 138.4, 138.6, 141.8, 141.9, 165.8. MS: M+H = 385.

4d: IR (KBr): 3443 (-NH), 1674 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH**),** 8.0-8.4, (d, 2H, -CH=CH). ^{13}C- NMR δ = 108.6, 109.6, 115.4, 116.8, 122.1, 123.2, 123.7, 124.6, 125.3, 128.3, 130.4, 130.7, 137.7, 137.8, 138.3, 138.5, 141.4, 141.5, 166.7. MS: M+H = 412.

4e: IR (KBr): 3433 (-NH), 1662 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ =109.7, 109.8, 116.3, 119.4, 123.2, 123.4, 123.5, 127.6, 128.2, 128.4, 130.2, 130.5, 137.6, 137.8, 138.1, 138.2, 141.4, 141.8, 166.3. MS: M+H = 401.

4f: IR (KBr): 3434 (-NH), 1653 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 109.5, 109.8, 116.1, 119.3, 123.3, 123.4, 123.4, 127.3, 128.0, 128.8, 130.0, 130.3, 137.1, 137.1, 138.1, 138.6, 141.1, 141.3, 167.1. MS: M+H = 400.

4g: IR (KBr): 3442 (-NH), 1663 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ =109.4, 109.6, 114.5, 119.6, 120.4, 123.3, 123.3, 127.5, 128.3, 128.8, 130.4, 130.6, 137.4, 137.5, 138.1, 138.6, 141.2, 141.3, 167.5. MS: M+H = 401.

4h: IR (KBr): 3425 (-NH), 1664 (-C=O) cm^{-1}. ^{1}HNMR δ = 4.3 (s, 3H, -CH_3), 7.4-8.0 (m, 16H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 109.7, 109.9, 116.1, 118.3, 121.9, 122.4, 123.4, 127.2, 128.2, 128.7, 130.2, 130.3, 137.1, 137.2, 138.1, 138.8, 141.1, 141.3, 167.2. MS: M+H = 432.

4i: IR (KBr): 3434 (-NH), 1664 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 16H, Ar-**H,** =CH-Ar and NH), 8.0-8.4,(d, 2H, -CH=CH). ^{13}CNMR δ = 108.6, 109.7, 116.4, 118.5, 123.5, 123.6, 123.3, 126.3, 128.3, 128.8, 130.2, 130.3, 137.1, 137.8, 138.1, 138.2, 141.1, 141.4, 167.4. MS: M+H = 420.

4j: IR (KBr): 3428 (-NH), 1650 (-C=O) cm^{-1}. ^{1}HNMR δ = 7.4-8.0 (m, 17H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 109.3, 109.5, 115.1, 118.3, 121.3, 122.4, 123.5, 126.3, 127.0, 128.3, 130.2, 130.3, 135.1, 136.2, 138.2, 138.5, 141.1, 141.2, 166.8. MS: M+H = 447.

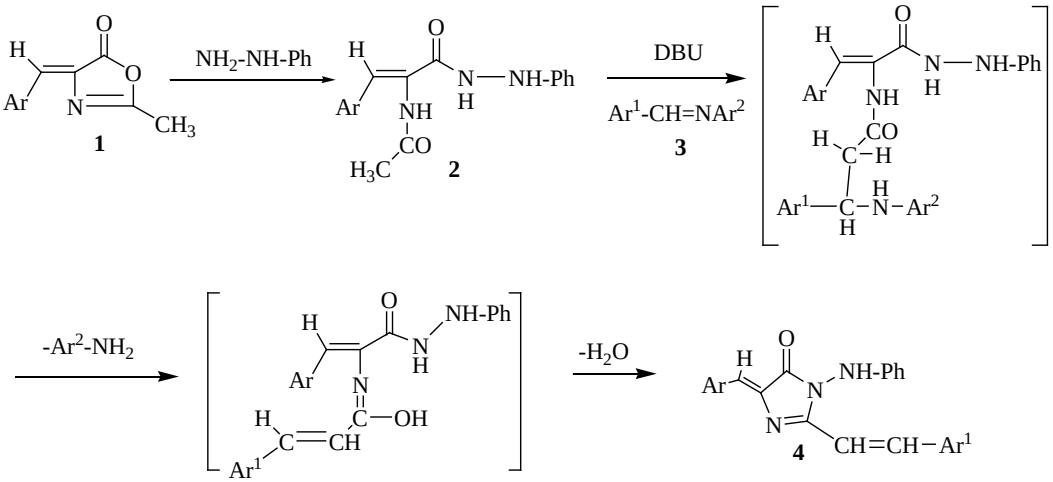
4k: IR (KBr): 3440 (-NH), 1662 (-C=O) cm^{-1}, ^{1}HNMR δ = 7.4-8.0 (m, 17H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 109.3, 109.6, 115.3, 116.3, 121.2, 122.2, 123.2, 124.5, 126.4, 127.6, 130.1, 130.3, 137.2, 138.2, 138.7, 138.9, 141.7, 141.8, 167.6. MS: M+H = 437.

4l: IR (KBr): 3430 (-NH), 1665 (-C=O) cm^{-1}, ^{1}HNMR δ = 7.4-8.0 (m, 17H, Ar-H, =CH-Ar and NH), 8.0-8.4, (d, 2H, -CH=CH). ^{13}CNMR δ = 109.2, 109.6, 116.5, 119.3, 123.2, 123.6, 123.8, 127.3, 128.3, 128.5, 130.3, 130.3, 137.5, 137.7, 138.1, 138.8, 141.2, 141.3, 167.3. MS: M+H = 437.

RESULTS AND DISCUSSION

As illustrated in scheme 1, the azalactone (*Z*)-4-benzylidene-2-methyloxazol-5(4*H*)-one **1(a-b)** were treated with phenylhydrazine and refluxed for 4-4.5 h in ethanol to produce (*Z*)-2-acetamido-N-phenyl-3-(phenyl/substituted phenyl)prop-2-enamides (**2a,2b)**. Then, **2a** and **2b** were reacted with the Schiff bases (benzylidine/substituted benzylidine)anilines (**3a-3f)** in the presence of DBU as a

catalyst in ethanol medium under reflux condition for 1-1.5 h to produce 4-(benzylidene/substituted benzylidene)-N-aryl amino-2-(styryl/substituted styryl)-1H-Imidazole-5(4H)-one derivatives (**4a-4l**)**.** A reasonable mechanism has been formulated for the formation of these imidazoline-5-ones (**4a-4l**). The IR spectrum of the compound showed absorption bands for NH, C=O, Ar, C=C, C-N and the ^{1}H NMR showed the signals for aromatic, two distinct signals for olefinic protons and signals for amide protons. ^{13}C NMR showed signals for -C=C, (-C=C) Ar, (C-N), (-C=N) and (C=O). The mass spectrum of the compound **4a-4l** showed the molecular ion peaks corresponding to molecular weight of the compounds. The spectral data confirms the structure of **4a-4l**.



Scheme 1. Synthesis of 4-(benzylidene/substituted benzylidene)-N-aryl amino-2-(styryl/substituted styryl)-1H-Imidazole-5(4H)-one derivatives.

The IR spectra of **1a** and **1b** showed the presence of NH-stretching absorptions for NH and absence of stretching absorptions of lactone ring. The ^{1}H NMR data showed signals for NH, which are D_2O exchangeable and mass spectra which confirms the molecular weight of the compounds.

The cyclocondensation of **1a** and **1b** with Schiff bases to produce (4Z)-4-(benzylidene/ substituted benzylidene-N-aryl amino-2-(styryl/substituted styryl)-1*H*-imidazol-5(4*H*)-one derivatives (**4a-4l**) is supported by IR spectra showing the absence of N-H stretching absorptions of the amide group. The ^{1}HNMR spectra showed the disappearance of peaks for NH and appearance of peaks for 2-styryl protons. ^{13}CNMR spectra of the compound **3a-3l** shows signals for the presence of C=C (C=C)Ar,C-N,C=N and C=O. Finally the mass spectrum of the compounds **4a-4l** confirmed the molecular weight of the compounds there by supporting the structure of the title compound (4Z)-4-(benzylidene/ substituted benzylidene-N-aryl amino-2-(styryl/substituted styryl)-1*H*-imidazol-5(4*H*)-one derivatives (**4a-4l**).

CONCLUSION

One pot green synthesis for the preparation of imidazolone derivatives (**4a-4l**) in high purity and excellent yields has been developed by making use of DBU as a catalyst.

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seco-acyclo-N- AND *-S-* NUCLEOSIDE ANALOGUES FROM CINNAMIC ACID: SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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Keywords: 1,3,4-Oxadiazole-5-thione; 1,2,4-triazole-5-thiol; 1,2,4-4*H* aminotriazole-5-thiol; cinnamic acid; synthesis; antibacterial activity, nucleoside analogues

Three seco-acyclo-*N*- and -*S*- nucleosides analogues, namely 2-phenyl-1,3-dioxan-5-yl 5-[(*E*)-2-phenylethenyl]-1,3,4-oxadiazole-2-sulfenate, 4-[(2-phenyl-1,3-dioxan-5-yl)amino]-5-[(*E*)-2-phenylethenyl]-4*H*-1,2,4-triazole-3-thiol and 2-[(2-phenyl-1,3-dioxan-5-yl)sulfanyl]-5-[(*E*)-2-phenylethenyl]-1,3,4-thiadiazole have been synthesized from cinnamic acid via a common synthetic pathway. Treatment of (2*E*)-3-phenylprop-2-enehydrazide with CS_2 under different conditions led to oxadiazole and thiadiazole derivatives on oxadiazole on treatment with hydrazine hydrate gave N-aminotriazole derivative. These diazolethiols gave the nucleoside analogues when treatment with freshly prepared 2-phenyl-1,3-dioxan-5-yl 4-methylbenzenesulfonate. All reaction intermediates and final products where characterized by IR, ^{1}H- and ^{13}C NMR spectroscopy**.** The antibacterial activities assessed against Gram-positive bacteria, *Staphylococcus aureus*, *Bacillus* c*ereus*, and Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. Some of the synthetic compounds showed promising activity against microorganisms under test in comparison to commercially available antibiotics Gentamycin.

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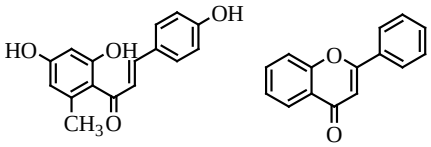
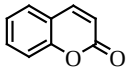
INTRODUCTION

Cis- and trans-cinnamic acid moiety is present in some important phenylpropanoid natural products such as lignins, naringenine chalcone (**A**), flavone (**B**) and coumarine (**C**).[1]



A

B



C

Figure 1. Chemical structures of some phenylpropanoids (cinnamic acid) natural products.

Cinnamic acid derivatives have been utilized in a wide range of biological activities such as anticancer,[2,3] antioxidant,[3,4] anti-inflammatory,[5] antibacterial,[1,4,6] antifungal,[7-9] antiviral,[10] dietary[11] and as a fragrance ingredient in many fragrance formulations.[12]

Cinnamic acid has the tendency to form metallic complexes[13] and various derivatives concerning their phenyl group and side chain moieties, those have shown pharmaceutical activity.[14] The natural piperidine derivative of cinnamic acid showed anticonvulsive effect.[15]

To our knowledge, there are only few cases where carboxylic group have been modified to heterocycles. The synthesis of N-(3-aryl-1,2,4-triazo-5-yl)cinnamide derivative and 2-[(*E*)-2-phenyethenyl]-4,5-dihydro-1,3-oxazole derivatives have been reported.[16,17] In this work we report first, the conversion of some modified cinnamic acid to 1,3,4-oxadiazolethiol, 1,3,4-thiadiazolethiol and N-amino-1,2,4-triazolethiol derivatives. The second stage of work concerns with the synthesis of sugar analogue molecule followed by the third stage to form seco-acyclo nucleoside analogues. All the compounds including starting material, intermediates and final nucleoside analogues were tested for biological activity.

EXPERIMENTAL

All reactions were monitored by TLC analysis (silica gel for TLC supplied by MERCK), iodine was used for visualization. The melting points were measured with a BÜCHI 540 melting point apparatus and are uncorrected. The IR spectra (ν cm^{-1}) were recorded using KBr discs in a JASCO V-530 spectrophotometer and a Bruker-Alpha (Platinium-ATR). The ^{1}H NMR and ^{13}C NMR were recorded on a Bruker AC 300 MHz spectrometer in DMSO-d_6 and expressed as δ (ppm) with reference to TMS.

Microorganisms in this study were supplied by the University Hospital of Oran and identified in our laboratory. The Mueller Hinton medium was supplied by Difco.

Syntheses

Methyl cinnamate (2)

The cinnamic acid **1** (1g, 6.75 mmol) and methanol (30 mL) in presence of concentrated sulfuric acid (1 mL) were refluxed for 6 h at 80 °C. The excess methanol was distilled out and the contents were cooled to a room temperature. The reaction mixture was diluted with 50 mL water and extracted by dichloromethane. The product was isolated in 90 % yield, m.p. 37 °C. The compound **2** was characterized by IR spectrum which showed absorption at 1717.3, 1278.57 and 1574.59 cm^{-1} for (C=O), (C-O) and (C=C) respectively.

(2E)-3-phenylprop-2-ene hydrazide(3)

The methyl cinnamate **2** (1 g, 6.17 mol) is dissolved in 30 mL methanol and hydrazine hydrate 99 % (5 mL) was added to it. The solution was refluxed for 10 h. After evaporating the solvent under reduced pressure, a solid appeared. This was recrystallized form ethanol to afford compound **3**. The product was isolated in 65 % yield, m.p. 98 °C. Its IR spectrum exhibited bands at 3293.82 and 3229.62 cm^{-1} for (NH) and (NH_2) respectively.

5-[(*E*)-2-Phenylethenyl]-1,3,4-oxadiazole-2-thione/thiol (4)

A mixture of compound **3 (**4.9 mmol in 20 mL ethanol) and 17.8 mmol of KOH in 10 mL ethanol was agitated continuously for a period of 30 min. To this, carbon disulfide (3 mL) was added dropwise. The mixture was refluxed for 14 h. The reaction mixture was then acidified with HCl (37 %) to pH 5 to give a yellow precipitate, which was filtered off, washed with ethyl acetate and recrystallized from ethanol. The product was isolated in 57 % yield, m.p. 158 °C. The IR spectrum showed the characteristic bands at 3423.03, 1622.8, 1051.98 and 1402.63 1687.41 cm^{-1} for (NH), (C=N), (C-O-C) and (C=S) respectively. ^{1}H NMR δ = 4.26, 4.97 (s, 2H, HC=CH), 7.45(s, 1H, NH), 8.46, 8.48, 8.52, 8.54, 8.56 (m, 5H, H_{arom}). ^{13}C NMR, δ = 111.4, 164.5 (C=C), 129.1, 129.2, 129.3, 129.5, 129.7, 140 (C_{arom}), 165.7 (N=CO), 179.6 (NC_2=S).

4-Amino-5-[(*E*)-2-phenylethenyl]-4*H*-1,2,4-triazole-3-thiol (5)

To a solution of 3.9 mmol of compound **4** in 20 mL of ethanol, 4 mL of hydrazine hydrate 99 % was added and the reaction mixture was refluxed for 14 h. After cooling, the mixture was filtered and washed by ethyl acetate. The product was isolated in 74 % yield, m.p. 215 °C. The IR spectrum showed absorption at 1637.27, 3242.72 and 3143.4 cm^{-1} for (C=N), (NH_2) and (NH) respectively. ^{1}H NMR δ = 2.98 (s, 2H, NH2), 5.16, 5.32 (s, 2H, HC=CH), 6.83, 6.84, 6.85, 6.87 (m, 5H, H_{arom}), 13.04(s, H, SH). ^{13}C NMR δ = 128.10, 128.31, 128.34, 140.3, (6C_{arom}), 126.16, 125.75 (2C, C=C), 151.4 (C1, N=CN), 165.8 (N=CSH).

5-[(*E*)-2-Phenylethenyl]-1,3,4-thiadiazole-2(3*H*)-thione/thiol (6)

KOH (5.8 mmol) was dissolved in ethanol (5 mL) and carbon disulfide (1.5 mL) was added drop-wise with stirring at 0 °C during 40 min. To this, 3 mmol of compound **3** in 20 mL of ethanol was added. After that, the mixture was refluxed for 10 h. After cooling the reaction mixture was acidified with H_2SO_4 to pH 2 to give a brown precipitate, which was filtered off, washed with ethyl acetate and recrystallized from ethanol. Yield 70 %. The IR spectrum showed the characteristic bands at 1620.88 and 649.89 cm-1 for (C=N) and (C-S-C). ^{1}H NMR δ = 5.91, 5.93 (s, 2H, C=C), 6.51, 6.54, 6.58, 6.73, 6.77 (m, 5H, H_{arom}), 13.66 (s, H, SH). ^{13}C NMR δ = 126.62, 1267.71 (2C, C=C), 128.51, 128.64, 128.76, 128.85, 129.09, 139.66 (6C_{arom}), 163.72 (N=CS), 177.9 (C-SH).

2-Phenyl-1,3-dioxan-5-ol (9)

A mixture of solution of glycerol (97 mmol) in chloroform (15 mL) and a solution of benzaldehyde (2 mol) in chloroform (10 mL) in presence of *p*-toluenesulfonic acid was refluxed for 6 h with magnetic stirring at 50-60 °C. After cooling to room temperature the product was extracted with dichloromethane and washed with $NaHCO_3$, then dried over $MgSO_4$ and filtered. Yield 50 %. The IR spectrum showed the characteristic bands at 3311.18, 2977.55 and 1600 cm^{-1} for (OH), ($CH_{aromatic}$) and (C =C) respectively.

2-Phenyl-1,3-dioxan-5-yl 4-methylbenzenesulfonate (10)

The compound **9** (22 mmol) was added to tosyl chloride (18 mmol) in the presence of pyridine. The mixture was refluxed for 6 h with magnetic stirring at 80 °C. After cooling to room temperature the product was extracted with dichloromethane and washed with $NaHCO_3$, then dried over $MgSO_4$ and filtered. Yield 60 %. The IR spectrum showed absorption at 1654.96 and 1080.62 cm^{-1} for (C=C) and (C-O-C).

Seco-acyclo glycosides and nucleosides

Treatment of compounds **4, 5, 6** with the equivalent amount of compound **10** in DMF (4 mL), in presence of dimethylamine (DEA) (2 mL), under reflux for 48 h and extraction with dichloromethane led to the formation of the desired compounds **11, 12** and **13.**

2-Phenyl-1,3-dioxan-5-yl-5-[(*E*)-2-phenylethenyl]-1,3,4-oxadiazole-2-sulfenate (11)

Yield 35 %. IR (KBr**):** 1656.27 (C=N, Ph), 1062.47 (C-S-C) cm^{-1}. ^{1}H NMR δ = 1.14 (m, H, H-CS), 2.43, 2.7, 3.04, 3.23 (d, 4H, 2CH_2), 3.83(s, H, H-CO_2), 6.44-6.49 (m, 2H, HC=CH), 7.29-8.19 (m, 10H, H_{arom}). ^{13}C NMR δ = 37(C, H-C-S), 41.99 (2C, CH_2), 101(C, Ph-C-H), 125.8-126.1 (2C, C=C); 127-142 (10C, $C_{aromatic}$),165(C, N=C-S), 170(C, N=C-C).

4-[(2-Phenyl-1,3-dioxan-5-yl)amino]-5-[(*E*)-2-phenylethenyl]-4*H*-1,2,4-triazole-3-thiol (12)

Yield 35 %. IR (KBr): 1656,27 (C=N, Ph) cm^{-1}. ^{1}H NMR δ = 2.18 (m, H, H-CNH), 2.86, 2.96 (d, 4H, 2CH_2), 3.4 (s, H, H-CO_2), 3.92 (s, H, H-NC), 5.55, 8.3 (d, 2H, HC=CH), 7.35-7.98 (m, 10H, H_{arom}), 10.55 (s, H, NH). ^{13}C NMR δ = 36.6(C, H-C-NH), 49,9 (2C, CH_2), 101(C, Ph-C-H), 127-

134.4 (10C,$C_{aromatic}$), 126.1, 143.9(2C, C=C); 162.9 (C, N=C-C), 173.7(C, N-C=S).

2-[(2-Phenyl-1,3-dioxan-5-yl)sulfanyl]-5-[(*E*)-2-phenylethenyl]-1,3,4-thiadiazole (13)

Yield 40 %. IR (KBr): 1631.19 (C=N, Ph), 1096.68 (C-S-C) cm^{-1}. ^{1}H NMR δ = 1.21 (m, H, H-CS); 2.93-3.48 (d, 4H, $2CH_2$), 4.40, 4.42 (m, 2H, HC=CH), 5.30 (s, H, H-CO_2), 7.20-8.05 (m, 10H, H_{arom}). ^{13}C NMR δ = 38.2 (C, H-C-S), 43.3-45.5 (2C, CH_2), 106 (C, Ph-C-H); 126.1, 129 (2C, C=C), 126.4-141.4 (10C, $C_{aromatic}$), 155.8(C, N=C-S), 172.3 (C, S-C-S).

Antibacterial evaluation

Antibacterial activity was determined by diffusion method on two strains of bacteria. The tests were carried out on Gram negative bacteria *Escherichia coli (*ATCC- 25924) and *Pseudomonas aeruginosa (*ATCC-27853), and Gram positive bacteria *Staphylococcus aureus (*ATCC-25923) sensitive and *Staphyloccocus aureus* resistant strains.

Table 1. Antibacterial activity of compounds **1-6** and **11-13**.

Comp-ounds	Gram positive, inhibition										GramnNegative, inhibition zone									
	S. aureus					*B. cereus*					*E. coli*					*P. aeruginosa*				
	a	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**
1	8	7	0	-	-	10	5	0	-	-	0	-	-	-	-	0	-	-	-	-
2	10	7	6	0	-	8	7	6	0	-	0	-	-	-	-	0	-	-	-	-
3	12	7	0	-	-	14	10	0	-	-	0	-	-	-	-	8	0	-	-	-
4	0	-	-	-	-	9	8	0	-	-	0	-	-	-	-	0	-	-	-	-
5	0	-	-	-	-	12	10	8	0	-	0	-	-	-	-	19	7	0	-	-
6	8	7	0	-	-	16	14	13	10	8	0	-	-	-	-	14	7	0	-	-
11	16	12	8	0	-	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
12	18	7	0	-	-	8	0	-	-	-	0	-	-	-	-	0	-	-	-	-
13	0	-	-	-	-	7	5	0	-	-	0	-	-	-	-	0	-	-	-	-
†	24	-	-	-	-	20	-	-	-	-	20	-	-	-	-	19	-	-	-	-
DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a, b, c, d, e: concentrations:(10, 10/2, 10/4, 10/8, 10/16) mg mL^{-1}, respectively; † Gentamycine; Key to the inhibition zones activities: Highly active = Inhibition zone > 15 mm, Moderately active = Inhibition zone 15-10 mm, Slightly active = Inhibition zone 9-6 mm, Inactive = Inhibition zone < 6 mm., O = not active, - = not tested.

The synthesized compounds were dissolved in DMSO to obtain a concentration of 30 mg mL^{-1}. A standard inoculum (10^5-10^7 c.f.u. mL^{-1}, 0.5 McFarland standards) was introduced on to the surface of sterile agar plates and a sterile glass spreader was used for even distribution of the inoculum. The discs measuring 5 mm in diameter were prepared and sterilized by dry heat at 140°C for 1 h. The sterile discs previously soaked in known concentrations of the test compounds were placed in Mueller Hinton agar medium. The inhibition zones were measured in mm at the end of an incubation period of 24 h at 37 °C and compared with the positive control (Gentamycin) and negative control DMSO, (Table 1). Compounds that showed good antibacterial activity tested by the diffusion method were further tested by dilution, when different concentrations of the tested compounds were prepared (15, 7.50, 3.75 and 1.80 mg mL^{-1} from 30 mg mL^{-1} in DMSO). After the incubation for 24 h, the last plate with no growth of microorganisms was taken to represent minimum inhibitory concentration (MIC) expressed in mg mL^{-1}. (Table 1).

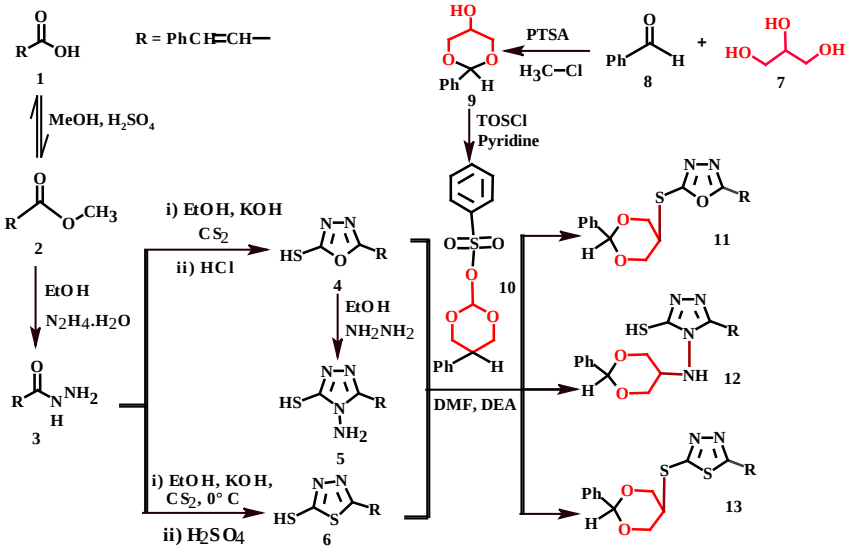
RESULTS AND DISCUSSION

Synthesis of diazole thiols **4-6,** and seco-acyclo N- and S-nucleosides analogues **11-13**, from cinnamic acid has been accompalished by a common synthetic pathway as described in Scheme 1 divided into three stages. All steps were monitored by TLC and products characterized were spectrally by IR, ^{1}H- and ^{13}C-NMR.

The key intermediate hydrazide **3** was obtained by classical procedures.[18,19] When **3** was refluxed with alcoholic solution of KOH and CS_2 for 14 h and finally treated by HCl, it gave the oxadiazole **4,** which on treatment with hydrazine hydrate yielded the N-amino triazole **5**.[20] On the other hand, when **3** was refluxed with alcoholic solution of KOH and CS_2 for 10 h and finally acidified by H_2SO_4 thiadiazole **6** was obtained.[21]

Synthesis of 2-phenyl-1,3-dioxan-5-yl 4-methylbenzenesulfonate **(10)**: Glycerol as a mimic for acyclic sugar molecule may be selectively protected to leave the desired OH group for further reactions. Glycerol was reacted with bezaldehyde to give a moderate yield of 2-phenyl-1,3-dioxan-5-ol **(9)**.[21] Treating **9** with *p*-toluene sulphonyl chloride yielded p-toluenesulphonate **10**. IR spectrum showed a reduction of OH absorption with the appearance of S=O absorption at 1373 cm^{-1}.

Synthesis of seco-acyclo glycosides **11, 12, 13**: Refluxing compounds **4, 5, 6** with an equivalent amount of **10** in DMF, in presence of dimethylamine, for 48 h led to the formation of the desired compounds **11, 12** and **13.**[21]



Scheme 1 : Summary of synthesis; *stage 1(left)* : synthesis of **4,5,6** ; *stage 2(middle)* : synthesis of tosylate **10** and *stage 3(right)* : synthesis of seco-acyclo glycosides **11, 12, 13**.

Antibacterial activity

Starting compound cinnamic acid **1**, synthetic intermediates **2-6** and final compounds **11-13** were assayed in vitro using the paper disk diffusion method for their antibacterial activities against Gram-positive bacteria, *S. aureus*, and *B. cereus*, and Gram-negative bacteria, *E. coli* and *P. aeruginosa*. In primary screening a concentration of 10 μg. mL^{-1} was maintained. Only compounds found active in this primary screening were further tested in a second set of concentration 5 μg mL^{-1} and lower against susceptible microorganisms as shown in Table 1.

Data in Table 1 showed that compounds under consideration (**1-6** and **11-13**) exhibited their effect mostly against Gram-positive bacteria *S. aureus*, *B. cereus*, and to less extent against Gram-negative *P. aeruginosa*, but non against *E. coli*. Gram-positive *S. aureus* is affected by compounds **1, 2, 3, 6, 11, 12** and **13**. The significant effect was exerted by seco-acyclo oxadiazole **11** and seco-acyclo amino triazole **12** and to less extent by **2** and **3**, whereas *B. cereus* was mostly affected by **6** and to a less extent by **1, 3** and **5.** Gram negative *E. coli* was affected by none of the compounds under consideration**.** *P. aeruginosa* was highly affected by heterocyclic clycon amino triazole **5** and to a lesser extent by **6**.

CONCLUSION

The three nucleobases: 5-[(*E*)-2-phenylethenyl]-1,3,4-oxadiazole-2-thione/thiol (**4**), 4-amino-5-[(*E*)-2-phenylethenyl]-4H-1,2,4-triazole-3-thiol (**5**) and 5-[(*E*)-2-phenylethenyl]-1,3,4-thiadiazole-2(3H)-thione/thiol (**6**) were successfully prepared from cinnamic acid and structures were confirmed by IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy. The seco-acyclo nucleoside analogues **11**, **12** and **13** were synthesized by two steps. First step concerned with the synthesis of 2-phenyl-1,3-dioxan-5-yl-4-methylbenzene-sulfonate **(10).** The second step including treatment of the latter with the nucleobases **4**, **5** and **6**. The substitution reactions were monitored by TLC and structure of the products were determined spectrally.

Biological evaluation between nucleobases **4**, **5** and **6** themselves and with their corresponding seco-acyclo nucleoside analogues revealed the following:

The 1,3,4-oxadiazole **4** exerted less biological effect upon both G(+) and G(-) bacteria under consideration. When 4-amino-1,2,4-triazole showed more antibacterial effect upon G(+) *b.cereus* and G(-) *p. aeruginosa***.** While the thiadiazole **6** showed a wider spectrum effect upon G(+) *S. aureus*, *B. cereus* and G(-) *P. aeruginosa.*

The nucleobases **4**, **5** and **6**, generally showed an more appreciable effect than their corresponding nucleosides analogues **11**, **12** and **13** did. Relatively, nucleosides **11** and **12** showed better biological effect than **13** as shown on G(+) *S. aureus* while **13** showed no antibacterial effect upon all bacteria under consideration.

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