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One pot Facile Synthesis of Anti-microbial *β*-Lactam Derivatives Catalysed by $Fe(acac)_3$

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ABSTRACT

We have developed novel route for the synthesis of *β*-Lactam by $Fe(acac)_3$ based catalytic system with >90% yield. It is one pot Knoevenagel condensation of aldehyde with ethyl cyanoacetate followed by chemoselective reduction of the nitrile substituted C=C bond and nitrile functional group with sodium borohydride. Anti-microbial activity of the compounds exhibited at lower concentration against gram positive, gram negative bacteria and yeast with minimum inhibitory coIncentration (MIC) as low as 16 µg/mL. Compound have low hemolytic activity at their respective MIC. These derivatives might be liable to be increase the permeability of the bacterial cell membrane, which was demonstrated by DAPI, FITC, PI uptake studies and atomic force microscopy imaging of the treated cells. One pot synthesis with anti-microbial activity of *β*-Lactam derivatives can be helpful to developed effective antibacterial agents.

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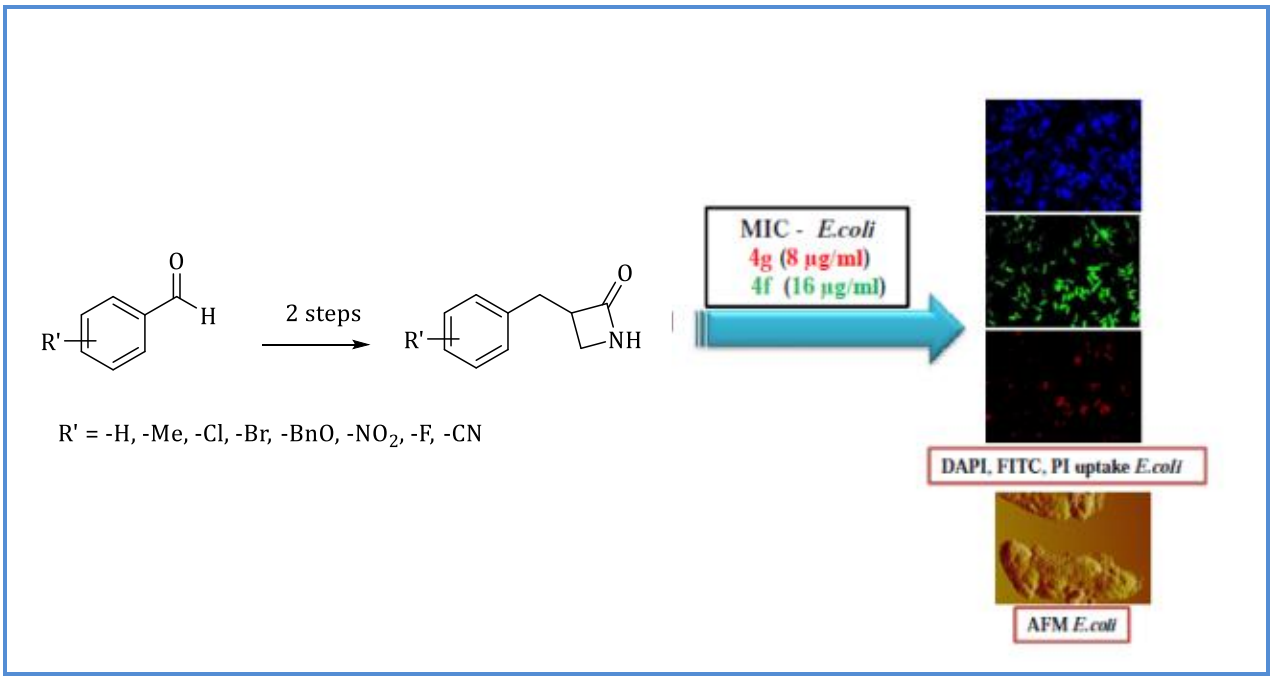
Graphical Abstract



Introduction

Knoevenagel condensation is one of the most useful synthetic strategy for the formation of C=C bond; It has been widely applicable for building desired molecules used as pesticide, in Pharmaceuticals, perfume and food industries [1-4]. It is primarily involve the basic weak amine, which has been used to carry out Knoevenagel condensation reaction of aldehyde with active methylene compound. Subsequently, several other bases and their salts have been developed including piperidine [5, 6], morpholine, DBU [7], DABCO [8], pyridine [9], DMAP, PPh_3 [10], LiBr [11], L-proline [12], *β*-alanine [13], ammonium acetate [14], ethylenediammonium acetate [15], and TEBA [16].

Furthermore, Lewis acid based catalysts such as $ZnCl_2$, $TiCl_4$ [17], CdI_2, $LaCl_3$ [18], $CeCl_3$, $7H_2O$-NaI [19] were also reported to carry out Knoevenagel condensations. The discovery of heterogeneous form catalysts like hydrotalcite [20], nanocrystalline MgO [21], MgO/ZrO_2 [22], methyl ammonium FAU-zeolite [23], $AlPO_4/Al_2O_3$ [24], Ni-SiO_2 [25], MgO/ZnO [26], poly (vinyl chloride) supported tetraethylene pentamine have shown to be better than the homogeneous catalytic system [27]. For the improvement in the Knoevenagel condensation has been realized looking at development of the ecofriendly routes. Herein, condensation shown too facilitated by aqueous medium [28, 29], ionic liquid [bmim] OH [30], microwave [31] and ultrasonic irradiation [32] respectively.

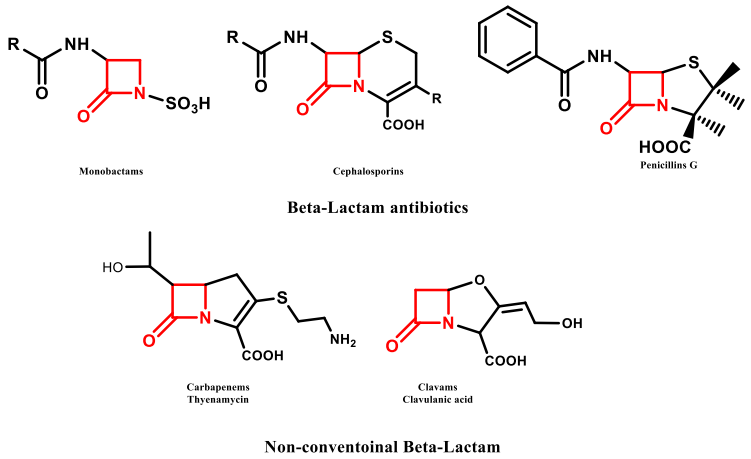


Figure 1. Structures of classical *β*-Lactam anti-biotics and non-conventional lactams

Knoevenagel condensation followed by one pot multiple functional group transromtions leads to a variety of useful products which are the most interesting aspect and have also been reported in the literature. Wherein, piperidine acetic acid, thylenediammonium diacetate [33], Pd-hydrotalcite [34], L-proline [35], glycine-K_2CO_3, Ru-catalystand water have been used for the condensation. Whereas molecular hydrogen, hantzsch pyridine, $NaBH_4$, NaTe hexcess of aldehyde with Ru-catalyst, ortho-phenylenediamine, BH_3-amine have been used for the reductions, several anti-microbial drugs have active core of *β*-lactone this class of drug known as beta-lactam antibiotics [36-39] e.g., monobatams [40], cephalosporins [41, 42], carbapenems [43, 44] and penicillin [45, 46] these type of antibiotic possesses, a four membered lactam ring attached to various functionalities.

Penicillin G is active against *Penicillium chrysogenum* [47] and cephalosporins types of antibiotics has six membered unsaturated ring attached to lactam, cephalosporins-C is active against *Streptomyces clavuligerus* [41, 42, 48, 49]. While monobactam type of antibiotics work only against aerobic gram negative bacteria e.g., *Neisseria*, *Pseudomonas* [50-52]. The nonconventional *β*-lactams contain a lactam ring with distinct bicyclic structure having oxygen as a heteroatom rather than sulphur [53].

Human cells don't have cell wall as bacterial cell possess it, hence the bacterial cell wall is one of the best targets for the antibiotics synthesis [54, 55]. Mechanism of these types of drugs were well studied which revealed the involvement of this type of antibiotics on bacterial cell wall synthesis [54, 55].

Herein we describe synthesis *β*-lactone functional derivatives by one pot synthetic approach. We tested these derivatives for their antibacterial and hemolytic activity. Further, we investigated sight of a compound action by using FITC assay and AFM imaging techniques.

Experimental

Chemical, reagents and biological materials

Reactions were carried out in dry solvents. Ingredients for media preparation were purchased from HiMedia, Mumbai. Propidium iodide (PI) and fluorescein isothiocyanate (FITC) were purchased from Sigma–Aldrich (USA). These test microorganisms were obtained from national collection of industrial microorganism (NCIM) Pune. Anti-microbial activities were assessed using gram positive *M. Luteus*, *S. aureus;* gram negative *P. fluorescence*, *E. coli*; bacteria and yeast *Candida albicans*.

Chromatographic procedures

Thin-layer chromatography (TLC) performed to monitored reaction progress on silica gel plates (Kieselgel 60 F_{254}, Merck). Migration of the compounds on TLC visualized in UV-light or by spraying it with a solution of in 2, 4-dinitrophenylhydrazine or 3.2% anisaldehyde, 2.8% H_2SO_4, 2% acetic acid in ethanol followed by heating for 1-2 min. Technical grade solvents were distilled before use for column chromatography. Silica gel (230-400 mesh) used for purification of compounds by column chromatography and ethyl acetate/petroleum ether gradient mixture as the eluent.

Spectral studies

^{1}H, ^{13}C, DEPT-135 NMR data were recorded on varian mercury (200-300 MHz). Chemical shifts are expressed in ppm values and tetramethylsilane (0.00 ppm) signal and CDCl3 (77.0 ppm) were designated as the internal standard. Chemical shifts are expressed in parts per million (ppm). IR spectra were recorded with the Shimadzu FTIR-8400 by loading samples as thin films on KBr plate, neat or in CH_2Cl_2. Mass spectra were collected on LC-MS/MS-TOF spectrometer and high resolution mass spectrometry (HRMS) data were collected on thermo scientific Q exactive quadrupole-orbitrap mass spectrometer (thermo scientific, pittsburgh, PA; Q exactive).

Fluorescent microscopy

Fluorescent microscopy performed on microscope (zeiss axiovert) was equipped with an AxioCam camera oil-immersion objective (64x) and images were processed with axiovision 4.7 software. The AFM images were acquired using a JPK Nano Wizard (Berlin, Germany) mounted on a zeiss axiovert 200 inverted microscope. Measurements were carried out in intermittent contact (IC) mode using tap 300 E-G (Cr/Pt conductive coating) cantilevers from silicon AFM probes (spring constant, k = 40 N/m, resonance frequency around 300 kHz, radius <10 nm). Height and size information were acquired using JPK data processing software.

Experimental Section

General reaction procedure

In a typical reaction, a mixture of aldehyde **1** (**a-h**) (500 mg, 4.7 mmol), $Fe(acac)_3$ (1 mol%) in DMSO (2 mL) were stirred at room temperature. Ethyl cyano acetate (4.7 mmol) was then added into the reaction mixture and stirred for 1 h. The reaction was monitored by TLC to see the complete consummation of aldehyde. It was then reduced in situ to saturated product **3** (**a-h**) by addition of 2 mL ethanol and cooled the reaction flask to 0 °C followed by addition of the 2.82 mmol of $NaBH_4$. In last step excess use of $NaBH_4$ (4.0 eq.) gave *β*-lactam derivatives reaction monitored by TLC. Reaction product **4** (**a-h**) was isolated by addition of the 10 mL saturated solution of NH_4Cl to reaction flask and extracted with (3×10 mL) of ethyl acetate. Combined organic layer was then washed with brine water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure on rota-evaporator to yield gummy crude **4** (**a-h**)**.** It was then directly subjected to flash column chromatography and yields of the pure products are given in the Table 1.

Anti-microbial activity

Broth micro dilution method used for anti-microbial activity in a 96-well microtitre plate [56]. Test compound with ten serial 2-fold dilutions of MHB media (a stock solution of 2.56 mg/mL compound in MHB in DMSO used for dilution) were carried out to obtain a concentration range of 256-0.5 µg/mL, in a sterile 96-well plate to a volume of 50 µL in each well. Final concentration range of compound (128-0.25 µg/mL) along with bacterial concentration (2×10^5 cfu/mL) made by addition of an aliquot of 50 µL bacterial suspensions (4×10^5 cfu/mL) and final volume obtained was 100 µL. The plate was incubated at 37 °C for 20 h. Sterility and growth control was upheld alongside; experiment conducted in triplicates. Minimum inhibitory concentration (MIC) is the concentration used to express for anti-microbial activity at which no growth obtained after 24 h of incubation. Thermo varian micro plate auto reader used to measure microbial growth and determine by measuring absorbance at 492 nm.

Cellular toxicity assay

Red blood cells (RBCs) lysis assay performed to determine cellular toxicity of compounds, assay was performed as mentioned earlier [57]. Fresh human red blood cells (hRBCs) were collected with ethylenediaminetetraacetic acid (EDTA) and washed with tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.2) five times, further diluted to 4% v/v of a final concentration. The assay was performed in a sterile 96-well plate 2-fold serial diluted of compounds 50 µL (100 µg/mL) in minimum amount of dimethyl sulfoxide (DMSO) diluted with, tris-buffer added to an aliquot of 50

μL of hRBCs suspension, incubated at 37 °C for 90 min, centrifuged for 20 min at 26000 rpm. hRBCs suspensions with tris-buffer, 1% triton-X comprised the negative and positive controls, respectively. Supernatant (50 μL) from each well transferred to another plate and diluted with 50 μL of water. Hemoglobin was monitored by measuring absorbance at 540 nm. The experiment was performed in duplicate. Hemolysis percentage was calculated as (A_{540} in the test–A_{540} in PBS)/(A_{540} in 0.1% triton-X 100–$A_{540\ in}$ PBS)×100.

FITC assay

DAPI FITC and PI staining procedure was performed to appraise the viability as well as membrane integrity of the cell. Procedure is similar as mentioned earlier [58]. After exposure to inhibitory concentrations of compound (g-h) at 37 °C for 60 min, the bacterial cells were immobilized on poly (L-lysine)-coated glass slides for 35 min at 30 °C. FITC (6 μg/mL suspended in buffer) was added and incubated at 30 °C for 30 min, the slide were washed, further 80 μL of PI (2 μg/mL) was added and incubation at 30 °C for 30 min, again slides were washed as described above. Controls were run in absence of compound. Fluorescent images were recorded using a zeiss axio-observer Z1 microscope equipped with an oil-immersion objective (64x) and an AxioCam camera. Image acquisition and processing were performed using Axiovision software.

Atomic force microscopy imaging

Cell integrity in bacteria after treatment with compounds were visualized by AFM method [59]. A culture of *E. coli* was grown; cells were centrifuged and resuspended in deionized water. The bacteria were treated with compound (50 μM) at 37 °C for 1 h. Droplets of each sample were applied onto poly-L-lysine (PLL)-pretreated glass slides and allowed to dry (at 24 °C/20 min). Cells without compound were treated as positive control. Measurements were carried out in intermittent contact (IC) mode using tap 300 E-G (Cr/Pt conductive coating) cantilevers from silicon AFM probes. The AFM images were acquired using a JPK Nano Wizard (Berlin, Germany) mounted on a zeiss axiovert 200 inverted microscope.

Compound characterization

3-Benzylazetidin-2-one (**4a**): Yield: colorless solid; m.p.: 75-78 °C (crystallized from $CHCl_3$); IR (KBr, cm^{-1}): 700, 750, 1070, 1218, 1454, 1496, 1602, 1641, 1718, 1955, 2245, 2887, 2935, 3028, 3064, 3421; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.26 (m, 5H), 3.79 (m, 2H), 2.97 (s, 3H); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 136.36, 128.77, 128.51, 127.01, 120.54, 61.29, 36.52, 34.13; *anal.* Calcd: $C_{10}H_{11}NO$, exact mass: 161.08, found m/z: 161; HRMS (ESI) m/z: $[M+Na]^+$ calcd. for $C_{10}H_{11}NO$ Na, 184.0733; found, 184.0732.

3-(4-Methylbenzyl)azetidin-2-one (**4b**): Yield: colorless solid; m.p.: 100-101 °C (crystallized from $CHCl_3$); IR (KBr, cm^{-1}): 667, 757, 1070, 1214, 1381, 1446, 1516, 1625, 1903, 2245, 2887, 2927, 3018, 3444; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.14 (m, 4H), 3.71 (m, 1H), 3.78 (s, 2H), 2.94 (s, 3H), 2.33 (s, 3H); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 136.82, 133.31, 129.37, 128.78, 120.65, 61.58, 36.83, 33.95, 20.93; anal. Calcd: $C_{10}H_{13}NO$, exact mass: 175.09, found m/z: 175; HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{10}H_{13}NO$ Na, 198.0889; found, 198.0886.

3-(4-Chlorobenzyl)azetidin-2-one (**4c**): colorless solid; m.p.: 44-46 °C (crystallized from $CHCl_3$); IR (KBr, cm^{-1}): ν_{max} 3433, 3020, 2400, 1492, 1215, 757, 669 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (d, *J* = 8.6 Hz, 2H), 7.23 (d, *J* = 8.6 Hz, 2H), 3.78 (m, 2H), 2.96 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 134.78, 133.22, 130.36, 128.94, 120.16, 61.53, 36.61, 33.69, anal. Calcd chemical formula: $C_{10}H_{10}ClNO$, exact mass, 195.05 found 195. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{10}H_{11}ONCl$, 196.0524; found, 196.0516.

3-(4-Bromobenzyl)azetidin-2-one (**4d**): Purified by column chromatography using eluent 95:5 hexane/EtOAc and isolated as colorless solid m.p. 50-53 °C; IR (KBr, cm^{-1}): ν_{max} 3432, 3012, 2417, 1482, 1213, 752, 667 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45 (d, *J* = 8.6 Hz), 7.12(d, *J* = 8.6 Hz, 2H), 3.71 (m, 2H), 2.89 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 135.32, 131.73, 130.63, 121.12, 120.28, 61.29, 36.43, 33.60; anal. Calcd: $C_{10}H_{10}BrNO$, exact mass: 238.99, found m/z: 239. HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{10}H_{11}ONBrNa$, 261.9838; found, 261.9825.

3-(2-(benzyloxy)benzyl)azetidin-2-one (**4e**): colourless liquid IR (neat, cm^{-1}): ν_{max} 3444, 3018, 2927, 2887, 2245, 1903, 1625, 1516, 1446, 1381, 1214, 1070, 757, 667 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.38 (5H, m), 7.34-730 (2H, m), 6.94-6.90 (2H, m) [Ar-H]; 5.04 (2H, s), 3.61 (m, 2H), 2.99 (s, 3H); ^{13}C NMR (75 MHz, CDCl3) δ 156.35 (C, NH-C=O), 136.40(C), 131.07, 128.52 (2 × CH), 127.97, 127.13 (2 × CH), 124.88, 121.01, 120.65, 111.70, 116.51, 61.35, 3485, 29.10; anal. Calcd: $C_{17}H_{17}NO_2$, exact mass: 267.13, found, m/z: 267. HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{17}H_{17}O_2NNa$, 290.1152; found, 290.1137.

3-(2-nitrobenzyl)azetidin-2-one (**4f**): colorless solid m.p.: 40-43 °C; IR (KBr, cm^{-1}): ν_{max} 3424, 3074, 2884, 2246, 1610, 1521, 1342, 1064, 735 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.13 (d, 1H, *J* = 8.2 Hz), 7.72 (t, 1H, *J* = 7.6 Hz), 7.63-7.34 (m, 2H), 4.07-4.00 (m, 2H), 3.98-3.41 (m, 2H), 3.39-3.15 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 148.90, 133.81, 133.33, 131.98, 128.80, 125.44, 119.96, 62.25, 36.03, 32.50; anal. Calcd: $C_{10}H_{10}N_2O_3$, exact mass: 206.0691, found m/z: 206. HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{10}H_{10}N_2O_3Na$, 229.584; found, 229.0574.

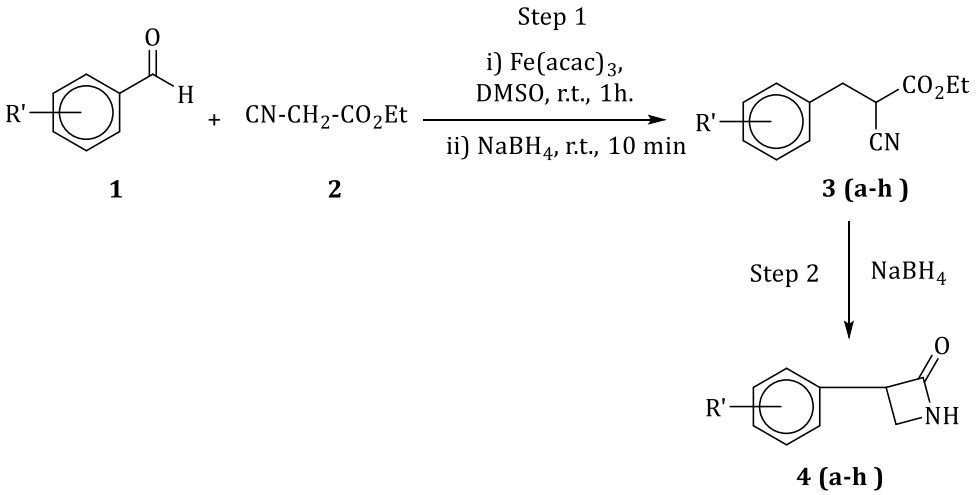
3-(4-fluorobenzyl)azetidin-2-one (**4g**): colorless liquid IR ($CHCl_3$, cm^{-1}): 705, 721, 754, 1070, 1220, 1457, 1498, 1602, 1649, 1722, 1958, 2248, 2877, 2945, 3032, 3068, 3423; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.23 (m, 2H), 7.11 (m, 2H), 3.80 (m, 2H), 2.99 (s, 3H); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 164.53

159.64, 132.05, 130.52, 120.22, 115.90, 61.61, 36.83, 33.61; anal. Calcd: $C_{10}H_{10}FNO$, exact mass: 179.07, found m/z: 179; HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{10}H_{10}FNO$ Na, 202.0639; found, 202.0638.

4-((2-oxoazetidin-3-yl)methyl)benzonitrile (**4h**): colorless solid; m.p.: 48-50 °C (crystallized from $CHCl_3$); IR (KBr, cm^{-1}): ν_{max} 3443, 3025, 2409, 1498, 1225, 761, 702 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.67 (d, J = 8.6 Hz, 2H), 7.38 (d, J = 8.6 Hz, 2H), 3.81 (m, 2H), 3.07 (m, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 142.01, 132.58, 129.93, 119.69, 118.51, 111.20, 61.45, 36.16, 34.36; anal. Calcd chemical formula: $C_{11}H_{10}N_2O$, exact mass 195.05, found m/z: 195 HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{11}H_{10}N_2O$ Na, 187.0866; found, 187.0865.

Result and Discussion

Herein, we would like to introduced $Fe(acac)_3$ based catalytic system for one pot Knoevenagel condensation followed by reduction of the C=C bond as well as CN group as shown in the Scheme 1. It is a prudent, cost-effective, simple and new methodology for tandem Knoevenagel condensation. It can help to overcome the demerits of the existing methods in terms of substrate selectivity, reaction time, temperature, yield, feasibility, and large scale synthesis.



Scheme 1. One-pot Knoevenagel condensation for synthesis of β-lactams

Table 1. gives an idea about varieties of substrates used and yields of the products obtained in the reaction. Yields reported in the Table 1. are of pure compounds obtained after column chromatographic purification. It is observed that rate of the reaction varied with respect to electron donating and the withdrawing substituents of the benzaldehyde. It realized that rate of the reaction is faster in case of strong electron withdrawing NO_2 and CN group (entry **f** and **h**). Furthermore, reduction of the CN group could be possible by excess addition of the $NaBH_4$ and it led to the

formation of valuable product 4 i.e., *β*-lactam through aminolysis of ester (Step 1, Scheme 1). Chemoselectivity of the reduction was controlled by amount of the $NaBH_4$ (Scheme 1). It was observed that for getting the *β*-lactam minimum 4.0 equivalent of $NaBH_4$ was required and below this amount the CN was found to be intact. The NO_2 group in the entry **f** fails to undergo reduction even after use of excess of $NaBH_4$ rather the CN group was reduced preferentially.

Table 1. Tandem Knoevenagel condensation followed by reduction with $NaBH_4$[a]

Entry	Substrate	Product	Time	Yield[b]
a	H	H	3.3	86
b	4-Me	4-Me	3.4	87
c	4-Cl	4-Cl	2.2	90
d	4-Br	4-Br	2.5	87
e	2-BnO	2-BnO	3.2	89
f	2-NO_2	2-NO_2	1.5	80
g	4-F	4-F	2.1	90
h	4-CN	4-CN	1.5	87

[a] Aldehyde (4.7 mmol), ethyl cyanoacetate (4.7 mmol) and $Fe(acac)_3$ (1 mol% DMSO 4 mL, r.t.; ii) EtOH 4 mL $NaBH_4$ (17.8 mmol). [b] yield of product after chromatographic purification

We tried varieties of polar protic and polar aprotic solvents for selecting the suitable solvent for reaction. It was observed that excellent results were obtained in DMSO than the other solvents tested for optimization of the reaction. The reaction was able to carry out at room temperature, required shorter reaction time and yields of the product lie in the range of 86-90% as mentioned in the Table 1.

We noticed that DMSO help to enhance rate of the tandem Knoevenagel condensation reaction in the presence of $Fe(acac)_3$ as catalyst. It is believed that enolization of ethyl cyanoacetate may takes placed in the DMSO. Therefore, we measured the ^{1}H-NMR spectrum of ethyl cyanoacetate in DMSO-D_6 as well as in $CDCl_3$ as solvent. In case of DMSO-D_6, singlet appearing at δ 3.99 while it was at δ 3.48 in $CDCl_3$. This difference in chemical shift of the singlet of ethyl cyanoacetate could be explain by presence of enolization in DMSO as solvent (Figure 2).

Previously, $Fe(acac)_3$ used as a catalyst in many chemical reactions we are reporting herein for the first time the use of $Fe(acac)_3$ as a catalyst for Knoevenagel condensation followed by reduction. We noticed that presence of $Fe(acac)_3$ is very much essential otherwise reaction fails to give product. Therefore, we optimized the quantity of $Fe(acac)_3$ which required to facilitate the reaction. It was observed that minimum 1 mol% of $Fe(acac)_3$ is sufficient to catalyze the above transformation.

Mechanistically, we propose that $Fe(acac)_3$ might be first transformed to species A as shown in the Figure 2**.** and it involved as catalyst in the reaction as depicted in the Figure 3. We believe that species A is like $Ti(acac)_3$, $VO(acac)_2$ or $VO(acac)_3$ species, which are already reported to have great potential for catalytic activity [43]. Besides, iron (Fe) and Ti, V are placed in the same row of the periodic table. Therefore, iron expected to have similar characteristic chemical behavior hence we propose species A as the active catalyst.

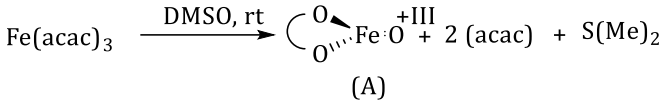


Figure 2. Formation of $Fe^{(+III)}$ from $Fe(acac)_3$

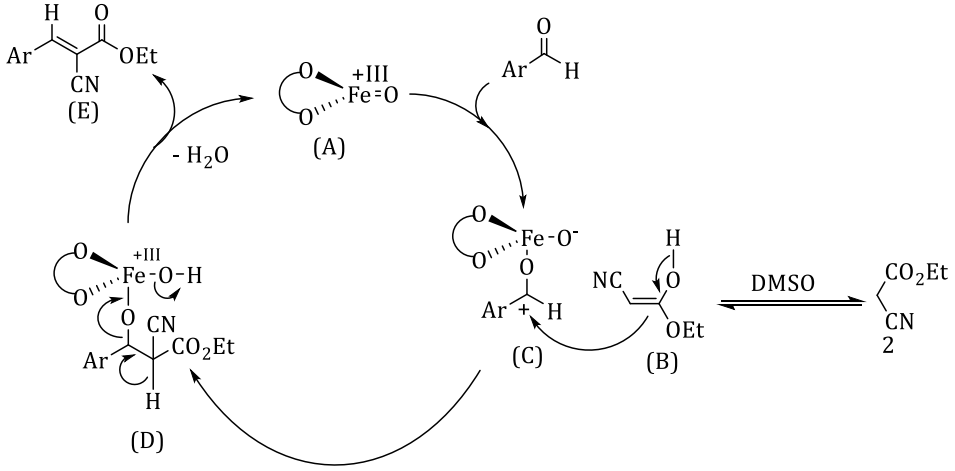


Figure 3. Probable mechanism of $Fe^{(+III)}$ catalysis

Mechanism

During the tandem Knoevenagel condensation, the aromatic aldehyde may get polarized (species **C**) while coming in contact with species **A**. It may also influence the attack of an enolate **B** to create the C-C bond as shown by species **C** (Figure 3). Finally, species **A** will be regenerated by deoxygenation with loss of most acidic proton from species **D** with releasing the product **E**. Furthermore, reduction of the C=C bond from the α, *β*-unsaturated product **E** could be predicted by coordination of this product with species **A**.

Anti-microbial activity

β-lactam compounds were exhibited antibacterial and antiyeast activity showed in Table 2. Compounds tested were all showed the anti-microbial activity but some at higher concentration. 3-

(4-fluorobenzyl)azetidin-2-one (4 **g**) and 4-(2-oxoazetidin-3-yl)-methyl)benzonitrile (4 **h**) showed antibacterial activity at lower concentration. 3-(4-fluorobenzyl)azetidin-2-one effective against *Staphylococcus aureus* (8 µg/mL) and *Micrococcus luteus* (16 µg/mL) it might be due to the fluorine functionality, which form strong hydrogen bonding and it will interact with some protein; 4-(2-oxoazetidin-3-yl)-methyl)benzonitrile showed MICs at lower concentration against *Staphylococcus aureus* (16 µg/mL) and *C.albicance* (16 µg/mL).

Hemolytic activity

Hemolytic activity study of the compounds showed up to maximum 50% but in case of 4 **g** and 4 **h**, it is less than 25% at their MICs concentration for *S. aureus*, *M. luteus* and *C. albicance*. Compounds showed anti-microbial activity at lower MIC and low hemolytic activity we can developed an anti-microbial agents. As the *β*-lactam containing antibiotics mainly targets inhibition of cell wall henceforth to investigate the mechanism of action we further study the effect of compounds on bacterial cell wall.

Effect on membrane integrity and cell viability

Fluorescent uptake assay used to investigate anti-microbial ability and cell membrane disruption. The assay performed as mentioned above. The entire bacterial cell permit DAPI to enter inside, hence it showed over all bacterial cells. But the entry of FITC requires significant membrane damage, FITC uptake assay suggested that the 4 **g** and 4 **f** may increase the permeability, and could accumulate FITC in cytoplasm. The images of treated *E. coli* cells before and after with the 4 **g** and 4 **h** are shown in Figure 3 (A). PI stain the cellular DNA, which indicated that compound might cause permanent damage to the cell membrane. FITC and PI uptake assay suggest that compounds treatment might be responsible for bacterial membrane disruption.

Table 2. Minimum inhibitory concentration of compounds (µM)

Microorganism	a	b	c	d	e	f	g	h	Gent. Sulf.[d]	Ben. Sod.[d]
Pseudomonas aeruginosa	>128	64	32	64	>128	16	32	64	nd	1
Escherichia coli	>128	>128	64	>128	32	32	8	16	0.5	2
Staphylococcus aureus	64	64	>128	>128	64	>128	>128	64	0.5	nd
Micrococcus luteus	>128	32	>128	32	32	>128	16	64	1	4
C.albicance	>128	>128	32	64	32	32	64	16	nd	nd
% Hemolysis (HD_{10})	48.2	43.1	36.2	28.4	55.4	60	22.4	25.1		

Gent.sulf. Gentamycine sulfate; ben. sod. Benzymide sodium. [a] The calculated average MIC values are presented ; [b] MIC>128 µg/mL was considered as inactive ; [c] nd : not determine ; [d] positive control

Visualation of cell damage by using atomic force microscopy (AFM)

The atomic force microscopic images of *E. coli* after treatment with compound and control without the treatment of compounds were shown in Figure 3 (B). Morphological change in the bacteria indicated that possible mode of action of compound through the cell membrane deformation.

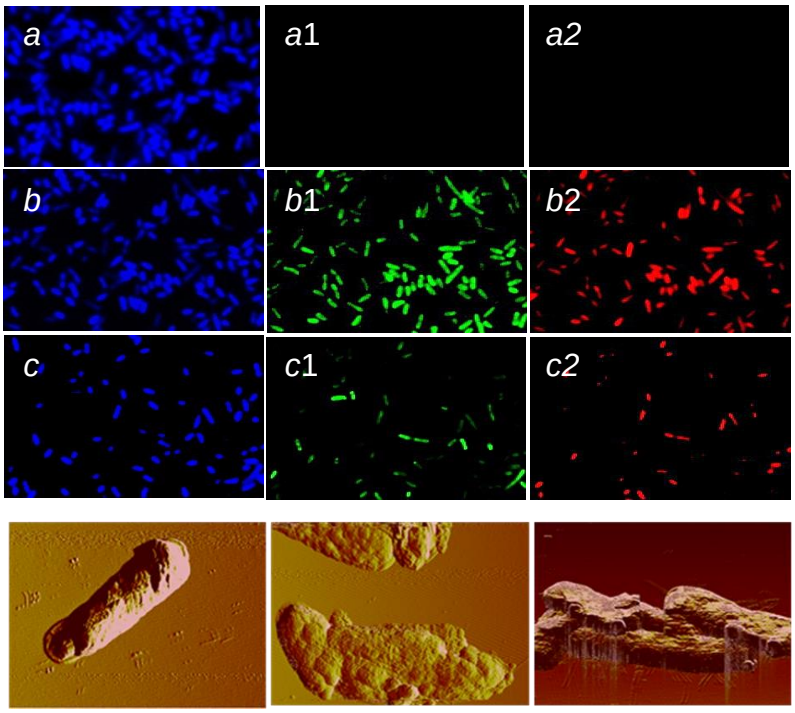


Figure 3. (a) Fluorescence microscope images bacterial cell membrane permeabilization of *E. coli* (DAPI, FITC and PI): control (a, a1 and a2), *E.coli* cell treated with 4 g (b, b1 and b2), *E.coli* cell treated with 4h (c, c1 and c2). (b) Atomic force microscopic images: *E. coli* untreated (d), treated with compound 4g (d1) and with treated with compound 4h (d2), the compound concentrations used were their MICs

Conclusion

We have developed an economical, accessible, environmentally friendly tandem Knoevenagel condensation followed by reduction of the C=C double bond and CN group at ambient reaction condition without formation of salt. Anti-microbial activity study of *β*-lactone derivatives showed MICs at lower concentration. Derivatives exhibited low hemolytic activities. Further investigation revealed that the derivative might act on the bacterial cell wall. Here, we developed a one pot Knoevenagel condensation and reduction for *β*-lactone derivative synthesis. Anti-microbial and hemolytic activity provide the basis to develop them as an anti-microbial agent. Furthermore, there is an urgent need to use this reaction for the synthesis of commercially important molecules and study the derivative activity at molecular level.

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