Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 3, 2012

Research Article

SYNTHESIS AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF SOME NOVEL CEPHEM ANTIBIOTICS

KUMAR GAURAV[a*], SUBIR KUNDU[a] AND RICHA SRIVASTAVA[b]

[a]School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi-221005, [b] Department of Applied Chemistry, Delhi Technological University, Delhi-110042, India. Email: gauravbiotech@rediffmail.com

Received: 31 Jan 2012, Revised and Accepted: 14 Apr 2012

ABSTRACT

In the present communication a number of semisynthetic cephalosporins (**1** - **9**) have been synthesized and characterized by UV, ^{1}H NMR, ^{13}C NMR spectra and also, evaluated for their antibacterial activities *in vitro* against a number of pathogenic bacterial strains. The compounds **7**, **8** and **9** have shown highly encouraging results as antibacterials with MIC in the range 0.391 to 0.097 µg/mL and molecules **1** – **6** have shown substantial degree of inhibitory action towards most of the bacteria screened in the present study. Thus, all the newly synthesized cephem antibiotics are acting as broad-spectrum antibacterial agents. The enhanced activity of these drugs may be due to increased concentration of drugs at the target site because all the molecules are having biodegradable ester bonds. Moreover, these newly synthesized cephem antibiotics are using enzymatically produced 7-ACA as an intermediate thus leading to more economical and environmental friendly synthesis.

Keywords: Semisynthetic cephalosporins; Antibiotics; Cephem antibiotics; β-lactamases; benzimidazole.

INTRODUCTION

Cephalosporins are β-lactam antibiotics and along with penicillin, constitute a major portion of pharmaceutical market all over the world[1-2]. In cephalosporin C, the four membered β-lactam ring (which is mainly responsible for the activity) is fused with the six membered dihydrothiazine ring to form the basic nucleus, 7-aminocephalosporanic acid (7-ACA), to which the α-aminoadipic acid side chain is attached through an amide bond[3] (**Fig 1**).

They are used to treat a variety of bacterial and fungal infections[4]. Although cephalosporin was found to be active against a large number of pathogenic bacteria[5], the main hindrance in its application is its low stability. Also, occurrence of bacterial strains that are resistant to already existing antibiotics such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* has led to the search of new semisynthetic cephalosporins with better solubility and new mechanism of action. Only cephalosporin C (CPC) is found naturally and itself exhibits negligible antimicrobial activity but substitutions at the C3 and C7 positions of its β-lactam ring along with other structures generate semisynthetic cephalosporins with diversified antimicrobial activity which are classified based on their activity profile, the antibacterial spectrum.[6] Each newer generation of cephalosporin has significantly greater Gram negative antimicrobial properties than the preceding generations[7-10] Fourth generation cephalosporins are known to have true broad spectrum activity[11-14].

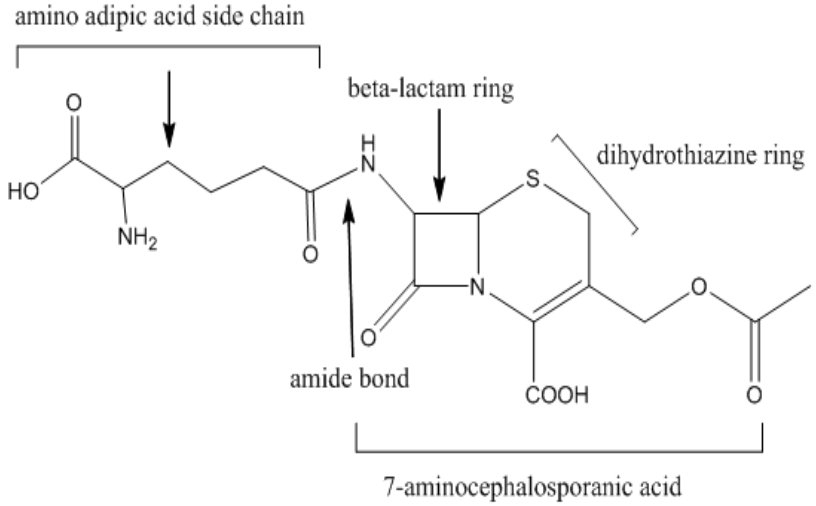


Fig. 1: Cephalosporin C

In the present work, the attempts have been taken to synthesize some new semi-synthetic cephalosporins and some by modifying already existing semi-synthetic cephalosporins such as cefotaxime which is a broad spectrum antibiotic with high resistance against β-lactamases[15]. But the main problem is that it is poorly soluble in water. Hence, the efforts have been made to prepare cephalosporins having better solubility using cefotaxime. All these semi-synthetic cephalosporins are derived from the key intermediate 7- ACA, a product derived from cephalosporin C hydrolysis. They differ in the nature of the substituents attached at the 3 and/ or 7- position of the cephem ring of bacteria and express various biological and pharmacological effects. 7α-Formamido cephalosporins were isolated as fermentation products of various gram negative bacteria. It is also known that incorporation of a MeO group in both cephalosporins and penicillins has led to a considerable increase in β-lactamase stability[6]. These findings prompted us to prepare methoxy and formamido derivatives of cephalosporins and screen them for their antibacterial activity.

7-ACA is required for the production of most of the clinically used cephalosporin derivatives i.e. semisynthetic cephalosporins[16-17]. It is produced from cephalosporin-C (CPC) either chemically or enzymatically. Chemical methods for 7-ACA production are time consuming and involve multiple step; hence, enzymatic approach is preferred by a number of workers[18-22]. These enzymatic processes have the advantage of generating less waste and requiring less expensive chemicals. Cephalosporin acylases are industrially important enzymes that hydrolyse cephalosporins to 7-aminocephalosporanic acid (7-ACA)[6, 23]. In the present work, this enzymatic method has been employed to produce 7-ACA, the key intermediate which is then utilized for the synthesis of new semi-synthetic cephalosporins. Nicotinic acid, benzimidazole, imidazole or substituted benzimidazole system has been shown to have different pharmacological effects including antifungal, antibacterial and antiviral effects[24-28]. 2-substituted benzimidazoles, with various types of biological activity, have a close relationship to nucleic acid metabolism. Hence, semi-synthetic cephalosporins containing these nucleuses were prepared and the assessment of these molecules has been checked to interfere with various cellular and metabolic processes.

MATERIALS AND METHODS

Chemistry

All reactions were performed in oven dried apparatus and the mixtures were stirred magnetically. Thiophene-2-carboxylic acid, phenyl acetic acid, hydroxybenzotriazole (HOBT), N-methylmorpholine (NMM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), nicotininc acid, pyrazine-2-carboxylic acid, imidazole-4- carboxylic acid, 2-methyl mercaptobenzimidazole, di-t-butyl dicarbonate, t-butyltrichloroacetimidate, dimethylaminopyridine (DMAP), p-nitrophenylchloroformate, 2,6-lutidine, 4-imidazole carboxylic acid, and all deuterated solvents were ordered from Sigma- Aldrich, USA and rest of the commonly used solvents and chemicals were obtained from Merck, India. UV measurements were carried out on a Hitachi 220S spectrophotometer. All NMR spectra were recorded on a Varian instrument at 300 MHz (^{1}H) and 75 MHz (^{13}C) with D_2O or $CDCl_3$ as solvent and Me_4Si (TMS) as an internal standard. Chemical shifts are expressed in δ values (ppm) from internal reference peaks of TMS. Multiplicities are indicated using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet). The characterization details of all the compounds are given in table-1. TLC analysis was performed on precoated silica plates (purchased from Merck) using solvents AcOEt and petroleum ether (PE) or CH_2Cl_2 and MeOH as mobile phases. The spots were visualized with UV light at λ = 254 nm and were confirmed by ninhydrin charring. All solvents were dried and distilled prior to use, THF was distilled from a mixture of Na and benzophenone, and MeCN and Et_3Nwere distilled from CaH_2. Organic solvents were dried over anh. Na_2SO_4 and concentration by evaporation was carried out *in vacuo*.

O-methyl ester of 7-aminocephalosporanic acid (A)

500 mg of 7- aminocephalosporanic acid was dissolved in 5-10 ml of dry methanol in a round bottom (RB) flask. 5 ml of thionyl chloride was added and the reaction mixture was refluxed for 5 – 6 h on oil bath. After the completion of the reaction, the solvent was evaporated on pump under reduced pressure and O-methyl ester of 7- aminocephalosporanic acid (**A**) thus obtained was used in next step without further purification.

General method for the synthesis of 1-4

Thiophene-2-carboxylic acid, phenyl acetic acid, nicotinic acid and pyrazine-2-carboxylic acid (1 equi.) were dissolved separately in four different round bottom flasks in 15 mL of MeCN at 0 ºC. N-hydroxybenzotriazole (HOBT, 3 equi.) along with the catalytic amount of NMM was added and the mixture was stirred for 15 min. To this suspension, EDC (4 equi.) and afterwards COOH protected 7-ACA (**A**, 1.5 equi.) was added and left stirring overnight. Next day, solvent was removed under vacuum and compound was taken in AcOEt, washed with citric acid solution, saturated $NaHCO_3$ soln, brine and dried on Na_2SO_4. The organic layer was concentrated and purified by cc (AcOEt/PE 3:7) to give **1-4** respectively.

Methyl ester of 7(-imidazole-2-acetamido) cephalosporanic acid (5)

To a suspension of 4-imidazole carboxylic acid (500 mg), in 10 mL of absolute benzene, powdered phosphorus pentachloride (PCl_5) was added and stirred at r. t. The reaction mixture first became clear and then cloudy due to the formation of acid chloride. The precipitated acid chloride was employed in the next step without further purification. This acid chloride was added to methyl ester of 7-ACA and mixture was stirred for 1 hr at 0 ºC and additional 2 h at r.t. The reaction mixture was acidified with 1N HCl and was extracted with AcOEt (thrice). Organic layer was washed with saturated $NaHCO_3$ solution and brine, dried over solid Na_2SO_4 and concentrated in *vacuo*. Methyl ester of 7(-imidazole-2-acetamido) cephalosporanic acid (**5**) was crystallized using ethanol.

7(pyrazine-formamido)-3'-mercapto methylbenzimidazole cephalosporanic acid (6)

Acid chloride of pyrazine-2- carboxylic acid was prepared as above was employed in the next step without further purification. To a solution of sodium salt of 7-ACA (540 mg) and $NaHCO_3$ (540 mg) in water (10 mL) and acetone (7 mL), the solution of acid chloride (formed previously) was added dropwise at 0 ºC with stirring. The reaction mixture was stirred for 1 hr at 0 ºC and additional 2 h at r.t. The reaction mixture was acidified with 1N HCl and was extracted with AcOEt (thrice). Organic layer was washed with saturated $NaHCO_3$ solution and brine, dried over solid Na_2SO_4 and concentrated in *vacuo* to give 7-(pyrazine-2-formamido) aminocephalosporanic acid. It was crystallized using ethanol. A solution of 7-(pyrazine-formamido) aminocephalosporanic acid (500 mg), $NaHCO_3$ (100 mg) and mercaptobenzimidazole (300 mg) in phosphate buffer was stirred for 6 h at 60 ºC. The reaction mixture was acidified with 1N HCl and extracted with AcOEt (thrice). Organic layer was washed with saturated $NaHCO_3$ solution and brine, dried over solid Na_2SO_4 and concentrated in *vacuo*. The final compound 7(pyrazine-formamido)-3'-mercapto methylbenzimidazole cephalosporanic acid (**6)** was purified by silica gel cc using DCM and MeOH as solvent and was crystallized using acetone.

$(t\text{-}BOC)_2O$ protection of cefotaxime (for analogues 7 - 9)

500 mg of cefotaxime (1 mmol, 1eq) was dissolved in saturated sodium bicarbonate solution and stirred at 0 ºC. 330 mg of $(t\text{-}BOC)_2O$ (1.5 mmol, 1.5 eq) was dissolved in dioxane and added to the stirred reaction mixture at 0 ºC and allowed to stir for 3-4 h. After the completion of the reaction, the reaction mixture was acidified using saturated citric acid solution (1N HCl can not be used otherwise BOC will cleave), extracted with AcOEt (thrice). Organic layer is washed with saturated citric acid solution and brine, dried over solid sodium sulphate and concentrated *in vacuo*. The solid compound thus obtained is used in next step without further purification.

(4-carboxamido)-cefotaxime methyl ester)-7-aminocephalos poranic acid (7)

Sodium salt of cefotaxime (**B**, 500 mg) is dissolved in methanol (10 mL) and 5 – 6 mL thionyl chloride was added, reaction mixture was refluxed for 4-5 h. All the solvent is removed using rotavapour and then it is treated with neat TFA for 2 h at r.t. TFA is removed and COOH protected cefotaxime (**C**) thus obtained is used further. 500 mg of 7-ACA (1.8 mmol, 1eq) was dissolved in saturated $NaHCO_3$ solution and stirred at 0 ºC. 590 mg of $(t\text{-}BOC)_2O$ (2.7 mmol, 1.5 eq) was dissolved in dioxane and added to the stirred reaction mixture at 0 ºC and allowed to stir for 3-4 h. After the completion of the reaction, the reaction mixture was acidified using saturated citric acid solution (1N HCl can not be used otherwise BOC will cleave), extracted with AcOEt (thrice). Organic layer is washed with saturated citric acid solution and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The solid compound thus obtained is used in next step without further purification. 744 mg of BOC protected 7-ACA (2 mmol, 1 eq) was taken in a round bottom flask and dissolved in MeCN at 0 ºC. 810 mg of HOBT (6 mmol, 3 eq) was added along

with the catalytic amount of NMM at 0 ºC and stirred for 15 min. To this suspension, 1.5 g of EDC (8 mmol, 4 eq) and afterwards 1.3 g of COOH protected cefotaxime **C** (3 mmol, 1.5 eq) was added and left for stirring overnight. Next day TLC was checked, solvent was removed under vacuum and compound was taken in AcOEt, washed with citric acid solution, saturated $NaHCO_3$ solution and brine (saturated sodium chloride solution), dried on solid Na_2SO_4. Organic layer was concentrated and purified by column chromatography in chloroform and methanol (10%) to give BOC derivative of analogue **7**. BOC protected analogue **7** (500 mg) is dissolved in methanol (10 mL) and 5 – 6 mL thionyl chloride was added, reaction mixture was refluxed for 4-5 h. All the solvent is removed using rotavapour and then it is treated with neat trifluoroacetic acid for 2 h at r. t. TFA is removed and (4-carboxamido)-cefotaxime methyl ester)-7-aminocephalosporanic acid or analogue **7** is obtained in good amount.

3'-(mercaptobenzimidazole) cefotaxime methyl ester (8)

BOC protected cefotaxime (500 mg) is dissolved in methanol (10 mL) and 5 – 6 mL thionyl chloride was added, reaction mixture was refluxed for 4-5 h. All the solvent is removed using rotavapour. A solution of BOC protected methyl ester of cefotaxime (500 mg), $NaHCO_3$ (100 mg) and mercaptobenzimidazole (300 mg) in phosphate buffer was stirred for 6 h at 60 ºC. The reaction mixture was acidified with 1N HCl and extracted with AcOEt (thrice). Organic layer was washed with saturated $NaHCO_3$ solution and brine, dried over solid Na_2SO_4 and concentrated in *vacuo*. The final compound was purified by silica gel column chromatography using dichloromethane and methanol as solvent. All the solvent is removed using rotavapour and then it is treated with neat trifluoroacetic acid for 2 h at r. t. TFA is removed and analogue **8** i. e. 3'-(mercaptobenzimidazole) cefotaxime methyl ester is obtained. It was crystallized using acetone.

3'-[1-(2-methylenebutyl)-4-nitrobenzene] cefotaxime methyl ester (9)

555 mg amino protected cefotaxime (1 eq, 1 mmol) was suspended in dichloromethane (1- 15 mL). Anhydrous hydrochloride (4 N in dioxane, 1.3 eq, 300 µl) was added, and the reaction mixture was stirred for 30 minat r. t. *tert*-Butyl trichloroacetimidate (3 eq, 200 µl, 3 mmol) was added, and the rection mixture was stirred overnight at r.t. Next day, the reaction mixture was washed consecutively with water, saturated sodium bicarbonate solution and brine. Organic layer was dried using solid sodium sulfate and solvent was removed under vacuum. O-tertiary butyl ester of (t-BOC)2O protected cefotaxime was purified by filter cc eluting with a solvent consisting of AcOEt and hexane. To a solution of this in dimethylformamide (DMF) and dichloromethane (DCM) was added trifluoroacetic acid (1 mL), and the solution was stirred for 2 h at r. t. The solvent was removed and ethyl ether was added. The solid was filtered and washed with ether to give O-tertiary butyl ester of cefotaxime which was (1.2 g, 1 eq, 2.2 mmol) dissolved in methanol (10 mL) and solid potassium carbonate (120 mg) was added. The mixture was stirred for 2 h at r.t. and acetic acid (200 µl) was added to quench the reaction. The solvent was removed and the product was purified flash cc eluting with 10% acetone in DCM to afford **D** in 28% yield. Compound **D** was dissolved in anhy THF, and DMAP, p-nirophenylchloroformate (0.2 g, 1 mmol) and 2,6-lutidine (120 µl, 1 mmol) were added sequentially. The reaction mixture was stirred overnight at r.t. The solvent was removed and the product was purified by cceluting with 5% AcOEt in DCM to afford final drug 3'-[1-(2-methylenebutyl)-4-nitrobenzene] cefotaxime methyl ester **(9)** in 65% yield.

Pharmocology

To determine zone of inhibition by Kirby-Baur's method

The antibacterial susceptibility test was done by determining zone of inhibition by Kirby- Baur's method. The stock solution of each of the semi-synthetic cephalosporin was prepared in water/DMSO and was serially doubly diluted (400, 200, 100, 50 , 25 µg/mL). Sterilized filter discs were dipped in these solutions and subsequently dried to remove excess solvent. Nutrient Agar medium plates were prepared using Muller- Hinton broth and allowed to solidify. Different bacterial strains were selected, viz. *Citrobacter freundii*, *Proteus mirabilis, Klebsiella pneumoniae*, *Escherichia coli* ATCC 25922, *Salmonella typhi* MTCC 3216, *Salmonella typhimurium*, *Salmonella paratyphi*, *Shigella flexinii*, *Vibrio cholerae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* and *Helicobacter pylori* (Gram-negative) as well as Gram – positive cocci like *Enterococcus facealis* ATCC 29912, *Staphlyococcus aureus* I ATCC 25923, *Listeria monocytogenes*, *Staphlyococcus aureus* II and *Streptococcus heamophila* and 1 mL of each bacteria and culture broth were added to the plates and spread with the help of sterile spreader. The filter paper discs soaked in bacterial strain were placed aseptically over the inoculated plates using sterile forceps. The plates were incubated at 37 ^{0}C for 24 h, in upright position. The zone of inhibition was measured using scale, table- 2.

To determine MIC by the Microdilution Broth Susceptibility Test method

The stock solution of 400 µg/mL each of the semi-synthetic cephalosporin was prepared in water/DMSO and was serially doubly diluted (200, 100, 50 , 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.391, 0.195, 0.978 µg/mL).Different concentrations of all the compounds were prepared in sterile dry test tubes to determine minimum inhibitory concentration (MIC). Nutrient broth was prepared and 2.9 mL of it was taken in each test tube and were sterlised after plugging. After cooling 0.1 mL of each dilution was added to the test tubes and the final volume was made upto 3.0 mL. To each of test tube 0.1 mL of bacterial culture broth was added. The test tubes were shaken to mix the inoculum with the broth uniformly. The tubes were incubated at 37^{0}C for 24 h. The lowest concentration at which there has been no visible growth of microorganism is reported as MIC, table- 3.

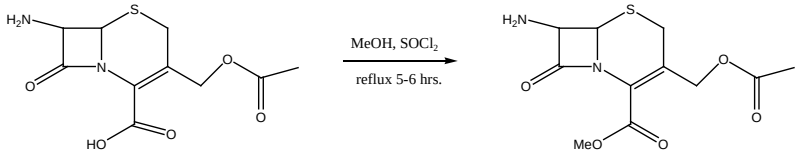
RESULT AND DISCUSSION

General synthetic routes employed for the synthesis of new semi-synthetic cephalosporins **1- 4,** methyl esters of 7-(thiophene-2-formamido) cephalosporanic acid (**1**), 7-(phenylacetamido) cephalosporanic acid (**2**), pyridine-3-yl -formamido) cephalosporanic acid (**3**) and 7-pyrazin-formamido cephalosporanic acid (**4**) is shown in scheme 1.

Literature survey revealed that in already existing second generation cephalosporins furan or thiophene (five membered heterocyclic ring) is present containing O or S as heteroatom. In the present work, we tried to find out the effect of five membered ring on the antibacterial activity by replacing it with six membered pyridine ring, having one N and pyrazine ring with two N atoms in the new cephalosporins **3** and **4,** respectively. For the development of better potency, we also synthesized the analogues 7-(imidazoleformamido) cephalosporanic acid (**5**) and 7-(pyrazine-formamido)-3'-mercapto methylbenzimidazole cephalosporanic acid (**6**), in which we tried to replace the thiazoline nucleus of third generation cephalosporins by the imidazole nucleus and the acetate group is replaced by the benzimidazole moiety, scheme 2.

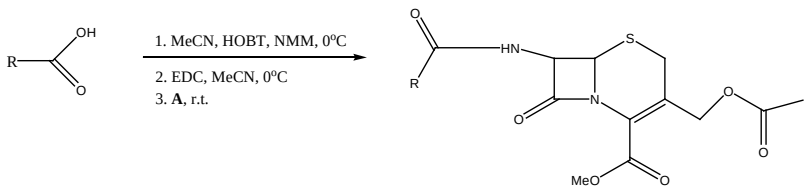
It is known that the benzimidazole nucleus has diverse biological properties and this nucleus is part of several existing antibacterial compounds, hence these analogues were expected to exhibit better broad spectrum activity as compared to existing third generation cephalosporins. Cefotaxim (**B**), first third generation cephalosporin, which was introduced in 1980, was known to be a drug that was able to combat the attack of nearly all β-lactamases. It had a higher affinity to penicillin binding proteins (PBPs) of gram-negative bacteria and was able to penetrate faster into the bacterial cell as compared to older generation cephalosporins. Therefore, it was considered to be a promising drug which was able to overcome the antibiotic resistance mechanism available at that time. But nowadays, newer mechanisms of resistance toward β-lactam antibiotics have been found due to which the activity of this valuable drug has reduced a lot. With the synthesis of analogues **7- 9** we tried to improve the antibiotic property of cefotaxime by modifying it at various positions. Acylation of 7-ACA or any semi-synthetic cephalosporin with an amino acid presents a problem common to peptide synthesis, *i.e.*, protection of the amino group by some function in such a manner that the rest of the molecule is not

affected. Review of literature showed that commonly used protecting group N-t-butoxycarbonyl could be used satisfactorily in the synthesis of cephalosporins. Although it can not be used in case of penicillin since it could not be removed because of instability of the resulting penicillin derivatives in acids. Greater acid stability of the β-lactam system in cephalosporin permitted removal of the t-butoxycarbonyl substituent under acidic conditions with a minimum of undesirable side reactions. So, for analogues **7 - 9**, amino group of cefotaxime is protected using (t-BOC)2O group and the product was converted to its methyl ester (in higher yield) by treating it with MeOH and $SOCl_2$. It can be employed to the next step without further purification. With an aim to discover a new generation cephalosporin with better efficiency as antibacterial drug, we tried to put two β--lactam rings together in case of **7**. For this purpose, carboxyl protected cefotaxime was coupled with amino protected 7-ACA, in presence of HOBT, EDC and NMM, scheme 3.



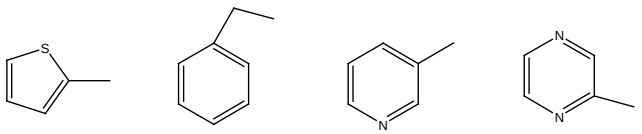
7- ACA — **Methyl ester of 7 - ACA**

A



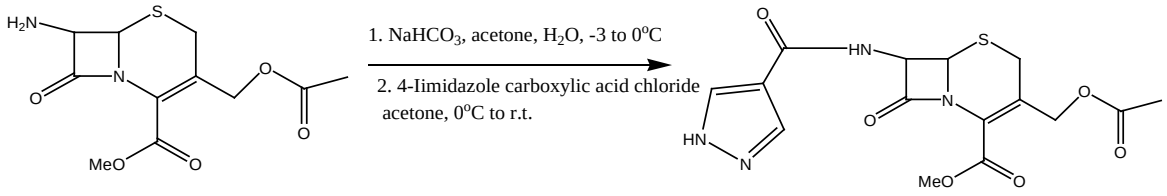
1 - 4

R =

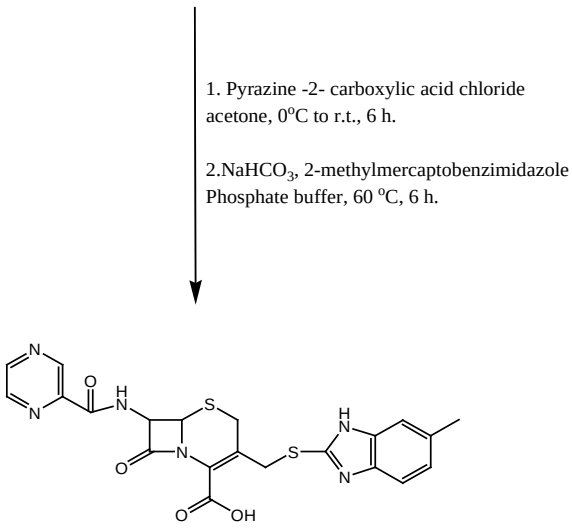


for 1 **for 2** **for 3** **for 4**

Scheme 1:

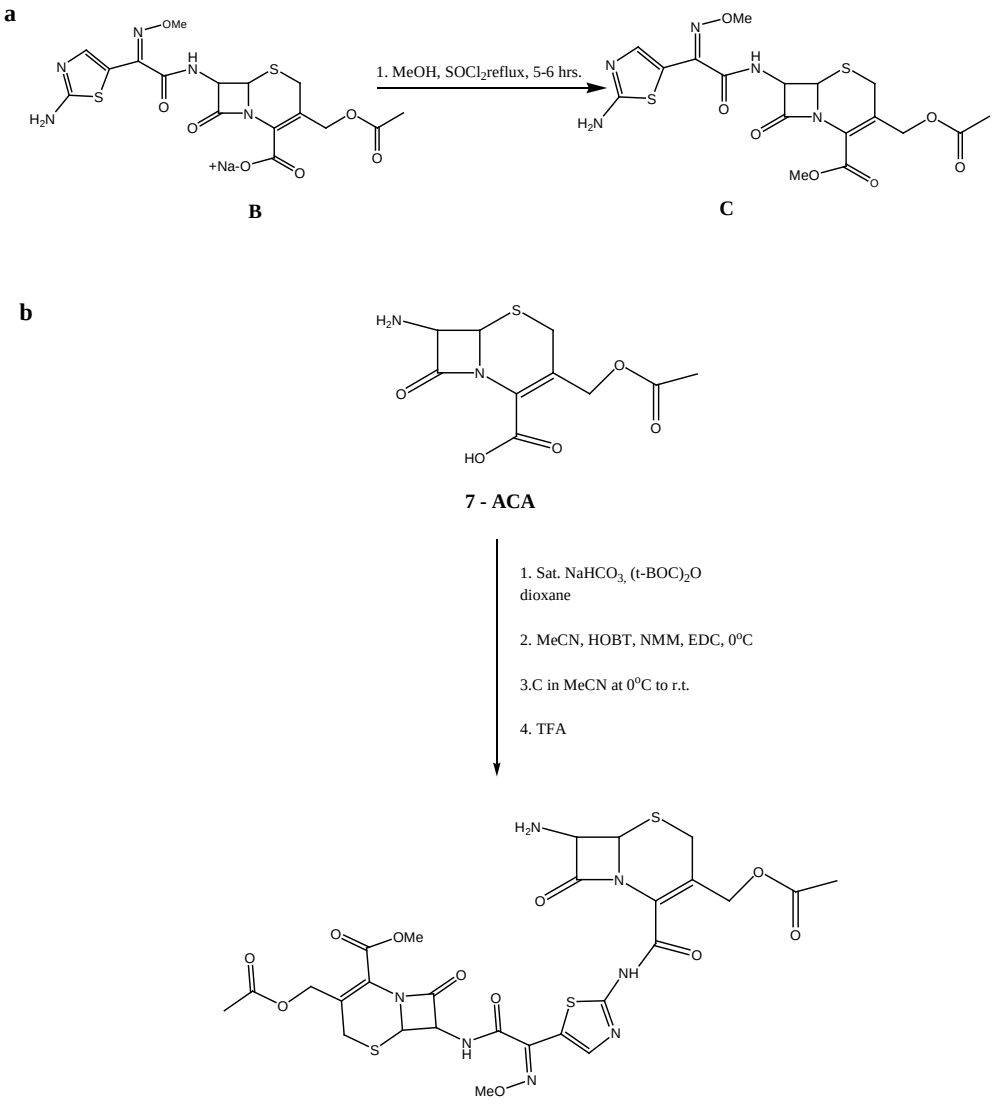


A — **5**



6

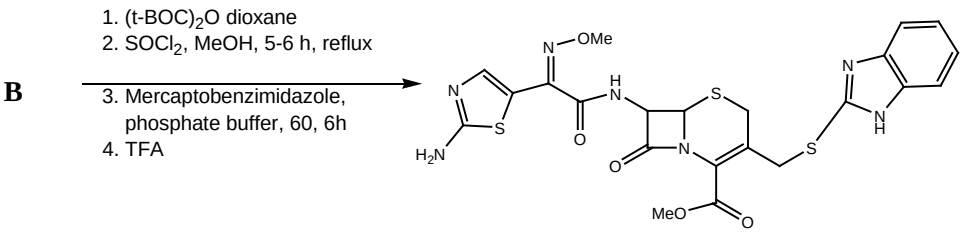
Scheme 2:



7

Scheme 3:

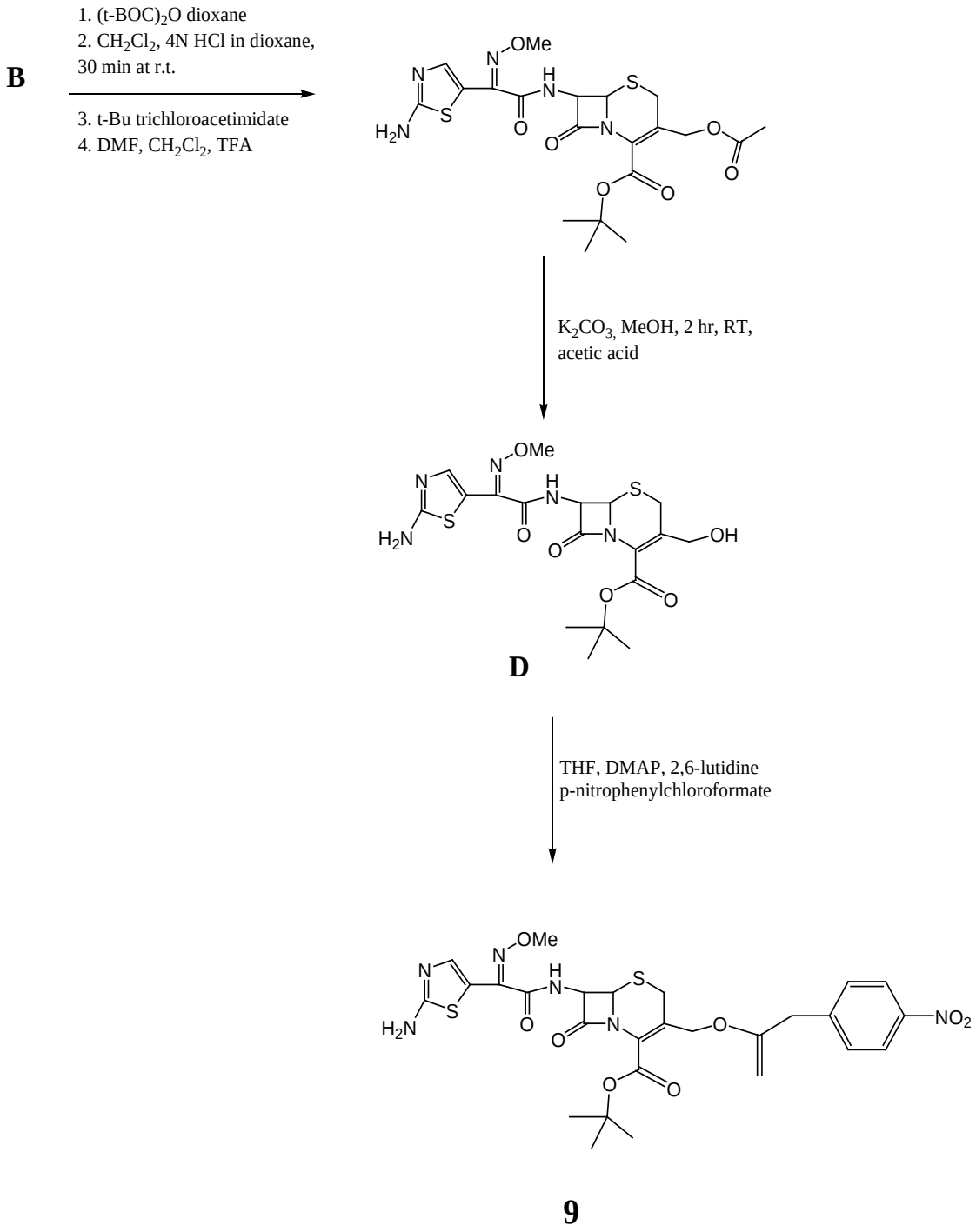
Finally, the (t-BOC)$_2$O group was removed using TFA to achieve the desired semi-synthetic cephalosporin **7**. The 3'-(mercaptobenzimidazole) cefotaxime methyl ester analogue **8** was obtained from the resulting ester by the displacement of the acetoxyl group by mercapto benzimidazole in a buffer solution and subsequently deprotecting (t-BOC)$_2$O by treating it with TFA, scheme 4.



8

Scheme 4:

3'-[1-(2-methylenebutyl)-4-nitrobenzene] cefotaxime methyl ester (**9**) is obtained by replacing 3-acetoxyl group from t-butyl ester of cefotaxime by p-nitrophenyl group Scheme 5.



Scheme 5:

All characterization details, given in table- 1, confirm the expected structures of the analogues. All the newly synthesized molecules were characterized by UV, ^{1}H NMR, ^{13}C NMR, MS and screened for their antibacterial activities against various pathogenic gram-positive and gram-negative bacterial strains using standard protocols.

All the newly synthesized semi-synthetic cephalosporins were evaluated *in vitro* for their antibacterial activity against a panel of Gram-negative as well as Gram-positive cocci which is shown in terms of zone of inhibition against 18 standard strains in table- 2. The minimum inhibitory concentrations (MIC) is defined as the lowest dilution of the new synthetic compound that inhibited visible growth of the micro-organism inoculated and the MIC for all the above mentioned antibiotics is summarized in table- 3. Each of the above-mentioned gram positive and gram negative bacteria were tested by Kirby Bauer Disc Diffusion (KBDD) method, as per standard protocol[29]. The compounds showing some inhibition zone by this method were further analysed by the broth dilution assay to determine their MIC values as per standard protocol[30, 31].

The synthesized cephalosporins were dissolved in DMSO-H_2O (50%) and doubling dilutions were subsequently prepared. Equal amount of bacteria was added into each tube to bring the turbidity level to 0.5 Mcfarlands. The tubes incubated at 37 ^{0}C for 18-24 hr and observed for any visible turbidity in the next day. Data were not taken for the initial solution because of the high DMSO concentration (12.5%).

Table 1: Characterization details of the compounds 1 - 9

Compound	Yield %	UV λ_{max}	^{1}H NMR (D_2O) δ	^{13}C NMR ($CDCl_3$)	MS m/z
1	85	262	7.6-7.2 (3H, m, thiophene ring), 8.9 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.11 (2H, d, H2), 4.75 (2H, s, CH_2O), 2.01 (3H, s, CH_3COO), 3.76 (3H, s, OMe)	136, 130, 137, 145, 168, 58.2, 58.5, 170, 126.6, 27, 122.8, 171, 50.5, 171, 17.4, 64	397.14
2	75	265	7.3-7.2 (5H, m, aro ring), 3.42 (2H, s, CH_2), 8.9 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.11 (2H, d, H2), 4.75 (2H, s, CH_2O), 2.01 (3H, s, CH_3COO), 3.76 (3H, s, OMe).	127-130, 136, 40, 171, 58.8, 59, 170, 27, 127, 122.8, 171, 63.5, 50.5, 171, 17.4.	405.11
3	85	267	8.7- 8.2 (4H, m, pyridine ring), 8.9 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.16 (2H, d, H2), 4.75 (2H, s, CH_2O), 2.21 (3H, s, CH_3COO), 3.76 (3H, s, OMe).	148, 125, 137, 131, 152, 168, 58.7 58.9, 170, 122.6, 27, 126.8, 171, 50.5, 171, 17.4, 64	392
4	70	258	8.90-8.5 (3H, m, pyrazine ring), 8.45 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.16 (2H, d, H2), 4.75 (2H, s, CH_2O), 2.21 (3H, s, CH_3COO), 3.76 (3H, s, OMe).	147.9, 143, 143.5, 145, 168, 58.9, 57.7, 170, 126.6, 27, 122.8, 171, 50.5, 171, 17.4, 64	393
5	65	272	13.6 (1H, s, NH of imidazole ring), 8.38 (2H, m, imidazole ring), 8.9 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.16 (2H, d, H2), 4.50 (2H, s, CH_2O), 2.21 (3H, s, CH_3COO), 3.76 (3H, s, OMe).	126, 122.8, 142.7, 63, 17.4, 27, 154, 166.5, 112, 58.5, 58.8, 50, 170, 171, 171	381.12
6	80	263	8.4-8.2 (3H, m, aro ring) , 8.5 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.16 (2H, d, H2), 3.80 (2H, s, CH_2O), 7.59 – 7.22 (3H, aro ring), 5.4 (1H, NH of benzimidazole ring), 2.3 (3H, s, CH_3), 11.0 (1H, s, COOH).	147.7, 143, 143.7, 145, 167, 58, 59, 170, 29.7, 31.8, 176, 142, 137, 135, 132, 116, 20.9, 123, 115	483.57
7	45	267	8.45 (2H, s, overlap, NH), 5.4 (2H, dd, overlap, H7), 5.1 (2H, d, overlap, H6), 3.16 (4H, d, overlap, H2), 4.75 (4H, s, overlap, CH_2O), 2.25 (6H, s, overlap, $COCH_3$), 9.15 (2H, s, NH_2), 7.7 (1H, thiazole ring), 4.0 (3H, s, $NOCH_3$), 3.77 (3H, s, $COOCH_3$).	171 (overlap), 170 (overlap), 162, 123.2, 172, 17.4 (overlap), 27, 26.6, 63.3, 61, 63, 123, 127, 50.5, 58.2, 58.9, 54.5, 59, 163, 164, 108, 139	724.23
8	78	260	9.5 (2H, s, NH_2), 7.5 (1H, s, thiazole ring), 8.9 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.16 (2H, d, H2), 4.50 (2H, s, CH_2O), 4.0 (3H, s, $NOCH_3$), 3.77 (3H, s, $COOCH_3$), 7.70-7.20 (4H, m, aro ring), 8.5 (1H, s, imidazole ring).	172, 139, 108, 164, 162, 123.2, 172, 58.2, 58.8, 29.7, 121.8, 50.5, 54.5, 31.8, 141.5, 138, 123, 115	560.1
9	65	265	9.9 (2H, s, NH), 7.5 (1H, s, thiazole ring), 8.4 (1H, s, NH), 5.4 (1H, dd, H7), 5.1 (1H, d, H6), 3.16 (2H, d, H2), 4.8 (2H, s, CH_2O), 4.1 (3H, s, $NOCH_3$), 8 -7.50 (4H, m, aro ring), 1.4 (9H, s, t-butyl group).	171, 162, 128.2, 172, 139, 17.4, 58.2, 58.9, 54.5, 59, 163, 164, 108, 121, 171, 128.1, 151, 122, 145, 160, 125, 75, 29.	633

Table 2: *In vitro* antibacterial activity in terms of zone of inhibition (mm, and is the average of 3 since every reading was taken in triplicate) of newly synthesized semisynthetic cephalosporins 1 - 9 against various grampositive and gram –ve bacteria.

Bacteria	Gram positive/negative	Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8	Drug 9
Citrobacter freundii	negative	8	9.2	11.5	10	13	14	14	15	20
Klebsiells pneuminoae	negative	5	6	15.5	14	5	9	9	10	11.5
E.coli ATCC 25922	negative	6	6.5	11	8	14	14.7	16	16	17
Entero. Facealis ATCC 29912 MTCC 3216	positive	8	8.2	10	7.5	13.5	14	16	15	16
Staph. Aureus ATCC 25923	positive	12	12	14	10	11	9	13	15	16
Acinetobacter 426	negative	6	6	8	6	7	9	10	10	12
Salmonella typhimurium	negative	-	-	2	-	-	-	3	4	2
Salmonella paratyphi	negative	-	-	1	-	-	-	4	2	2
Shigella flexinii	negative	6	6	8	6	8	10	13	12	16
Vibrio cholerae	negative	4	4	6	4	6	10	13	12	12
Pseudo.aeru. ATCC 27853	negative	6	6	8	6	10	12	14	15	16
Proteus vulgaris	negative	6	6	10	8	9	10	10	12	14
Listeria monocytogenes	positive	-	-	2	-	-	4	4	3	3
Helicobacter pylori	negative	-	-	2	-	-	6	4	-	-
Staph. Aureus	positive	8	8	10	6.5	10	11.4	14	12	15
Strptococcus heamophila	positive	-	-	2	-	-	6	6	5	4

Table 3: In vitro antibacterial activity in terms of MIC (µg/ml) of semisynthetic cephalosporins 1 - 9 against various gram positive and gram –ve bacteria.

Bacteria	Gram positive/negative	Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8	Drug 9
Citrobacter freundii	negative	3.125	3.125	1.563	1.563	0.781	0.391	0.391	0.391	0.097
Proteus mirabilis	negative	6.25	6.25	1.563	1.563	0.781	0.391	1.563	0.391	0.097
Klebsiells pneuminoae	negative	12.5	6.25	0.391	0.391	12.5	3.125	3.125	3.125	1.562
E.coli ATCC 25922	negative	6.25	6.25	1.562	3.125	0.390	0.390	0.390	0.390	0.195
Entero. Facealis ATCC 29912	positive	3.125	3.125	3.125	3.125	0.390	0.390	0.390	0.390	0.390
Salmo. Typhi MTCC 3216	negative	1.562	1.562	3.125	3.125	1.562	1.562	1.562	3.125	3.125
Staph. Aureus ATCC 25923	positive	0.781	0.781	0.781	1.562	0.390	1.562	0.390	0.390	0.195
Acinetobacter 426	negative	6.25	6.25	3.125	3.125	3.125	6.125	3.125	1.562	1.562
Salmonella typhimurium	negative	100	100	50	100	100	50	25	50	25
Salmonella paratyphi	negative	100	100	50	100	100	25	25	50	25
Shigella flexinii	negative	6.25	6.25	3.125	3.125	1.562	3.125	0.781	0.781	0.391
Vibrio cholerae	negative	12.5	12.5	6.25	12.5	1.562	6.25	0.781	0.781	0.781
Pseudo.aeru. ATCC 27853	negative	6.25	6.25	6.25	6.25	1.562	1.562	0.781	0.391	0.391
Proteus vulgaris	negative	6.25	6.25	3.125	3.125	3.125	1.562	3.125	3.125	0.781
Listeria monocytogenes	positive	100	100	25	100	50	50	50	25	25
Helicobacter pylori	negative	100	100	50	50	50	25	25	50	50
Staph. Aureus	positive	3.125	3.125	3.125	12.5	1.562	1.562	0.781	0.781	0.390
Strptococcus heamophila	positive	100	100	25	25	6.25	50	50	6.25	6.25

Further studies were focused on determining Minimum Bactericidal Concentration (MBC) of short-listed molecules. Following the usual procedure of subculturing from each tube showing MIC, we observed that MBC was equal to MIC and the drug molecules were found to be effective bactericidal. The drug has a bactericidal effect when given in concentration exceeding the bacteriostatic dose. Since the molecules under study are acting as bactericidal agent, it may be proposed that they destroy the system that controls the osmotic properties of the bacterial cell wall. The mode of action by which known bacteriocidal agents kill bacteria is varied and includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components. It may be possible that they inhibit enzyme transpeptidase which is essential for bacterial cell wall synthesis. Bacteria eventually lyse due to ongoing activity of cell wall autolytic enzymes (autolysins and murein hydrolase) while cell wall assembly is arrested. In many cases the exact mechanism of killing is not known. In this work, further studies are going on to clarify the mechanism of action of newly synthesized semi-synthetic cephalosporins, but it can be hypothesized that these semi-synthetic cephalosporins are also working on any one of these mechanisms.

CONCLUSION

In general, attempts to modify the β- lactam thiazolidine ring system of penicillin without loss of antibacterial activity had been unsuccessful. The discovery, structure elucidation and modification of cephalosporin C, which led to the marketing of important new antibiotics, have provided additional impetus for the study and synthesis of the β- lactam antibiotics. In the past decade, even though cephalosporin antibiotics have made remarkable progress and contribution in the treatment of acute disease originated from pathogenic infection in clinics, many efforts still exist to achieve the well-balanced broad-spectrum and to improve β--lactamases stability. In a search for unique and potent cephalosporin antibiotics we have prepared new semi-synthetic cephalosporins **1- 9.** The motivation for synthesizing these semi-synthetic cephalosporins was to increase the availability of drug at the target site and their oral absorptivity, increased stability. Thus, recurring need for an easily cleaved blocking group for the carboxylic acid in the cephalosporin synthetic chemistry forms the basis of the present work. All the synthesised cephalosporins were having easily hydrolysable esters for oral absorption studies; they were also having such suitable blocking groups for the carboxyl, which might be removed later without disruption of the β--lactam ring. Although simple esters, like the methyl ester, are known to possess diminished antibiotic acitivity compared to the free acids, the possibility exists that more easily hydrolysable esters (by enzymatic or chemical means) might exhibit significant *in vivo* activity. A therapeutic advantage might be anticipated from derived compounds if the structural environment of the carboxyl group is a bar to absorption through the gastric or intestinal walls. Activity could be inherent in the derivative or be produced a result of enzymatic cleavage to the parent compound after absorption has occurred. Variations of the cephalosporin C at the three possible positions, which is exploited in the present work, lead to highly active, acid stable, penicillin resistant, nontoxic antibiotic with increased potency against a wide range of bacteria. There still remain several technical considerations which require resolutions. However, the result establish, for the synthesis of new generation cephalosporins with different processing strategies, will be an useful concept, which is likely to be used with considerable advantage.

ACKNOWLEDGEMENT

One of the authors (Kumar Gaurav) is thankful to University Grants Commission, New Delhi, for financial assistance as senior research fellowship and the NMR Centre, Indian Institute of Science, Bangalore, India for providing spectral data.

REFERENCES

1. Elander RP, Industrial production of beta-lactam antibiotics. Applied Microbiology and Biotechnology, 2003; 61 (5-6): 385-392.
2. Nigam VK, Verma R, Kumar A, Kundu S, Ghosh P, Influence of medium constituents on the biosynthesis of cephalosporin-C. Electronic Journal of Biotechnology ISSN: 0717-3458, 2007; 10 (2) issue of April 15.
3. Mandell GL, Sande MA, In G. A. Goodman, L. S. Goodman, and A. Gilman (eds.), Goodman and Gilman's The Pharmaceutical Basis of Therapeutics, 8th edn., MacMillan Publishing Company Inc., New York, 1991, pp. 1065-1098.
4. Dasari VRRK, Donthireddy SRR, Nikku MY, Garapati HR, Optimization of medium constituents for *Cephalosporin C*production using response surface methodology and artificial neural networks. J Biochem Tech., 2009; 1(3):69-74.
5. Medeiros AA. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis., 1997; 24 (Suppl.1): 9-45.
6. Nigam VK, Kundu S, Ghosh P, Continuous production of 7-Aminocephalosporanic acid by immobilized cells of

Pseudomonas diminuta. Biocatalysis and Biotransformation, 2009; 27(1): 60-65.

7. Stan C, Dumitrache M, Diaconu DE. Means of purification of cephalexin with a view to therapeutic use. Rev Med Chir Soc Med Nat Iasi., 2004; 108 (3):718- 720.
8. Jones RN. The antimicrobial activity of cefotaxime: comparative multinational hospital isolate surveys covering 10 years. Infection, 1994; 22 (Suppl 3): 152-160.
9. Jacoby BK. Amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant β-lactamases. http://lahey.org/studies/webt.htm, 2000.
10. Babini GS, Livermore DM. Antimicrobial resistance amongst Klebsiella spp. collected from intensive care units in southern and western europe in 1997-1998. J.Antimicrob Chemother, 2000; 45: 183-189.
11. Wilson WR. The role of fourth-generation cephalosporins in the treatment of serious infectious diseases in hospitalized patients. Diagn Microbiol Infect Dis., 1998; 31: 473- 477.
12. Sanders CC. Cefepime: the next generation. Clin Infect Dis., 1993; 17: 369-379; (b) Garau J, Wilson W, Wood M, Carlet J, Fourth generation cephalosporins: a review of in vitro activity, pharmacokinetics, pharmacodynamics, and clinical utility Clin Microb Infect., 1997; 3:(Suppl 1): S87-S101; (c) Jones RN. Important and emerging β-lactamases-mediated resistances in hospital-based pathogens: the Amp C enzymes. Diagn Microbiol Infect Dis. 1998; 31: 461-466.
13. Gottlieb T, Wolfson C. Comparison of the MICs of Cefepime for extended-spectrum β-lactamase-producing and non-extended-spectrum β-lactamase-producing strains of *Enterobacter cloacae*. J Antimicrob Chemother, 2000, 46: 323-342.
14. Tzouvelekis LS, Tzelepi E, Prinarakis E, Gazouli M, Katrahoura A, Giakkoupi P, Paniara O, Legakis NJ. Sporadic emergence of Klebsiella pneumoniae strains resistant to cefepime and cefpirome in Greek hospitals. J. Clin. Microbiol, 1998; 36: 266-268.
15. Rodriguez JC, Hernandez R, Gonzalez M, Lopez MA, Fini A. IL Farmaco, 2000; 393–396.
16. Park SW, Choi SY, Chung KH, Hong SI, Kim SW, New method for the immobilization of glutaryl 7-aminocephalosporanic acid acylase on a modified epoxy/silica gel hybrid. J Biosci Bioeng., 2002; 94:218-224.
17. Pollegioni L, Lorenzi S, Rosini E, Marcone GL, Molla G, Verga R, Cabri W, Pilone MS, Evolution of an acylase active on cephalosporin C. Protein Sci., 2005; 14: 3064-3076.
18. Egorov AM, Kurochkina VB, Sklyarenko AV, Nys PS. Enzymatic transformation of beta lactam antibiotics; Trends of development and approaches to practical implementation. Biocatalysis, 2000; 41: 43-46.
19. Monti D, Carrea G, Riva S, Baldaro E, Frare G, Characterization of an industrial biocatalyst: immobilized glutaryl-7-ACA acylase. Biotechnol Bioeng., 2000; 70: 239 -244.
20. 20 Oh B, Kim M, Yoon J, Shin Y, Lee D, Kim Y Deacylation activity of cephalosporin acylase to cephalosporin C is improved by changing the side-chain conformations of active-site residues. Biochem Biophys Res Comm., 2003; 310: 19 -27.
21. Takimoto A, Takakura T, Tani H, Yagi S, Mitsushima K, Batch production of deacetyl 7-aminocephalosporanic acid by immobilized cephalosporin-C deacetylase. Appl Microbiol. Biotechnol., 2004; 65: 263-267.
22. Gallego FL, Batencor L, Hindalgo A, Mateo C, Lafuente RF, Guisan JM, One pot conversion of cephalosporin-C to 7-aminocephalosporanic acid in the absence of hydrogen peroxide. Adv Synth Catalysis, 2005; 347: 1804-1810.
23. Nigam VK, Kundu S, Ghosh P. Single-step conversion of cephalosporin-C to 7-aminocephalosporanic acid by free and immobilized cells of *Pseudomonas diminuta*. Appl. Biochem. Biotechnol, 2005; 126: 13–21.
24. Canan KUS. Synthesis of New Substituted 6-(morpholin-4-yl)-1H- benzimidazole derivatives. Turk. J. Chem, 2002; 559-564; (b) G¨oker H, Ku C. Synthesis of 1,2,5(6)-Trisubstituted Benzimidazoles and Evaluation of Their Antimicrobial Activities. Arch.Pharm.(Weinheim), 1995; 328: 425-430;
25. Garuti L, Roberti M, Gentilomi G. Synthesis and antiviral assays of some 2-substituted benzimidazole-*N*-carbamates. Il Farmaco, 2000; 55: 35-39; (b) Coburn RA, Clark MT, Evans RT, Genco RJ. Substituted 2-(2-hydroxyphenyl) benzimidazoles as potential agents for the control of periodontal diseases. J. Med. Chem., 1987; 30: 205-208.
26. Roth T, Morningstar ML, Boyer PL, Hughes SH, Buckheit RW, Michejda CJ. Synthesis and Biological Activity of Novel Nonnucleoside Inhibitors of HIV-1 Reverse Transcriptase. 2-Aryl-Substituted Benzimidazoles. J.Med. Chem., 1997; 40: 4199-4207; (b) Denny WA, Rewcastle GW, Baguley BC. Structure activity relationship for 2-Phenylbenzimidazole-4-carboxamide, a new class of "minimal DNA" intercalating agent which may not act via topoisomerase. J. Med.Chem., 1990; 33: 814-819; (c) Evans D, Hicks TA, Williamson WRN, Dawson W, Meacock SCR, Kitchen EA. Synthesis of a group of 1H-benzimidazoles and their screening for antiinflammatory activity. Eur. J. Med. Chem., 1996; 31: 635-642.
27. Guckian KM, Morales JC, Kool ET. Structure and Base Pairing Properties of a Replicable Nonpolar Isostere for Deoxyadenosine. J. Org. Chem., 1998; 63: 9652–9656; (b) Morales JC, Kool ET. Efficient replication between non-hydrogen-bonded nucleoside shape analogs. Nat. Struct. Biol., 1998; 5: 950–954.
28. Gudmundsson KS, Tidwell J, Lippa N, Koszalka GW, Draanen NV, Ptak, Drach JC, Townsend LB. Synthesis and antiviral evaluation of halogenated beta-D- and -L-erythrofuranosylbenzimidazoles. J. Med. Chem., 2000; 43: 2464–2472.; (b) Zou R, Ayres KR, Drach JC, Townsend LB. Synthesis and antiviral evaluation of certain disubstituted benzimidazole ribonucleosides. J. Med. Chem., 1996; 39: 3477–3482; (c) Zou R, Drach JC, Townsend LB. Design, synthesis, and antiviral evaluation of 2-substituted 4,5-dichloro- and 4,6-dichloro-1-D-ribofuranosyl benzimidazoles as potential agents for human cytomegalovirus infections. J. Med. Chem., 1997; 40: 802–810.
29. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotics susceptibility testing by a standardized single disk method. Amer. J. Clinical pathology, 1966; 45: 493-496.
30. Sumana MN, Rajgopal V. A study of dermatophytes and their in-vitro antifungal sensitivity. Ind. J. Pathol. Microbiol, 2002; 45 (2): 169- 172.
31. A guide to sensitivity testing. Report of the Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. J. Antimicrobial Chemotherapy, 1991; 27: supplement D, 1-50.