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Antimicrobial Activity of Some Diaminobenzoic Acid Derivatives

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ABSTRACT

Heterocyclic compounds constitute the largest family of organic compounds, regardless of structure and functionality. Heterocyclic compound are of particular interest in medicinal chemistry and this has catalyzed the discovery and development of many new heterocyclic compounds. The compound bearing thiazole and oxazole moiety have been found to possess antimicrobial, antitubercular and anti-inflammatory activity.

Benzimidazole derivatives of 3,4-diaminobenzoic acid derivative (IV a, b), have been synthesized which are tautomer of each other, after the synthesis of 4-isothiocyanato-3-methylbutanal (CIT), a reagent. All the synthesized derivatives have been screened with various bacterial and fungal strains viz. Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Bacillus typhi, Candida albicans, Penicillium chrysogenum, Saccharomyces cerevisiae, Penicillium chrysogenum, Aspergillus niger.

After the antimicrobial studies, it were found that compound (IV)a can be act as a standard drug against fungal strain Candida albicans, Sacharomyces cerevisiae, as it showed more inhibition zone than the standard drug Ketoconazole respectively. Compound (IV) a showed very good activity against fungal strains Aspergillus niger and Penicillium chrysogenum as these derivatives showed comparable inhibition zone than the standard drug Ketoconazole. Thus these compounds can be used as a standard drug having fewer side effects.

Keywords: 3,4-diaminobenzoic acid, Antibacterial activity, Antifungal activity

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INTRODUCTION

Heterocyclic compound is one which possesses a cyclic structure with at least two different kinds of hetero atoms in the ring. Nitrogen, oxygen, sulphur are most common hetero atoms. Most of the hetero atoms exist in the form of five membered (furan) or six membered (pyran) rings containing one atom. Some member of vitamin B group possess heterocyclic ring containing nitrogen. Pyridoxine is an example of vitamin B_6, which is a derivative of pyridine essential in amino acid metabolism [1]. Nitrogen containing heterocycles with a sulfur atom are an important class of compounds in medicinal chemistry and has been considerable interest in the development of preparative methods for the production of pyrimidines. It is because pyrimidines represent one of the most active classes of compounds, possessing a wide spectrum of biological activity, known as the heterocyclic core of the nucleic acids bases. These ring systems are often incorporated into drugs designed for anticancer, antiviral, antihypertensive, analgesic, antipyretic, anti-inflammatory, antisporiasis agents and some are active on the blood circulation system. They can stimulate the skin preparative regeneration and increase the efficacy of antibiotic therapy of *Staphylococcus* and *Proteus* infected wounds.

Some new heterocyclic compounds containing isooxazole, pyrazole, and oxadiazole ring systems were prepared from various chalcones and were very potent for their antimicrobial activities [2]. Chalcones and its derivatives synthesized by considering ketones with aromatic aldehydes in the presence of suitable bases and are very useful intermediates for the synthesis of five (1,2),

six (1,3) and seven membered heterocyclic compounds exhibit diverse pharmacological activities. The preparation of heterocycles starting from chalcone precursors that have been tested for their antimicrobial activities [3]. Various heterocyclic derivative exhibit a wide range of biological activities and exhibits activities such as anti-inflammatory and analgesic [4-6], antitumor [7-13] and antimicrobial [14-22] etc. In bio organic and medicinal chemistry, 2-aminobenzothiazole derivatives are broadly found with the applications in drug discovery and development of the treatment of diabetes, epilepsy, inflammation, amyotrophic lateral sclerosis, analgesic, tuberculosis and viral infection.

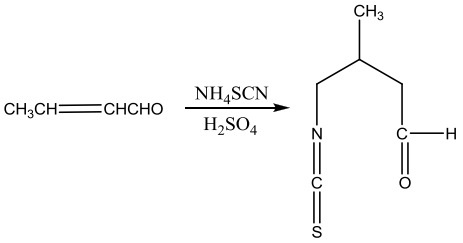
The benzimidazole ring is an important pharmacophore in modern drug discovery. A variety of benzimidazole is in use, like thiabendazole and flubendazole (anthelmintic), omeprazole and lansoprazole (antiulcerative) and astemizole (antihistaminic). Imidazoles are generally known as anticancer agents and are heterocyclic compounds containing 5-membered planar ring, soluble in water and other polar solvents. Imidazoles are of two equivalent tautomeric forms because of hydrogen atom which is located on either of two nitrogen atoms. They are amphoteric and therefore can function as both acid and base. The Thiadiazole & their derivatives have shown the number of pharmacological activity as antimicrobial, anti-inflammatory activity, anti-tubercular activity, ant diabetic activity, diuretics, antidepressant & cytotoxic activity. These thiadiazole are the heterocyclic compound which contain the five member ring, nitrogen & sulphur. The biological activities of 1,3,4-thiadiazole derivatives have been studied by Singh et al. [23].

The chemical synthesis of new molecules with biological activity is very interesting and opens new fields of research, important in molecular biology and pharmacology. In continuation of above research work we are trying to synthesize some potent heterocyclic compounds in our laboratory for their antimicrobial activity.

METHODOLOGY

STEP I: Synthesis of 4-isothiocyanato-3-methylbutanal (CIT)

4-isothiocyanato-3-methylbutanal was prepared by adding H_2SO_4 (49 g; 0.5 mol), diluted with distilled water (50 ml) to croton aldehyde (70 g; 1 mol) over a period of 25 min at 15ºC. Ammonium thiocyanate (76 g; 1 mol) dissolved in water (100 ml), was added to the mixture at 21ºC. After stirring for 15 min the upper oily layer was separated with the help of separating funnel and washed with aqueous sodium carbonate (saturated solution) and finally with water to free it from acid. The content were left over fused calcium chloride for 24 hrs and subjected to fractionation. The yield was 25.68 g.



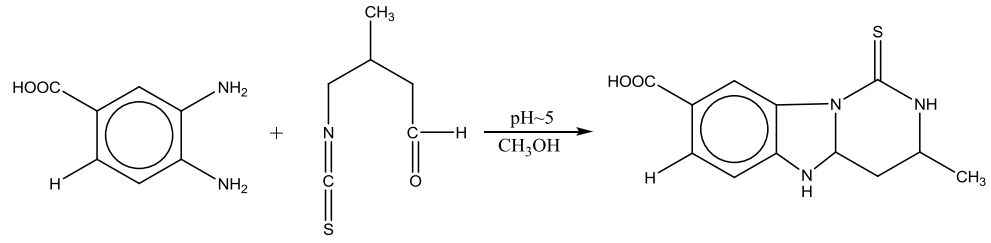
but-2-enal
Molecular Weight: 70.09
I

4-isothiocyanato-3-methylbutanal
Chemical Formula: C_6H_9NOS
Molecular Weight: 143.21
II

Scheme 1: Schematic representation of the synthesis of 4-isothiocyanato-3-methylbutanal

STEP II: General procedure for the condensation of 3,4-diaminobenzoic acid with CIT

4-isothiocyanato-3-methylbutanal (0.8 ml) was added to a solution of 3,4-diaminobenzoic acid (1 g) in methanol (10 ml). The pH of the reaction medium was adjusted to about ~5 by adding few drops of 10% H_2SO_4 (10% H_2SO_4 in methanol). The reaction mixture was heated under reflux for 8 hrs under reduced pressure and gets no compound. The rest mixture was used in packing the column and performed the column chromatography. At last we get the final compound of yield 0.315 g.



3,4-diaminobenzoic acid
Chemical Formula: $C_7H_8N_2O_2$
Molecular Weight: 152.15
III

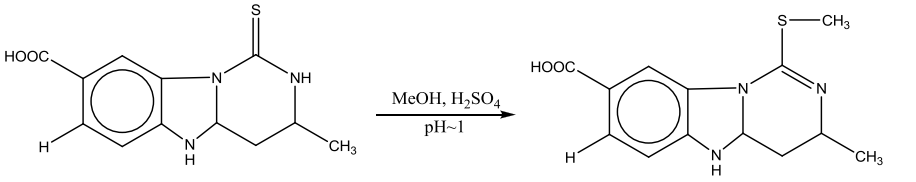
4-isothiocyanato-3-methylbutanal
Chemical Formula: C_6H_9NOS
Molecular Weight: 143.21
II

3,4-diaminobenzoic acid derivative
Chemical Formula: $C_{12}H_{13}N_3O_2S$
Molecular Weight: 263.32
IV

Scheme 2: Schematic representation of condensation of 3,4-diaminobenzoic acid

STEP III: General procedure for S-Methylation of 3,4-diaminobenzoic acid derivative

3,4-diaminobenzoic acid derivative (0.2 g) was dissolved in methanol (20 ml) and add conc. H_2SO_4 til the pH becomes 1. Reaction contents having pH~1 was heated under reflux for 8 hrs under reduced pressure and gets no compound. The rest mixture was used in packing the column and performed the column chromatography. At last we get the final compound of yield 0.015 g.



3,4-diaminobenzoic acid derivative
Chemical Formula: $C_{12}H_{13}N_3O_2S$
Molecular Weight: 263.32
IV

S-Methylated derivative of 3,4-diaminobenzoic acid
Chemical Formula: $C_{13}H_{15}N_3O_2S$
Molecular Weight: 277.34
V

Scheme 3: Schematic representation of S-methylation 3,4-diaminobenzoic acid derivative

STEP IV–Detection of elements

Test for sulphur

Sodium extract take in small test tube, add in excess of acetic acid and few drops of lead acetate, black precipitate obtained. Sulphur is confirmed.

Test for nitrogen

Treat 2 ml sodium extract with 2-3 drops of dil H_2SO_4. Now added a small amount of solid $FeSO_4$, persian blue colour appears. Nitrogen is present.

Test for halogen

Taking about 2-3 ml of sodium extract and acidity with some drops of dilute HNO_3. Now evaporates the solution to almost 1 ml. This will expel hydrogen cyanide and hydrogen sulphide. Dilute the solution with equal volume of water and add 2-3 ml of silver nitrate solution. If no precipitate is found, it indicate, no halogen atom is present.

STEP V: General experimental procedure

Melting Points (m.p.) were determined by a JSGW apparatus and are uncorrected. Only principle sharply defined I.R. peaks are reported. Thin Layer Chromatography (TLC) was performed on silica gel-G for TLC and spots were visualized by iodine vapour. Column chromatography was performed by using Molychem silica gel for column chromatography (100-200 mesh). For all compounds yield, m.p. and spectral data are reported.

STEP VI: Antimicrobial activity

In this study we reported the antimicrobial activity of 3,4 diaminobenzoic acid against various pathogenic microorganism.

Microorganisms

The bacterial and fungal cultures *Escherichia coli, Bacillus cereus, Salmonella, Pseudomonas, Aspergillus niger, Candida albicans, Penicillium chrysogenum, Saccbaxomyces* were provided by department of Microbiology, Dolphin (PG) Institute of Bio-Medical and Natural Sciences, Manduwala, Dehradun. The bacterial and fungal cultures were stored on Nutrient Broth and Sabouraud Dextrose Broth respectively at 4ºC. The bacterial and fungal cultures were grown on the Muller Hinton and Sabouraud Dextrose Agar respectively.

Culture media

For bacterial culture purpose Nutrient Broth and Muller Hinton Agar media (HIMEDIA) were used. For fungal culture purpose, Sabouraud Dextrose Broth and Sabouraud Dextrose Agar (HIMEDIA) were used.

Antimicrobial assay

The *in-vitro* antibacterial and antifungal effect of 3,4-diaminobenzoic acid derivatives were determined by Disc and Hole method. The bacterial strains were sub-cultured in Muller-Hinton Agar and incubated at 37ºC for 24 hrs. Turbidity of the suspension was adjusted to the Mac Farland Standard (0.5) and 100 µl of suspension plated on Muller Hinton Agar; wells were made with the help of (6 mm) borer. Prepare the solution of each derivative (1^{st} and 2^{nd}) and standard drug in 200 mg/ml concentration and 100 µl of each solution (Dimethyl sulfoxide) loaded in each well against the control (solvent) and standard drug Amoxicillin. Plates were incubated at 37ºC for 24 hrs and recorded the zone of inhibition or sensitivity against *Escherichia coli, Bacillus cereus, Pseudomonas Salmonella.*

For antifungal test, the fungal cultures were grown in Sabouraud Dextrose Agar for 96 hrs adopting the above procedure, made suspension of subcultured organisms. Plates were incubated at 27ºC for 72 hrs and recorded the zone of inhibition or sensitivity against *Aspergillus niger, Candida albicans, Penicillium chrysogenum, Saccbaxomyces* against the standard drug Ketoconazole (Scheme 1-3).

RESULTS AND DISCUSSION

4-isothiocyanato-3-methylbutanal (II) on condensation with 3,4-diaminobenzoic acid (III) by refluxing for eight hrs in methanol at pH~5. During the reaction no compound separated out. After refluxing of eight hrs, reaction mixture was used in packing column and performed column chromatography. In column chromatography, we used different polarity combination of several solvents (Table 1).

Table 1: Polarity during column chromatography

S. No.	Combination of different solvents	Observations
1.	Petroleum ether	Absence of any spot
2.	Petroleum ether: CCl_4 (5:5)	Absence of any spot
3.	CCl_4 (pure)	Absence of any spot
4.	Petroleum ether: Ethyl acetate (8:2)	Absence of any spot
5.	Petroleum ether: Ethyl acetate (5:5)	Single spot
6.	Petroleum ether: Ethyl acetate (2:8)	Single spot compound
7.	Ethyl acetate (pure)	Mixture
8.	Methanol: Petroleum ether (1:9)	Mixture
9.	Methanol: Petroleum ether (5:5)	Mixture
10.	Methanol (pure)	Mixture

From the column chromatography, we get 0.384 g compound (IV). The compound (IV) having following properties:

1. Yield - 0.384 g
2. % Yield - 22.19%
3. Melting point - 230ºC
4. Solubility - DMSO, THF, Partially soluble in methanol
5. Element detection - Nitrogen and sulphur are present and halogen are absent
6. Elution - Petroleum ether: Ethyl acetate, Ethyl acetate: Petroleum ether
7. Solvent of crystallization - Methanol
8. I.R. Spectra

From the column chromatography, we get 0.257 g (IVa) compound. After column chromatography, we get different compounds, i.e., compound (IV) and compound (IV) a which are tautomer of each other (Table 2).

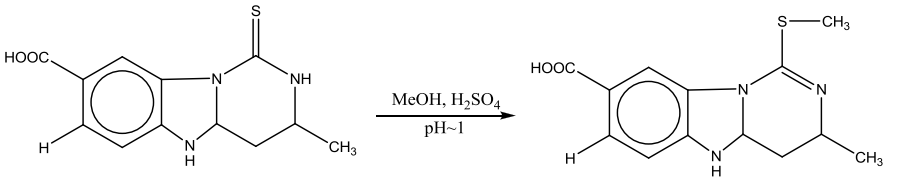
Table 2: Column chromatography

Wave number (cm^{-1})	Type of vibrations
3392.75	N-H Stretching
3021.53	C-H Stretching
2969.32	C-H Stretching
2917.19	O-H Stretching
1674.84	C=O Stretching
1460.83	C-H Def in methyl
1304.79	C-H Def
899.96	C-N Stretching

The compound (IV) a having following properties:

1. Yield - 0.257 g
2. % Yield - 14.85%
3. Melting point - 222ºC
9. Solubility - DMSO, THF, Partially soluble in methanol
4. Element detection - Nitrogen and sulphur are present and halogen are absent
5. Elution - Petrolium ether: Ethyl acetate, Ethyl acetate: Petrolium ether
6. Solvent of crystallization - Methanol
7. I.R. Spectra

Step 3: S-Methylation of 3,4-diaminobenzoic acid derivative



3,4-diaminobenzoic acid derivative
Chemical Formula: $C_{12}H_{13}N_3O_2S$
Molecular Weight: 263.32
IV

S-Methylated derivative of 3,4-diaminobenzoic acid
Chemical Formula: $C_{13}H_{15}N_3O_2S$
Molecular Weight: 277.34
V

3,4-diaminobenzoic acid derivative (IV) was dissolved in methanol and add conc. H_2SO_4 till the pH becomes 1. Reaction contents having pH~1 was heated under reflux for 8 hrs under reduced pressure and gets no compound. The rest mixture was used in packing the column and performed the column chromatography. At last we get the final compound S-Methylated derivative of 3,4-diaminobenzoic acid. In column chromatography, we used different polarity combination of several solvents (Table 3).

Table 3: Column chromatography

Wave number (cm^{-1})	Type of vibrations
3393.84	N-H Stretching
3034.57	O-H Stretching
1739.02	C=O Stretching
1600.36	C=N Stretching
1510.19	C-C Stretching (Due to aromatic ring)
1082.55	C=N Stretching

From the column chromatography, we get 0.015 g compound (V).

The compound (V) having following properties:

10. Yield - 0.015 g
11. % Yield - 1.42%
12. Melting point - 234ºC
13. Solubility - DMSO, Partially soluble in methanol
14. Element detection - Nitrogen and sulphur are present and halogen are absent
15. Elution - Petrolium ether: Ethyl acetate, Ethyl acetate: Petrolium ether
16. Solvent of crystallization - Methanol
17. I.R. Spectra

NMR was also done and reported in one of our published paper (Tables 4 and 5) [24,25].

Table 4: Polarity during column chromatography

S. No.	Combination of different solvents	Observations
1.	Petroleum ether	Absence of any spot
2.	Petroleum ether: $CHCl_3$ (5:5)	Absence of any spot
3.	$CHCl_3$ (pure)	Absence of any spot
4.	Petroleum ether: Ethyl acetate (8:2)	Absence of any spot
5.	Petroleum ether: Ethyl acetate (5:5)	Absence of any spot
6.	Petroleum ether: Ethyl acetate (2:8)	Absence of any spot
7.	Ethyl acetate (pure)	Absence of any spot
8.	Methanol: Petroleum ether (2:8)	Absence of any spot
9.	Methanol: Petroleum ether (5:5)	Absence of any spot
10.	Methanol: Petroleum ether (5:5)	Absence of any spot
11.	Methanol (pure)	Single spot

Table 5: Column chromatography

Wave number (cm^{-1})ti	Type of vibrations
2921.74	O-H Stretching
1739.13	C=O Stretching
1634.78	C=N Stretching
782.61	C-S Stretching weak band

Antimicrobial activity of prepared compounds

The prepared 3,4-diaminobenzoic acid derivatives were screened for antibacterial and antifungal action. The compounds under investigation were found active against both bacterial and as well as fungal strains tested.

Microorganism used

Bacteria

1. *Escherichia coli*
2. *Bacillus cereus*
3. *Salmonella typhi*
4. *Pseudomonas pneumonia*

Fungi

1. *Aspergillus niger*
2. *Candida albicans*
3. *Penicillium chrysogenum*
4. *Saccharomyces cerevisiae*

Antibacterial activity

The agar well diffusion method was used. The test organisms (bacterial cultures) were spread on the prepared Muller Hinton Agar plates. Well of 6 mm diameter were punched into the agar medium. The each well 100 µl of each compound (IV) and (IV) a and standard drug Ciprofloxacin were added and allowed to diffuse. The plates were then incubated at 37ºC for 24 hrs.

The antibacterial activities for different strains of bacteria were tested for each compound against the standard drug Ciprofloxacin and recorded the zone of inhibition in millimeter (Table 6).

Table 6: Antibacterial activity of 3,4-diaminobenzoic acid derivatives and standard drug Ciprofloxacin

S. No.	Name of the test organisms	Antibacterial activity of compound (IV)	Antibacterial activity of compound (IV)[a]	Antibacterial activity of standard drug Ciprofloxacin
1.	*Escherichia coli*	14 mm	10 mm	43 mm
2.	*Bacillus cereus*	(-)	10 mm	50 mm
3.	*Salmonella typhi*	10 mm	12 mm	42 mm
4.	*Pseudomonas pneumonia*	8 mm	(-)	40 mm

Antibacterial activities indicated that the both compounds possess a less spectrum of activity against the tested bacterial strains. Compound (IV) was in active against *Bacillus cereus* and showed very mild activity against *Escherichia coli* (14 mm inhibition zone), *Salmonella typhi* (10 mm inhibition zone) and *Pseudomonas pneumonia* (8 mm inhibition zone).

Compound (IV) a was inactive against *Pseudomonas pneumonia*. This compound was shown mild activity against *Escherichia coli* (10 mm inhibition zone), *Bacillus cereus* (10 mm inhibition zone) and *Salmonella typhi* (12 mm inhibition zone).

Antifungal activity

The agar well diffusion method was used. The test organisms (fungal cultures) were spread on the prepared Sabouraud Dextrose Agar plates. Well of 6 mm diameter were punched into the agar medium. The each well 100 µl of each compound (IV) and (IV) a and standard drug Ketoconazole were added and allowed to diffuse. The plates were then incubated at 37ºC for 72 hrs.

The antifungal activities for different strains of fungal were tested for each compound against the standard drug Ketoconazole and recorded the zone of inhibition in millimeter (Table 7).

Antifungal activities indicated that the both compounds possess a mild spectrum of activity against the tested fungal strains. Compound (IV) was in active against *Penicillium chrysogenum* and showed very mild activity against *Aspergillus niger* (15 mm inhibition zone), *Candida albicans* (10 mm inhibition zone) and *Saccharomyces cerevisiae* (12 mm inhibition zone).

Compound (IV) a good activity against against *Candida albicans* (20 mm inhibition zone) and *Saccharomyces cerevisiae* (15 mm inhibition zone). This compound showed significant activity against *Aspergillus niger* (19 mm inhibition zone) and *Penicillium chrysogenum* (17 mm inhibition zone).

Table 7: Antifungal activity of 3,4-diaminobenzoic acid derivatives and standard drug Ketoconazole

S.No.	Name of the test organisms	Antifungal activity of compound (IV)	Antifungal activity of compound (IV)[a]	Antifungal activity of standard drug Ketoconazole
1.	*Aspergillus niger*	15 mm	19 mm	23 mm
2.	*Candida albicans*	10 mm	20 mm	12 mm
3.	*Penicillium chrysogenum*	(-)	17 mm	21 mm
4.	*Saccharomyces cerevisiae*	12 mm	15 mm	13 mm

CONCLUSION

Antibacterial activity

In bacterial strains the compound (IV) was inactive against: *Bacillus cereus* and mild active against *Escherichia coli, Salmonella typhi, Pseudomonas pneumonia.* In bacterial strains the compound (IV) a was inactive against: *Pseudomonas pneumonia,* and mild active aganist *Escherichia coli, Salmonella typhi, Bacillus cereus.*

Antifungal activity

In fungal strain compound (IV) was inactive against: *Penicillium chrysogenum* and mild active *Aspergillus niger, Candida albicans, Saccharomyces cerevisiae.* In fungal strain compound (IV) a showed good activity: *Aspergillus niger*, *Penicillium chrysogenum* and excellent activity: *Candida albicans*, *Saccharomyces cerevisiae*

So we concluded that compound (IV) a can be act as a standard drug against fungal strain *Candida albicans*, *Sacharomyces cerevisiae,* as it showed more inhibition zone than the standard drug Ketoconazole respectively. Compound (IV) a showed very good activity against fungal strains *Aspergillus niger*, *Penicillium chrysogenum,* respectively.

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