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SYNTHESIS OF NOVAL DERIVATIVES OF 2-HYDROXY-5-SULFO-BENZOIC ACID & BIOLOGICAL AND PRESERVATIVE EVALUATION.

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ABSTRACT

A series of substituted anilides were synthesized and tested *in vitro* for antibacterial activity against Gram positive *B. subtilis, S. aureus* and Gram negative *E.coli* and as well as for antifungal activity against *C. albicans* and *A. niger*. The effective antimicrobial anilide derivatives from our earlier study were subjected to preservative efficacy testing in an official antacid preparation, (Aluminium Hydroxide Gel-USP) against *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli*, *Candida albicans* and *Aspergillus niger* as representative challenging microorganisms as per USP guidelines. A Nitro and Bromo group was found to be effective preservative against *E. coli, S .aureus, A. niger* and *C. albicans* and against *B. subtilis.* This study showed the potential of anilides to be used as a preservative in pharmaceutical products.

Keywords: Anilides, antimicrobial activity, preservative, Log CFU/ml.

INTRODUCTION

It is well known in literature that nitrogen and sulphur containing compounds are essentially used in medicinal purpose for the treatment of different kinds of fungal, bacterial infections along with treatment of gastric ulcer and cancer[1-3].The introduction of nitrogen and sulphur atom in organic moiety resulting towards higher efficacy against various

diseases[4-5].Since sulphur is capable of forming both σ and π bonds therefore the studies of their binding interaction with receptor moiety was also an interesting field of research during last few years[6-9].It is also revealed in literature that amines and amino compounds plays excellent role in controlling of various pathogenic diseases[10]. The present communication deals with the synthesis of some novel anilides of 2-Hydroxy-5-sulfo-benzoic acid along with their antimicrobial activity against pathogenic microbial strains.

Experimental Section

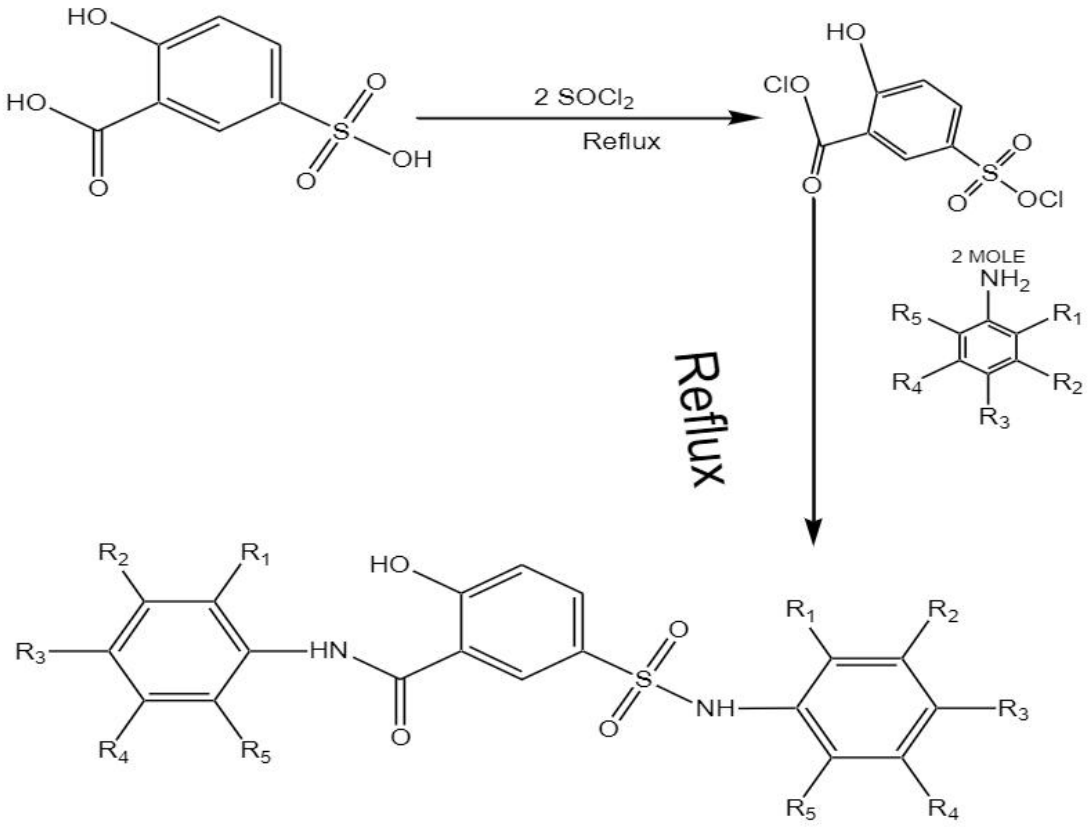
General Procedures. All chemicals used were of Ranbaxy Laboratories Ltd., Delhi; Qualigens, Mumbai and S.D. Fine Chemicals, Mumbai. Melting points in degree Celsius were determined with Elico melting point apparatus and are uncorrected. The FTIR spectra were recorded in KBr pellets on Perkin Elmer IR spectrophotometer. The 1H-NMR were recorded on Brucker Avance II 400 NMR spectrophotometer using $CDCl_3$ as solvent and TMS as internal standard (chemical shift in δ ppm). The purity of compounds was checked by thin layer chromatography (TLC) on silica gel plates. The spots were detected by exposure to iodine vapours.

2. Results and Discussion

A series of new 2-Hydroxy-5-sulfo-benzoic acid anilides were synthesized by reaction of substituted anilines with ester in moderate to good yield (Scheme 1). The intermediate esters were prepared by treatment of 2-Hydroxy-5-sulfo-benzoic acid with ethanol. The IR and ^{1}H NMR spectral data of the synthesized compounds were found in agreement with the assigned molecular structures. The physicochemical parameters of synthesized anilide derivatives used in present study are given in Table 1. Substituted anilides were evaluated for their in vitro antibacterial activity against Gram-positive *Staphylococcus aureus*, *Bacillus subtilis,* Gram-negative *Escherichia coli* and antifungal activity against *Candida albicans* and *Aspergillus niger* by serial dilution method [11] using ciprofloxacin and cotrimazole as reference standards for antibacterial and antifungal activity respectively and the results are presented in Table 2.

Compounds	Name of compound
1.	5-(3-Chloro-benzenesulfonyl)-N-(3-chloro-phenyl)-2-hydroxy-benzamide
2.	2-Hydroxy-5-(toluene-3-sulfonyl)-N-m-tolyl-benzamide
3.	2-Hydroxy-5-(4-methoxy-benzenesulfonyl)-N-(4-methoxy-phenyl)-benzamide
4.	5-(4-Bromo-benzenesulfonyl)-N-(4-bromo-phenyl)-2-hydroxy-benzamide
5.	2-Hydroxy-5-(2-nitro-benzenesulfonyl)-N-(2-nitro-phenyl)-benzamide

Scheme 1



Comp. 1= R1, R3, R4, R5, = H R2= Cl

Comp. 2= R1, R3, R4, R5, = H R2= CH_3

Comp. 3= R1, R3, R4, R5, = H R3= OCH_3

Comp. 4= R1, R3, R4, R5, = H R3= Br

Comp. 5 = R1, R3, R4, R5, = H R1= NO_2

Table 1. Physicochemical characteristics of substituted anilides.

S .No	Molecular formula	M.wt	%Yeild	Rf value	Melting point	Compound analysis		
						C	H	O
1.	$C_{19}H_{13}Cl_2NO_4S$	422	70	0.71*	132-135	54.04	3.10	3.32;
2.	$C_{21}H_{19}NO_4S$	381	67	0.69*	128-131	66.12	5.02	3.67;
3.	$C_{21}H_{19}NO_6S$	413	65	0.71**	131-134	61.01	4.63	3.39;
4.	$C_{19}H_{13}Br_2NO_4S$	511	60	0.62*	139-143	44.64	2.56	2.74;
5.	$C_{19}H_{13}N_3O_8S$	443	63	0.65**	126-129	51.47	2.96;	9.48;

TLC mobile phase - **Toluene: chloroform (1:1), *ethyl acetate: hexane (1:3).

All synthesized compounds were screened for antibacterial activity against E. coli, S. aureus and B. subtilis. Most of the compounds showed moderate activity at low concentration. Against *E. coli*, comp. 1 and comp.5 were found to have better activity than other titled compounds. Against *S. aureus*, all compounds were found to have moderate activity while comp. 4 and comp. 5 were found to have good activity. Against *B. subtilis*, almost all comp. 1, 4 and 5 were found to have better activity.

Table 2. Antibacterial and antifungal activity of substituted anilides.

Compounds	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
1	6.25	3.125	3.125	6.25	6.25
2	12.50	12.50	12.50	12.50	12.50
3	12.50	6.25	6.25	6.25	12.50
4	3.125	3.125	6.25	3.125	6.25
5	3.125	3.125	3.125	3.125	3.125
DRUG	0.15*	0.25*	0.01*	0.10**	0.30**

DRUG = CIPROFLXACIN* COTRIMAZOLE**

Against *A. niger*, Comp.5 show the excellent activity and Comp.4 were found to good activity. Against *C. albicans*, almost all titled compounds were found to good activity but the comp. 4 and 5 were found to have excellent activity. Comp.4 and 5 have great antimicrobial activity than other anilide derivatives. But none of the activity was comparable to standard.

3. Preservative Evaluation:

3.1 Preparation of Aluminum Hydroxide Gel-USP [12] **Formula**

Aluminium hydroxide gel	- 36 g
Mannitol	- 7 g
Methyl paraben	- 0.2 g
Propyl paraben	- 0.02 g
Saccharin	- 0.05 g
Peppermint oil	- 0.005 ml
Alcohol	- 1 ml
Purified water q.s.	- 100 ml

The weighed quantity of aluminum hydroxide gel and mannitol were triturated with 50 ml of water in a mortar. Methyl paraben, propyl paraben, saccharin and peppermint oil were dissolved in alcohol and added to above mixture and triturated well. The volume was made upto 100 ml with purified water. For preservative efficacy testing, the Aluminium hydroxide gel was prepared using the preservatives mentioned in Table 3.1 by replacing methyl paraben and propyl paraben from the above formula. The equimolar amount of selected preservatives (Table 3) were calculated with reference to the amount of methyl paraben (0.0013 mol) and added into the pharmaceutical products.

Table 3 Amount of selected preservatives added in Aluminum Hydroxide Gel-USP

Preservative Amount (g)

Preservative	Amount (g)
2-Hydroxy-5-sulfo benzoic acid	0.232
Comp. 4	0.399
Comp. 5	0.329

Preparation of inoculums

The representative microorganisms were inoculated in nutrient agar I.P. (S. aureus, B. subtilis, E. coli) and sabouraud agar I.P. (C. albicans, A. niger). The seeded plates were incubated at 37°C for 24 h (S. aureus, B. subtilis, E. coli), 37°C for 48 h (C. albicans) and 25°C for 7 d (A. niger). After the inoculation period, suspensions of microorganisms were prepared in sterile saline solution (0.9% w/v NaCl) to give a microbial count of 1x 10^4 CFU/ml.

Test Procedure

Aluminium hydroxide gel-USP in their final container was used in the challenge test. The preparation was inoculated with the microbial cell suspension with a cell count of 1 x 10^4 CFU/ml. The inoculums never exceeded 1% of the volume of the product sample. Inoculated samples were mixed thoroughly to ensure homogeneous microorganism distribution and incubated. The CFU/ml of the product was determined at an interval of 0, 7, 14, 21 and 28 days on agar plate. The log values of number of CFU/ml (Table 3 - Table 3.4) of Aluminium hydroxide gel was calculated and compared as per the guidelines of USP 2004.

Criteria of acceptance for preservative system

As per USP NF 2004 the requirement for antacid made with an aqueous base, preservative effectiveness are met if there is no increase from initial calculated count at 14th and 28th days in case of bacteria, yeast and moulds. Where, no increase is defined as not more than 0.5 $\log^{10}$ higher than previous value measured (USP 2004).

Preservative efficacy testing in Aluminum Hydroxide Gel-USP: [13,14]

Aluminum Hydroxide gel with different preservatives were prepared as described in 3.1.1. The products were sterilized in autoclave at 120^0 C for 15 minutes. The products were

then inoculated separately with 10^4 to 10^5 CFU/ml of *S. aureus, B. subtilis, E. coli, C. albicans* and *A. niger* and stored at room temperature (25^0C). As per USP NF 2004 the requirement for antacid made with an aqueous base, preservative effectiveness are met if no increase from initial calculated count at 14^{th} and 28^{th} days in case of bacteria, yeast and molds. Where, no increase is defined as not more than 0.5 log^{10} higher than previous value measured. The 2-Hydroxy-5-sulfo benzoic acid, Comp. 4 and Comp. 5 were selected for preservative efficacy testing in pharmaceutical product (Aluminium Hydroxide Gel-USP).

Table 4. Bacterial count (CFU/ml) of *B. subtilis* in Aluminium Hydroxide Gel-USP supplemented with preservatives.

Comp.	LOG CFU/Ml (time in days)				
	0	**7**	**14**	**21**	**28**
2-Hydroxy-5-sulfo-benzoic acid	0.471	0.305	0.305	0.000	0.471
Comp 4	0.471	0.305	0.000	0.471	0.000
Comp 5	0.000	0.000	0.000	0.000	0.305
Methyl paraben and propyl paraben	0.000	0.000	0.000	0.000	0.000
control	0.698	0.602	1.113	0.305	0.845

Table 5. Bacterial count (CFU/ml) of S. *aureus* in Aluminium Hydroxide Gel- USP supplemented with preservatives

Comp.	LOG CFU/Ml (time in days)				
	0	**7**	**14**	**21**	**28**
2-Hydroxy-5-sulfo-benzoic acid	0.000	0.000	0.000	0.000	0.305
Comp 4	0.471	0.305	0.000	0.305	0.000
Comp 5	0.778	0.305	0.000	0.305	0.000
Methyl paraben and propyl paraben	0.602	0.305	0.000	0.305	0.471
control	0.903	0.471	0.602	0.778	0.845

Table 6. Bacterial count (CFU/ml) of *E. coli* in Aluminium Hydroxide Gel-USP supplemented with preservatives.

Comp.	LOG CFU/Ml (time in days)				
	0	**7**	**14**	**21**	**28**
2-Hydroxy-5-sulfo-benzoic acid	0.471	0.305	0.305	0.698	0.471
Comp4	0.000	0.000	0.000	0.305	0.000
Comp5	0.000	0.305	0.000	0.305	0.305
Methyl paraben and propyl paraben	0.778	0.000	0.602	0.305	0.698
control	0.845	0.602	0.778	0.954	1.041

Table 7.Bacterial count (CFU/ml) of *C. albicans* in Aluminium Hydroxide Gel-USP supplemented with preservatives.

Comp.	LOG CFU/Ml (time in days)				
	0	7	14	21	28
2-Hydroxy-5-sulfo-benzoic acid	0.305	0.305	0.305	0.000	0.305
Comp 4	0.000	0.000	0.305	0.000	0.000
Comp 5	0.305	0.000	0.305	0.305	0.305
Methyl paraben and propyl paraben	0.305	0.471	0.602	0.778	0.000
control	0.471	0.778	0.845	0.845	0.903

Table 8. Bacterial count (CFU/ml) of A. niger in Aluminium Hydroxide Gel- USP supplemented with preservatives.

Comp.	LOG CFU/Ml (time in days)				
	0	7	14	21	28
2-Hydroxy-5-sulfo-benzoic acid	0.471	0.698	0.305	0.000	0.000
Comp 4	0.000	0.305	0.000	0.305	0.000
Comp 5	0.305	0.305	0.305	0.000	0.471
Methyl paraben and propyl paraben	0.305	0.305	0.698	0.000	0.471
control	0.698	1.079	0.954	1.000	1.079

The results of preservative evaluation indicated that out of two selected Comp.4 and 5 were found to be the most effective preservatives against different representative microorganisms (*S. aureus, B. subtilis E. coli, A. niger* and *C. albicans).*

ANTIMICROBIAL EVALUATION.

The synthesized compounds were evaluated for their *in vitro* antimicrobial activity against Grampositive *S. aureus, B. subtilis,* Gram negative *E. coli* and also against fungi *C. albicans* and A.*niger*. The MIC (µg/ml) was determined by serial dilution technique[15] using double strength nutrient broth IP and Sabouraud dextrose broth IP as media for bacterial and fungal growth respectively. Ciprofloxacin and Cotrimazole were used as reference compounds in case of antibacterial and antifungal activity respectively. The compounds were dissolved in Dimethyl Sulfoxide to give a concentration of 100 µg/mL, which was serially diluted to give concentrations of 50.0, 25.0, 12.5, 6.25, 3.125 µg/mL in culture tubes containing 1 ml of nutrient medium. To all the tubes including standards and controls, 0.1 mL of fresh inoculum was added and the tubes were incubated at 37 ± 1°C for 24 h (bacteria), 37 ± 1°C for 48 h (*C. albicans*) and 25°C for 7 d (*A. niger*). The MIC was recorded in each case as the minimum concentration of compound, which inhibited the growth of tested

microorganism. From the MIC observed, the intermediate concentrations between MIC values were prepared by suitable dilution of stock solution and the accurate MIC values were determined.

General procedure (Preparation of anilides).

Acid chloride was prepared by the reaction of 0.15 mol of 2-Hydroxy-5-sulfo-benzoic acid with thionyl chloride. Anilides were prepared by drop wise addition of a solution of corresponding substituted aniline (0.3mol) in ether (50 mL) to a solution of respective acid chloride (0.3 mol) in ether (50 ml). An immediate reaction took place and the mixture was stirred for 30 min at room temperature which resulted in the precipitation of crude anilides. The resulting mixture was washed successively with 5% Hydrochloric acid and water to remove excess of aniline. The crude anilide was recrystallized from ethanol.

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