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Full Length Research Paper

Antipyretic, analgesic, anti-inflammatory and cytotoxic effects of four derivatives of salicylic acid and anthranilic acid in mice and rats

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Four-Substituted derivatives of salicylic and anthranilic acids: 2-hydroxy-5-azidosulfonylbenzoic acid (HASBA, 1), 2-acetyloxy-5- azidosulfonylbenzoic acid (AASBA, 2), 2-acetamido-5- azidosulfonylbenzoic acid (AMASBA, 3) and 2-acetamido-5-sulfonamidobenzoic acid (AMSABA, 4) were synthesized and evaluated for their analgesic, antipyretic, anti-inflammatory and cytotoxic activities. HASBA, AASBA and AMASBA showed higher analgesic activity than aspirin (ASA) at 100 mg/kg dose, while AMSABA showed the least analgesic property. AMASBA exhibited higher antipyretic activity than paracetamol (PCM), while HASBA, AASBA and AMSABA also showed antipyretic effects which were of equal potency to that of PCM. The order of anti-inflammatory effects of the four compounds is: AASBA > AMASBA > HASBA > AMSABA. The effects of the substituents on the biological activities of the synthesized compounds are discussed.

Key words: Salicylic acid derivatives, anthranilic acid, analgesic, anti-inflammatory, antipyretic, cytotoxicity.

INTRODUCTION

Salicylates are the class of compounds that are widely valued for their pain killing, antipyretic and anti-inflammatory properties (Moncada and Vane 1979; Insel, 1991). The most commonly known and used salicylates are salicylic acid (also called 2-hydroxybenzoic acid), aspirin (acetylsalicylic acid -ASA) and sodium salicyclates. They are used extensively for the relief of headache, inflammation, arthritis pain, and some are employed in the treatment of heart attacks and strokes in the elderly (Rainsford, 1984).

Their mode of action is the inhibition of the synthesis of prostaglandin and its derivatives that cause inflammation, pain, rise in temperature and related diseases (Moncada and Vane, 1979; Meade et al., 1993). Recently, salicylic acid has been used primarily as an intermediate in the production of agrochemicals, dyes and colorants products (Cremlyn, 1991; Raskin, 1992). Salicylate toxicity and poisoning are rare in recommended doses, however, salicylate poisoning and its side effects are prominent problem in developing countries where they are used as antipyretic in the management of infectious malaria, both in children and in elderly people. Meanwhile, there are development and introduction of new analgesic, antipyretic and anti-inflammatory agents that compete with aspirin. This has made chemists to search for a better tolerable drug which are devoid of toxic and side effects of aspirin has been shown in the CNS, respiratory, gastro-intestinal tract, hepatic, metabolic and coagulation systems of the body (Goldfrank et al., 1990). This work is the first stage in

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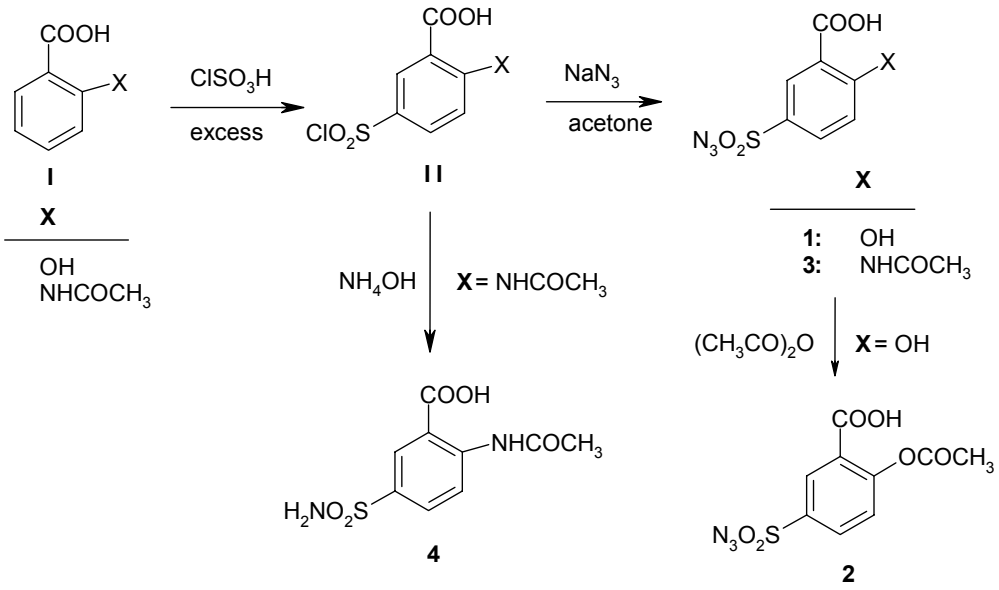

Figure 1. Outline for the Synthesis of Compounds **1-4**

Table 1. Ortho-Substituted Benzoic Acid Derivatives (**1 – 4**).

Compd.	Yield (%)	Mp (^{0}C)	X	Y	Formula	C (%)	H (%)	N (%)
1	76	176 – 179[a]	OH	N_3	$C_7H_5N_3O_5$ S	34.57 (34.21)	2.07 (1.98)	17.28 (17.10)
2	48 (68)	131 - 132	$OCOCH_3$	N_3	$C_9H_7N_3O_6$ S	37.89 (38.05)	2.47 (2.58)	14.74 (14.58)
3	46	279 – 281 (dec)	$NHCOCH_3$	N_3	$C_9H_8N_4O_5$ S	38.02 (38.11)	2.84 (2.55)	19.71 (19.42)
4	68	>300 (dec)	$NHCOCH_3$	NH_2	$C_9H_{10}N_2O_5$ S	41.85 (41.96)	3.90 (3.68)	10.85 (10.44)

a: Obafemi and Onigbinde, 1991.

evaluating the various biological activities produced by four derivatives of salicylic acid synthesized in our laboratories. These compounds are tested to examine their potency, efficacy and cytotoxicity with a view of developing a compound having lower toxicity and less side effects. This communication therefore describes the synthesis of 2-hydroxy-5-azidosulfonylbenzoic acid (HASBA, **1**), 2-acetyoxy-5- azidosulfonylbenzoic acid (AASBA, **2**), 2-acetamide-5- azidosulfonylbenzoic acid (AMASBA, **3**) and 2-acetamido-5-sulfonamidobenzoic acid (AMSABA, **4**) and their effects on pain, temperature (pyrexia), inflammatory processes and cytotoxicity in-vitro.

MATERIALS AND METHODS

Synthesis of derivatives of Salicylic and anthranilic acids

The four sulfonyl derivatives of salicylic acid and anthranilic acid, **1-4,** were synthesized following a procedure described earlier (Obafemi and Onigbinde, 1991). This involves chlorosulfonation of 2-amino or 2-hyroxybenzoic acid with excess chlorosulfonic acid, to give the corresponding 2 substituted 5 chlorosulfonylbenzoic acid derivatives. This is followed by reaction with either sodium azide in aqueous acetone or with ammonium hydroxide to afford **1-4** (Figure 1, Table 1).

Animals

Swiss albino mice (18-24g) and Wistar albino rats (180-220g) of either sex were obtained and bred in the Faculty of Pharmacy Animal House. Food and water were provided *ad libitum*. Experiments were carried out between 10.00 and 17.00 h.

Antipyretic activity in rats

Rats were given s.c. 20 ml/kg of a 20% aqueous suspension of sterilized brewer's yeast powder. After 18 h, animals showing an increase of rectal temperature > 0.5^{0}C were selected. Control group received normal saline; treated groups received 25, 50 and 100 mg/kg of compounds **1** to **4,** respectively. Rectal temperature was determined by thermal-probe (Ellab thermistor thermometer) 30 min before (pre-treatment) and at 30 min., 1, 2 and 4 h after administration.

Analgesic Activity

Tests were carried out by the following methods:

Table 2. The Effects of Salicylic acid and Anthranilic acid Derivatives on Acetic acid (chemical) induced writhing, hot-plate and tail immersion (thermal) induced pain response in mice.

Treatment	Dose (mg/kg)	Writhing response (No/20 min)	Tail immersion reaction time (sec)	Hot - Plate reaction time (sec)
Control (DMSO)	0.3 ml	58.4 ±1.75	3.6 ± 0.36	9.6 ±0.92
HASBA	25	54.8 ± 3.91	2.4 ± 0.22	8.0 ± 0.49
[**1**]	50	36.8 ± 8.66*	8.8 ± 1.72**	11.3 ±1.78
	100	30.4 ± 8.52**	4.2 ± 0.44	14.6 ± 1.04**
AASBA	25	48.3 ± 1.98**	5.6 ± 1.12	19.6 ± 0.83**
[**2**]	50	32.8 ±7.7**	10.2 ± 2.94*	24.2 ± 1.43**
	100	24.4 ± 5.44**	7.0 ± 1.66*	24 8 ± 0.77**
AMASBA	12.5	41.5 ±4.98**	3.8 ± 0.33	16.6 ± 0.73**
[**3**]	25	38.0 ± 5.87**	4.8 ± 0.18	15.2 ±1.01**
	50	24.5 ±9.42**	7.0 ±2.02	19.8 ±0.77**
AMSABA	25	42.7 ± 3.72**	3.8 ±0.18	13.4 ±0.61**
[**4**]	50	38.8 ±5.95**	2.6 ± 0.22	13.6 ±0.78**
	100	38.0 ± 2.83**	2.6 ± 0.2	12.8 ±0.52**
Acetylsalicylic acid (ASA)	100	32.0 ± 2.5**	36.0 ± 1.5**	16.2 ±2.9**

Values represent the Mean ± S.E.M, (n = 5). *p < 0.05; **p < 0.01.

Hot Plate Test: Male and female albino mice showing reaction time of 10 sec to thermal stimulus of 55 ± 1^0C were selected. Groups of mice (5 mice per group) were given doses of 25, 50 and 100 mg/kg. i.p., and saline to the control group. The reaction time for control and treated mice were recorded after 1 h of drug administration.

Acetic acid induced writhing: Using the method of Siegmund et al. (1957), three graded doses (25, 50 and 100 mg/kg) of the compounds **1 - 4** were administered intraperitoneally to 16 h fasted mice, divided into 5 groups of 5 mice each. The first three groups received 3 doses of each compound, the 4th and 5th groups served as the negative and positive controls and they received 0.3 ml normal saline and 100 mg/kg ASA, respectively. One hour after treatment, animals in each group received 0.1 ml of 3% acetic acid to induce the characteristic writhing response. The number of writhings occurring for 30 min was recorded. This same procedure was repeated for the other three compounds.

Tail immersion test: The tail of the mice (about 5 cm long) was immersed into water bath of 55 ± 1^0C, the time (s) taken to withdraw the tail clearly out of the water bath was taken as the reaction time. The mice were first treated with different doses of the compounds (25, 50 and 100 mg/kg s.c.), and after one hour of compound administration, their tails were immersed in the hot water bath and the reaction time recorded. ASA (100 mg/kg s.c.) was used as the reference drug.

Anti-Inflammatory Activity

Inflammation in the hind paw of albino rat was induced as described by Winter et al. *(1*962). 0.1 ml of 1% carrageenan suspension was injected into sub-plantar surface of the right hind limb of each rat. The control group received 0.3 ml normal saline, the treated and positive control groups received 25, 50 and 100 mg/kg of compounds **1 - 4** and 100 mg/kg of phenylbutazone (PBZ) respectively by subcutaneous route, 30 min before carrageenan injection. The volume of each paw was measured by using a thread to determine the diameter of oedema formation size at 0, 0.5, 1, 2, 3 and 4 h. The difference in diameter of the left and the right hind paws was taken as a measure of oedema every 30 min for 4 h.

Cytotoxicity Test

The cytotoxicity of the 4 compounds was monitored by haemagglutination activity using formaldehyde fixed bovine erythrocytes as described by Peumans et al. (1982), Sadique et al. (1989) and Wang et al. (1995).

Statistical analysis

Student's t-test was employed for statistical analysis of the data. A probability value less than 0.05 or 0.001 was considered statistically significant. Values in the text and tables are represented as mean ± SEM.

Drugs

Acetylsalicylic acid (Bento Pharmaceuticals), carrageenan (Sigma), DMSO (BDH), yeast (Food Science and Technology Department, O.A.U, Ile-Ife), paracetamol (Bento Pharmaceuticals), Phenylbutazone (KGN Pharmaceuticals), acetic acid (Sigma)

RESULTS

Four substituted derivatives of salicylic and anthranilic acid possess analgesic activity. The compounds **1- 4** (HASBA, AASBA, AMASBA and AMSABA respectively) significantly reduced pain induced by acetic acid writhing responses. The number of writhing episode in treated mice decreased significantly (P<0.05) compared to ASA. HASBA, AASBA and AMASBA were more active in decreasing the number of writhing than ASA, with AMASBA showing the highest activity (Table 2). Likewise all the compounds exhibited potent analgesic effect against thermal noxious stimuli in hot plate model. In this model, AASBA and AMASBA exhibited higher analgesic

Table 3. Antipyretic activities of Acetylsalicylic and Anthranilic acids Derivatives and Paracetamol on Yeast induced pyrexia in rats.

Treatment	Dose (mg/kg)	Before drug (^{O}C)		After drug (^{O}C)				
		- 18 h	0 h	0.5 h	1 h	2 h	3 h	4 h
Control (DMSO)	0.3 ml	37.8±0.14	38.2±0.05	38.7±0.03	38.5±0.06	38.5±0.05	38.5±0.05	38.3±0.04
HASBA	25	37.0±0.23	37.5±0.23	37.4±0.24*	37.2±0.39*	37.3±0.23*	37.3±0.25*	37.3±0.24*
[**1**]	50	36.9±0.19	37.4±0.12	37.4±0.11*	37.3±0.13*	36.7±0.15*	36.6±0.16*	37.0±0.12*
	100	37.2±0.26	37.5±0.20	37.3±0.20*	37.1±0.17*	37.0±0.2*	36.9±0.12*	37.2±0.20*
AASBA	25	37.5±0.07	37.7±0.11	37.4±0.05*	37.1±0.16*	37.1±0.13*	37.1±0.19*	37.4±0.26*
[**2**	50	37.8±0.07	38.0±0.11	37,9±0.11*	37,8±0.24*	39.9±0.26*	37.5±0.19*	37.6±0.19*
	100	37.7±0.06	37.9±0.19	37.6±0.17*	37.6±0.18*	37.4±0.18*	36.9±0.21*	37.4±0.13*
AMASBA	25	37.3±0.5	37.7±0.19	37.1±0.10*	36.8±0.23*	37.1±0.22*	37.2±0.30*	37.5±0.23*
[**3**]	50	37.3±0.39	37.8±0.09	37.4±0.13*	36.6±0.29*	35.8±0.36*	35.7±0.32*	36.9±0.51*
	100	-	-	-	-	-	-	-
AMSABA	25	37.5±0.18	37.6±0.28	37.4±0.27*	37.4±0.24*	37.3±0.23*	37.3±0.19*	37,6±0.06*
[**4**]	50	37.2±0.09	37.5±0.09	37.5±0.15*	37.5±0.10*	37.5±0.22*	37.8±0.15*	37.9±0.10*
	100	37.3±0.15	37.5±0.19	37.3±0.26*	37.1±0.23*	37.4±0.13*	37.9±0.16*	37.9±0.24*
Paracetamol (PCM)	100	37.4±0.3	37.6±0.4	37.5±0.3*	37.2±0.2*	37.5±0.2*	37.3±0.3*	37.2±0.4*

Values represent the rectal temperature mean ± S.E.M, (n = 5). *p < 0.05.

effect against thermal stimuli than ASA. However, in the tail immersion technique ASA was more potent than all the synthesized compounds.

The results of the antipyretic activity of the compounds are presented in Table 3. Administration of the yeast to the rats produced significant increase in rectal temperature 18 h after yeast injection. All the compounds showed antipyretic effect like the reference drug (PCM). However, AMASBA showed the highest activity even more than that of the reference drug.

The anti-inflammatory effects of the compounds on carageenan-induced oedema in the rat's right hind paw are presented in Table 4. There was a gradual increase in oedema paw volume of rats in the control group. However, in the treated groups, all the 4 compounds produced a significant reduction in oedema formation except AMSABA. The order of potency is: AASBA > AMASBA > HASBA > AMSABA. However, the effects of these compounds were less compared to that of phenylbutazone. The cytotoxicity (haemagglutination) assay performed for HASBA, AASBA, AMASBA, AMSABA and ASA exhibited no agglutination on the formalin fixed bovine RBC.

DISCUSSION

Pain, swelling (oedema) and fever (pyrexia) are the signs and symptoms of both acute and chronic inflammation, malaria and stress. Acetylsalicylic acid (ASA) and other non-steroidal anti-inflammatory drugs (NSAID) are prominent agents used for or in combination with agents used in treating these symptoms. The study revealed that synthesized HASBA, AASBA, AMASBA and AMSABA compounds were active and possess antipyretic, analgesic, anti-inflammatory properties. It was also detected that some of the compounds evaluated were more potent, and less toxic than ASA except AMASBA, which killed all the experimental animals at 100 mg/kg dose level.

According to these findings, analgesic effects were assessed in chemical and thermal models of nociception using acetic acid induced writhing, hot plate and tail immersion tests. The results show that the compounds did not exhibit significant analgesic property in tail immersion test. In acetic acid induced writhing, a dose-related analgesic effect of the compounds **1 – 4** was observed. Collier et al. (1968) proposed that acetic acid acts indirectly by inducing the release of endogenous mediators peripherally and stimulates the pain neurons sensitive to NSAID agents. Likewise, in the hot plate model, the compounds also exhibited dose-related analgesic effect. These models are useful tools to access the potency of the compounds, their probable sites and mechanism of action. It seems therefore, according to Martins Do Monte et al. (2004), that these compounds may be producing their effects both
peripherally (through acetic acid induced writhing) and centrally (using both tail immersion and hot plate tests).

The compounds tested also produced significant antipyretic and anti-inflammatory effects (Tables 3 and 4). These compounds may exhibit both central and

Table 4. Anti-inflammatory activities of Salicylic acid and Anthranilic acid derivatives on Carrageenan-induced oedema in the right hind limb paw of rats.

Treatment	Dose (mg/kg)	Time (h)					Average Oedema formation
		0.5 h	1 h	2 h	3 h	4 h	
Control (DMSO)	0.3 ml	0.26±0.08	0.70±0.21	1.12±0.12	0.90±0.16	0.84v0.16	0.76±0.15
HASBA [**1**]	25 50 100	0.28±0.05 0.26±0.07 0.34±0.06	0.40±0.06 0.36±0.08 0.42±0.05	0.44±0.05 0.62±0.07 0.46±0.08	0.44vv0.07 0.58±0.08 0.50±0.11	0.56±0.07 0.50±0.04 0.52±0.10	0.42±0.04* 0.46±0.06* 0.45±0.03*
AASBA [**2]**	25 50 100	0.24±0.10 0.22±0.05 0.15±0.05	0.44±0.06 0.28±0.21 0.37±0.20	0.52±0.09 0.36±0.16 0.22±0.12	0.72±0.08 0.36±0.09 0.32±0.19	0.84±0.04 0.40v0.20 0.22±0.13	0.55±0.07 0.25±0.15* 0.26v0.10*
AMASBA [**3**]	25 50 100	0.28±0.05 0.20±0.07 -	0.34v0.17 0.26±0.07 -	0.48±0.09 0.32±0.05 -	0.72±0.03 0.19±0.09 -	0.72±0.03 0.42±0.13 -	0.51±0.08 0.28±0.07*
AMSABA [**4]**	25 50 100	0.22±0.11 0.23±0.02 0.20±0.03	0.34±0.17 0.43±0.03 0.40±0.07	0.64±0.02 0.58±002 0.60±0.08	0.78±0.04 0.68±0.05 0.78±0.07	0.90±0.04 0.78v0.04 0.78v0.06	0.58±0.12 0.54v0.02 0.55±0.07
Phenylbutazone (PBZ)	100	0.20±0.05	0.20±0.09	0.21±0.07	0.23±0.05	0.14±0.05	0.17±0.03**

Mean ± S.E.M, (n = 5). *p < 0.05; **p < 0.01.

peripheral actions because pyrexia and inflammation are central and peripheral processes, respectively. The compounds show suppressant activity on acute inflammatory model of carrageenan-induced paw oedema in rats and produced significant antipyretic activity higher than that exhibited by PCM. From these observations it can be indeed inferred that the endogenous chemical substances liberated during pain, inflammation and fever like histamine, serotonin and arachidonic cascade metabolites are inhibited to produce various activities highlighted in this study.

In order to establish some structural activity relationship from the results presented in the study, the activity of the compounds are correlated with the functional groups at the C-2 and C–5 positions on the benzene ring, compared to aspirin (ASA). It has been suggested by Insel (1991) that substitution on carboxyl or hydroxyl group changes the potency or toxicity of ASA compounds. The ortho position of the OH group is an important feature for the action of salicylates, even benzoic acid shares many of the actions of salicylic acid but is much weaker.

In general, the introduction of the sulfonyl azide functional group ($-SO_2N_3$) at position 5 of the benzene ring (as in structures **1**, **2** and **3**) results in a higher analgesic activity compared to ASA (Table 2). Introduction of the acetamido ($-NHCOCH_3$) functional group as in **3**, instead of the acetyloxy functional group ($-OCOCH_3$) as in **2** seems to increase the analgesic activity in acetic acid-induced writhing test only. However, compound **2** exhibited higher activity over **3** at 50 mg/kg in hot plate, tail immersion analgesic tests (Table 2) and in the anti-inflammatory test (Table 4). Our observation from this study is that few minutes after compound **3** was administered, all the animals died at 100mg/kg dose level only. On the other hand, changing the sulfonyl azide group in **3** into the sulphonamide group ($-SO_2NH_2$), as in **4** decreased the analgesic and anti-inflammatory activities and did not show such toxicity exhibited by compound **3.**

The cytotoxicity assay was used to assess the effect of compounds **1 – 4** and ASA on membrane stability of bovine RBC in relation to its ability to maintain the integrity or preventing the lysing of the cells. It is one of the mechanisms to explain their anti-inflammatory property. NSAID has been shown to stabilize the cell membrane protein (Bowman and Rand, 1980). Even though acetylsalicylic acid (1 mg/ml) is known to possess a weaker membrane stabilizing activity (Sadique et al., 1989), the results in this study indicated that not all the compounds and ASA lysed the RBC hence they are capable of preventing membrane damage caused by injury during inflammation.

In conclusion, the results of the present investigation suggest that derivatives of acetylsalicylic and anthranilic acids such as HASBA, AASBA and AMSABA

(Compounds **1, 2** and **4)** have promising analgesic, antipyretic and anti-inflammatory properties. However, AMASBA (Compound **3**) produced potent and promising similar pharmacological properties, but was found to kill the animals at 100 mg/kg probably due to the presence of the acetamido (-$NHCOCH_3$) group. Further studies on the potential toxicity of AMASBA are being carried out in our laboratories.

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