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SYNTHESIS AND EVALUATION OF ANALGESIC, ANTI-INFLAMMATORY AND ANTI-BACTERIAL ACTIVITY OF SYNTHETIC PORPHYRIN DERIVATIVES

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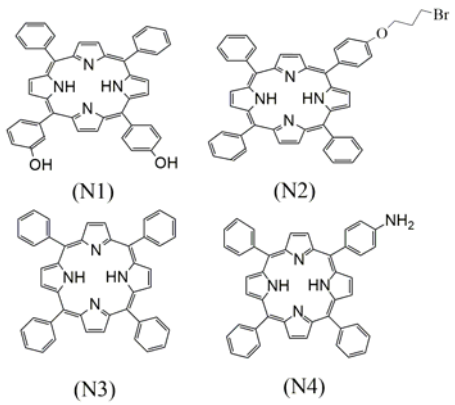
In this work porphyrins compounds (N1-N4) were synthesized from monohydroxy tetraphenylporphyrin, and then characterized on the basis of their chemical properties and spectral data. They were further tested for their potential analgesic and anti-inflammatory activities in acetic acid induced writhing test in mice and carrageenan induced paw edema in rats. The compounds were also evaluated for antibacterial activity in disc diffusion method. Compounds N1, N2 and N4 showed significant analgesic and anti-inflammatory activity at 10 and 30 mg/kg (b.w), comparable to the standard reference drugs. Furthermore, all the tested compounds possessed significant anti-bacterial activity against both gram positive and gram negative bacteria. The analgesic, anti-inflammatory and anti-bacterial activities of the tested compounds were found comparable to reference drugs. These compounds can serve as precursors for the development of clinically useful analgesics, anti-inflammatory and anti-bacterial agents.



INTRODUCTION

Porphyrins (derived from Greek πορφυροσ means "purple, scarlet") are very important class of naturally occurring molecules. Many biological molecules work with prosthetic groups basically consisting of these units. Heme, a component of hemoglobin which transports oxygen to animal tissues and chlorophylls of chloroplasts responsible for the process of photosynthesis in plants, both have active sites essentially made of porphyrin core.[1] Porphyrins play vital role in biological systems and they also have useful applications in material science and medicine. Infectious diseases have shown exponential growth recently. The problem is becoming more complex due to the emergence of resistance to current anti-bacterial therapy.[2] Infections are caused by microorganisms and are usually accompanied by pain and inflammation which is the response of the organism towards the pathogen. Although inflammation is a protective response of the body and constitutes a form of innate immunity but chronic inflammation causes a number of serious disorders such as hay fever, periodontitis, atherosclerosis rheumatoid arthritis, and even cancer (e.g. gallbladder carcinoma). Thus there is an urgent need to develop new anti-bacterial agents

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with analgesic and anti-inflammatory properties. Porphyrins have previously been shown to possess anti- inflammatory and antibacterial activities.[3, 4] In the present study we have synthesized porphyrin compounds that could be used as suitable agents in the treatment of bacterial infections accompanied by pain, inflammation and other related disorders.

EXPERIMENTAL

Synthesis and pharmacological screening

Synthesis of N1 (Dihydroxytetraphenylpophyrin)

4-hydroxybenzaldehyde (2 g, 16.38 mmol) was dissolved in propanoic acid (60 mL) in 500 mL round bottom flask. Benzaldehyde (2.3 mL, 22.54 mmol) was added to this mixture and then stirred at 140 ^{o}C. A solution of pyrrole (4.6 mL, 66.39 mmol) and propionic acid (15 mL) was prepared. This solution was added to the first one constantly with the help of syringe and canola. The whole mixture was then heated to 140 ^{o}C for 2 h.

The reaction mixture was cooled and neutralized with NaOH solution. The neutralized mixture was mixed with Ethyl acetate in a separating funnel and shaked intensely. Two layers were formed when the mixture was allowed to stand for 5 minutes. The upper layer of dihydroxytetraphenyleporphyrin and ethyl acetate was separated. Ethyl acetate was evaporated by mean rotary evaporator to get compound N1. The compound was identified by spectroscopic techniques.

Characterization of N1

EMIS 646 (M^+)

Major fragmentation peaks in EIMS 323, 55, 630.

^{1}HNMR ($CDCl_3$, 300 MHz) δ 8.86 (s, 8H, Pyrrole–H), δ 8.22 (m, 8H, aromatic), δ 7.77 (m, 12H, aromatic), δ -2.31 (2H, pyrrole NH), δ 4.1(2H, Hydroxyphenyl 2OH).

Major peaks in UV Spectrum

648 nm (Abs 0.0037), 590.4 nm (Abs0.0019), 553 nm (Abs 0.0099), 516.4 nm (Abs 0.0358), 420 nm (Abs 1.4541).

Synthesis of N2 (Bromoalkylation of Hydroxyphenylporphyrin)

Monohydroxytetraphenylporphyrin (3 g, 4.76 mmol) was dissolved in DMF (10 mL) then K_2CO_3 (3g, 21.73 mmol) was added to this mixture and then stirred at 75 ^{o}C for 1 h. Dibromopropane (3 mL, 90.89 mmol) was added to the reaction mixture which was then stirred overnight at 75 ^{o}C. After 15 h, distilled water (50 mL) and CH_2Cl_2 (50 mL) were added to the reaction mixture. The organic layer was separated from aqueous layer and the aqueous layer was extracted three times with CH_2Cl_2. The combined organic phases were evaporated on rotary evaporator and the residue was loaded on column Hex: $CHCl_3$ 8:2 resulting 53 % N2 as a major product.

Characterization of N2

EMIS 752(M^+)

Major fragmentation peaks in EIMS 315 614 672.

^{1}HNMR ($CDCl_3$, 300 MHz) δ 8.86 (s, 8H, Pyrrole–H), δ 8.22 (m, 8H, aromatic), δ 7.77 (m, 12H, aromatic), δ -2.31 (2H, pyrrole NH), δ 1.2 (2H, Bromoproxy 1CH_2), δ 1.5 (2H, Bromoproxy 1CH_2), δ 2.0 (2H, Bromoproxy 1CH_2).

Major peak in UV 648.6nm (0.0369 Abs), 591.2nm (0.0291Abs), 516.2nm (0.0951Abs), 419.2 nm (2.3605Abs)

Synthesis of N3 (*meso*-Tetraphenylporphyrin), $C_{44}H_{30}N_4$ (H_2TPP)

Acetic acid and propionic acid were used as solvents for the synthesis of H_2TPP. More pure H_2TPP with improved yield was obtained when propionic acid was used as solvent.

Acetic/propionic acid (75 mL) was placed in a 250 mL round bottom flask. Benzaldehyde (5.0 mL, 49 mmol) was dissolved in propionic acid. The benzaldehyde solution was added during a time period of 3-4 minutes. The reaction mixture was refluxed for 30 minutes and then cooled to room temperature. After cooling, the reaction mixture was neutralized by using 50 mL of 10 N NaOH. The fractions were extracted by column chromatography using hexane: CH_2Cl_2 (8:2). An additional product formed from this reaction was chlorin with a yield of 15 %.

Characterization of N3

EIMS 614 (M^+)

^{1}HNMR ($CDCl_3$, 300 MHz) δ 8.86 (s, 8H, Pyrrole–H), δ 8.22 (m, 8H, aromatic), δ 7.77 (m, 12H, aromatic), δ -2.31 (2H, pyrrole NH).

Synthesis of N4 (5-(4-Aminophenyl)-10, 15, 20- triphenylporphyrin)

5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (297 mg, 0.45 mmol) was dissolved in concentrated hydrochloric acid under nitrogen atmosphere. Tin (II) Chloride dihydrate (500 mg, 2.21 mmol) was added to the solution and the reaction mixture was heated to 70 ^{o}C for 1 h. The porphyrin solution was cooled and added to 100 mL of cold water. The reaction mixture was then adjusted to pH 8 with concentrated ammonium hydroxide. The aqueous phase was extracted with 5 × 100 mL of chloroform. The chloroform fractions were combined together and dried over magnesium sulfate.

The organic phase was then concentrated on a rotary evaporator and this solution was chromatographed through silica column with methylene chloride as an eluent. The first and only band eluting from the column was the desired 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (197 mg, 0.31 mmol) which was obtained in 70 % yield as analytically pure material.

Characterization of N4

EIMS m/z 629.8 (M+)

1HNMR (300 MHz, $CDCl_3$) δ 8.96 (s, 2H, pyrrole-H), δ 8.84 (s, 6H, pyrrole-H), δ 8.21 (m, 6H, aromatic), δ 7.98 (d, 2H, 4-aminophenyl), δ 7.75 (m, 9H, aromatic), δ 7.03 (d, 2H, 4-aminophenyl), δ 4.02 (s, 2H, amino), δ -2.37 (s, 2H).

Pharmacological experiment

Chemicals and reagents

Acetic acid, diclofenac sodium, indomethacin, DMSO and carrageenan were purchased from local suppliers of Sigma-Aldrich. All media and standard discs of meropenem (2 µg) was obtained from Oxoid (pvt) Ltd. Pure cultures of microbial strains were obtained from the department of microbiology, Quaid-e-Azam University Islamabad, Pakistan.

Animals

Male swiss mice (25-30 g) and adults *Sprague Dawley* rats (150 to 200 g) were purchased from department of pharmacy, University of Peshawar. Animals were housed in animal house with fresh water and standard food available *ad libitum*. The animals were maintained at 12 h light and dark cycle and with

room temperature maintained at 22-25 °C in the animal house. All the experiments were conducted according to the UK animal scientific procedure act, 1986.

Analgesic activity

Acetic Acid-induced writhing test

The analgesic effect was tested according to the method described by Gawade.[5] Abdomen writhing is a model of visceral pain and was produced by i.p. injection of 0.2 mL of a 0.8% aqueous solution of acetic acid to each mouse 1 h after i/p administration of diclofenac sodium (40 mg/kg) and test compounds N1 (1, 10 & 30 mg/kg), N2 (1, 10 & 30 mg/kg) and N3 (1, 10 & 30 mg/kg). Immediately after the injection of acetic acid, each mouse was isolated in an individual observation box and the number of abdominal contortions per mouse was counted over a 20 min period. Finally, percentage (%) analgesic activity was calculated using the following formula:

$$\% \text{ Analgesic activity} = \frac{\{\text{Mean writhing count (Control group- treated group)}\}\ 100}{\text{Mean writhing count of control group}}$$

Carrageenan-induced paw oedema in rats

Anti-inflammatory activity was assessed by the method described by Nanthakumar and Winter.[3, 6] The rats were divided into five groups of eight animals each. First group (negative control) received 0.2 mL of normal saline, second group (positive control) received aspirin (100, 150 & 200 mg/kg p.o.) third group received compound N1 (1, 10 and 30 mg/kg) and fourth and fifth groups received compounds N2 (1, 10 and 30 mg/kg) and N3 (1, 10 and 30 mg/kg) respectively. After 1 h, the rats were challenged with subcutaneous injection of 0.1 mL of 1% w/v solution of carrageenan into the plantar side of the right hind paw. Oedema was assessed for 3 hours, at 1-hour intervals after administration of the compounds, in terms of an increase in circumference of the carrageenan-injected paw compared to the saline.[7]

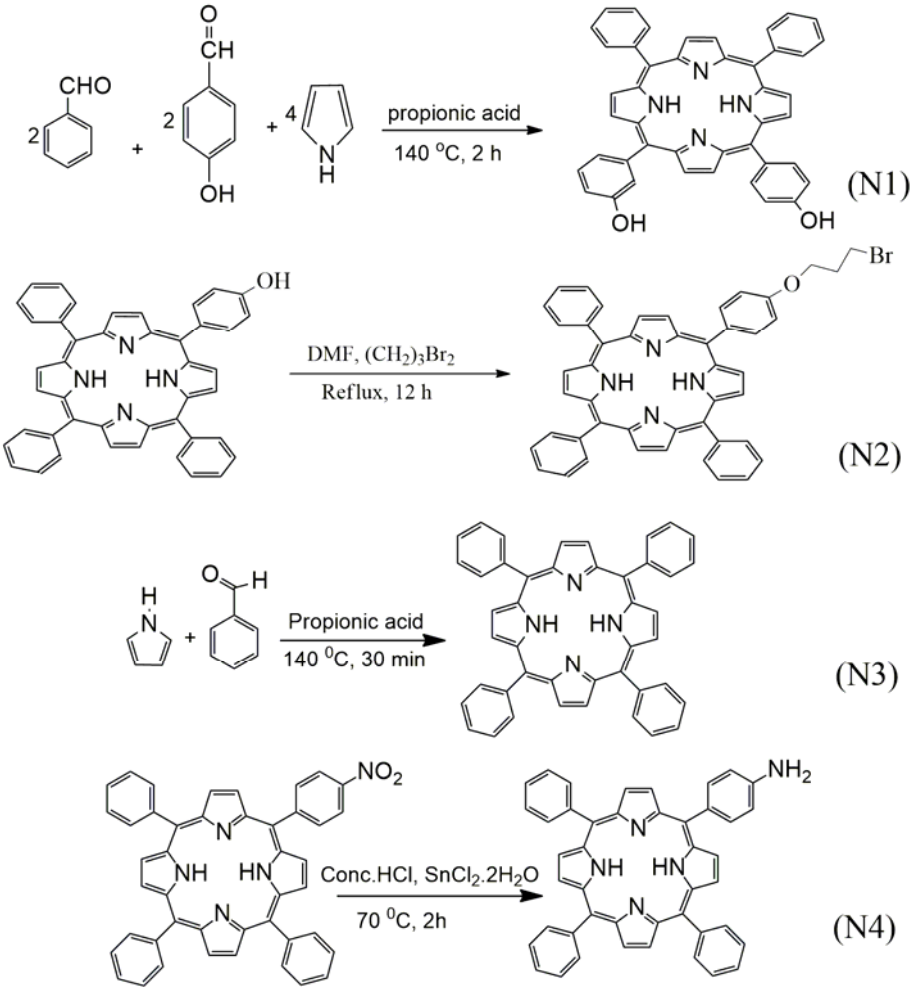
Inflammatory effect was assessed as the percentage reduction in oedema level when drug was present, relative to control.[4]

Activity = 100 – (100 × average drug treated/average for control)

Anti-bacterial activity

Disc diffusion method

The anti-bacterial activity was screened against three gram negative bacteria *Neisseria gonorrhea*, *Salmonella typhi* and *Pseudomonas aureginosa* and two gram positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae* by agar disc diffusion method.[2]



Scheme 1 – Synthesis of compounds N1-N4.

The inoculum was introduced onto the surface of sterile Nutrient agar plate and evenly distributed by using a sterile glass spreader. To find out the antibacterial activity 0.5 g of porphyrin compounds were dissolved in DMSO (2%) and were tested using 6 mm sterilized filter paper discs. Discs were impregnated with test sample (3 mL), allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 ^{o}C for 2 h before incubation with the test microbial agents at 37 ^{o}C for 24 h. Following this incubation the diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Mean values (± standard deviation) are reported in this study. The standard disc of meropenem (2 µg) was served as positive control for antimicrobial activity. The control measurements were carried out using DMSO.

Statistical analysis

All the values were represented as mean ± S.E.M. Results were analyzed by one way ANOVA followed by Dunnett's posthoc multiple comparison tests. Differences between groups were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Analgesic activity

The results presented in Table 1 show that the porphyrins derivatives injected i/p caused significantly reduced the number of writhes compare to normal saline control. The N1 (1-30 mg/kg) reduced the writhing count from 70.5 ± 3 to 35 ± 3, 24.5 ± 2 and 21 ± 2 /20 minutes respectively. Similarly the N2 analogue (10 & 30 mg/kg) reduced the writhing count from 70.5 ± 3 to 55 ± 4 and 39 ± 3 /20 minutes respectively and the N3 analogue (10 & 30 mg/kg) reduced the writhing count from 70.5 ± 3 to 45 ± 3 and 38 ± 2 /20 minutes respectively. The N1 analogue was found to be significantly ($P < 0.05$) more efficacious than N2 and N3 analogues. The N1, N2 and N4 derivatives and Diclofenac Sodium showed significant ($P<0.01$) reduction of pain in comparison with control group (Table 1). Opioid such as codeine[8] and anti-inflammatory substances[9] were able to produce analgesic activity in acetic acid induced pain. Since the porphyrin analogues N1, N2 and the N3 showed significant inhibition ($P < 0.05$, $P < 0.01$ and $P < 0.001$) of acetic acid induced writhing response of mice, so it could be suggested that porphyrin analogues had potential analgesic activity.

Anti-inflammatory activity

The results of anti-inflammatory activity are shown in Table 2. The porphyrin analogues N1, N2 and N4 at doses 10 and 30 mg/kg after oral administration exerted significant (**$P < 0.01$, $n = 6$) anti-inflammatory effect in carrageenan-induced paw edema in rats at 3rd hour after carrageenan injection. The anti-inflammatory effect of the porphyrin analogues at doses 10 & 30 mg/kg was comparable to standard drug indomethacin (5 mg/kg) on paw edema in rats. Development of edema in the paw of rat after injection of carrageenan was a biphasic event.[10] the result of the study indicated that the porphyrin analogues possess significant anti-inflammatory activity.

Antibacterial activity

Porphyrin derivatives have been reported to possess significant anti-bacterial activity.[11] We synthesized porphyrin analogues in an attempt to find newer and better anti-bacterial agents. Here we report the antibacterial activity of porphyrin analogues (N1-N4) against gram positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae* and gram negative bacteria *Neisseria gonorrheae*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The results are shown in Table 3. The porphyrin analogues N1, N2, N3 and N4 were found to be effective against both gram positive and gram negative bacteria. The anti-bacterial activity of the analogues N1, N2, N3 and N4 against gram positive bacteria *Staphylococcus aureus* and streptococcus *pneumonia* was comparable to broad spectrum antibiotic meropenem. The porphyrin analogues N1,N2, N3 and N4 showed 17 mm, 18 mm, 19 mm and 18 mm in diameter of zone inhibition against *Staphylococcus aureus* and 19 mm, 17 mm, 20 mm and 17 mm in diameter of zone inhibition against *Streptococcus pneumonia*. Meropenem showed 20 mm and 18 mm in diameter of zone inhibition against *Staphylococcus aureus* and *Streptococcus pneumonia* respectively. However the activity against gram negative bacteria was moderate compared to standard drug meropenem. The porphyrin analogues N1, N2, N3 and N4 produced 9 mm, 10 mm, 8 mm and 11 mm inhibition against *Neisseria gonorrheae*, respectively, 10 mm, 9 mm, 11 mm and 12 mm against *Salmonella typhi*, respectively and 13 mm, 12 mm, 11 mm and 12 mm inhibition against *Pseudomonas aeruginosa* respectively. Meropenem showed 18 mm, 19 mm and 20 mm inhibition against *Neisseria gonorrheae, Salmonella typhi* and *Pseudomonas aeruginosa*, respectively.

Table 1

Effect of porphyrin analogues and Diclofenac Sodium on acetic acid induced writhing response in Swiss albino mice

Treatment Groups	Dose (mg/kg)	Writhing count	% Activity
Saline Control	2 mL	70 ± 3	-
Diclofenac Sodium	40	20 ± 2***	71.4
N1	1	35 ± 3**	50.0
	10	24 ± 2***	65.7
	30	21 ± 2***	68.5
N2	1	65 ± 3	52.8
	10	55 ± 4*	62.8
	30	39 ± 3**	72.5
N3	1	67 ± 2	42.8
	10	45 ± 3*	50.0
	30	38 ± 2**	60.0

Test groups were compared with the control group and significant differences in response were noted. * P < 0.05, **P < 0.01, ***P < 0.001, n = 6; ANOVA followed by posthoc Dunnett's multiple comparison test.

Table 2

Effect of porphyrin analogues and indomethacin on carrageenan induced paw edema wistar albino rat

Group (n=6)	Dose (mg/kg)	Mean Paw size (cm)	Inhibition (%)
Control	-	3.4 ± 0.18	-
N1	1	3.4 ± 0.3	-
N1	10	2.4 ± 0.2**	29.4
N1	30	2.3 ± 0.3**	32.4
N2	1	3.3 ± 0.4	-
N2	10	2.6 ± 0.3**	23.4
N2	30	2.4 ± 0.2**	29.4
N3	1	3.2 ± 0.3	-
N3	10	2.3 ± 0.2**	32.3
N3	30	2.2 ± 0.3**	35.2
Indomethacin	5	2.5 ± 0.2**	26.4

Test groups were compared with the control group and significant differences in response were noted. **P<0.01 n = 8; ANOVA followed by posthoc Dunnett's multiple comparison test.

Table 3

Anti-bacterial activity of and meropenem against gram positive and gram negative bacteria in disc diffusion method

Type of bacteria	**Name of bacteria**	**Zone of inhibition (mm)**					
		N1	**N2**	**N3**	**N4**	**Merop**	**DMSO**
Gram positive	*Staphylococcus aureus*	17	18	19	18	20	-
	Streptococcus pneumonia	19	17	20	17	18	-
Gram negative	*Neisseria gonorrheae*	9	10	8	11	18	-
	Salmonella typhi	10	9	11	12	19	-
	Pseudomonas aeruginosa	13	12	11	12	20	-

CONCLUSIONS

The synthesized porphyrin analogues exhibited promising analgesic, anti-inflammatory and anti-bacterial activity. Thus these porphyrin analogues can serve as leads for clinically useful analgesics, anti-inflammatory and anti-bacterial agents.

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