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Original Research Article

Synthesis, Anti-inflammatory and Anti-nociceptive Evaluation of Palmitoyl Benzamides

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Abstract

Purpose: *To synthesize and characterize palmitoyl amino benzamides, and to evaluate them for possible anti-inflammatory and anti-nociceptive activities.*
Methods: *Palmitoyl amino benzamides were synthesized by the opening of isatoic anhydride ring with respective amino acids (glycine, β-alanine and γ-aminobutyric acid) and the condensation of the product with palmitoyl chloride. The final products were purified on column chromatography, eluting with dichloromethane/ethyl acetate. All the compounds were unequivocally characterized using the combination of infra red (IR), 1H and 13C (nuclear magnetic resonance (NMR), mass spectrometry (MS) and elemental analysis. In vivo anti-inflammatory and anti-nociceptive activities of the synthesized compounds at 20, 50 and 100mg/kg doses were carried out using carrageenan-induced paw oedema in rat and acetic acid-induced writhing in mice, respectively. Aspirin was used at a dose of 100mg/kg as the reference drug.*
Results: *The compounds were obtained in high yield (70 – 90 %) and purity. The anti-inflammatory results showed a poor activity for the compounds except o-palmitoylamino N-carboxyethyl benzamide which produced significant inhibition ($p < 0.05$) at a dose of 50 mg/kg (43.8 % oedema inhibition) while the reference drug, aspirin, showed 51.3 % inhibition. The anti-nociceptive study, however, showed good inhibition ($p < 0.05$) of acetic acid-induced writhing, with o-palmitoylamino N-carboxymethylbenzamide producing 86.2 % inhibition at 100 mg/kg dose compared with the reference drug (aspirin) which gave 74.3 % inhibition at 100 mg/kg.*
Conclusion: *The findings of this study indicate that the synthesized compounds, though displaying poor anti-inflammatory activity, do possess promising anti-nociceptive activity.*

Keywords: *Anti-inflammatory, Analgesic, Benzamide, Palmitoyl, Glycine, β-Alanine, γ-Aminobutyric acid, Aspirin*

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INTRODUCTION

Inflammation is a necessary and beneficial host response to foreign challenge or tissue injury that leads ultimately to the restoration of tissue structure and function [1]. Prolonged inflammation can cease to be a beneficial event and it contributes to the pathogenesis of many disease states. Recent studies have shown that certain lipid mediators might have a crucial role in the resolution of inflammation as endogenous anti-inflammatory mediators [2]. The discovery of anandamide (AEA) created an avenue for the search of other important endogenous fatty acid amides which have been shown to have interesting anti-inflammatory and anti-nociceptive activities [3].

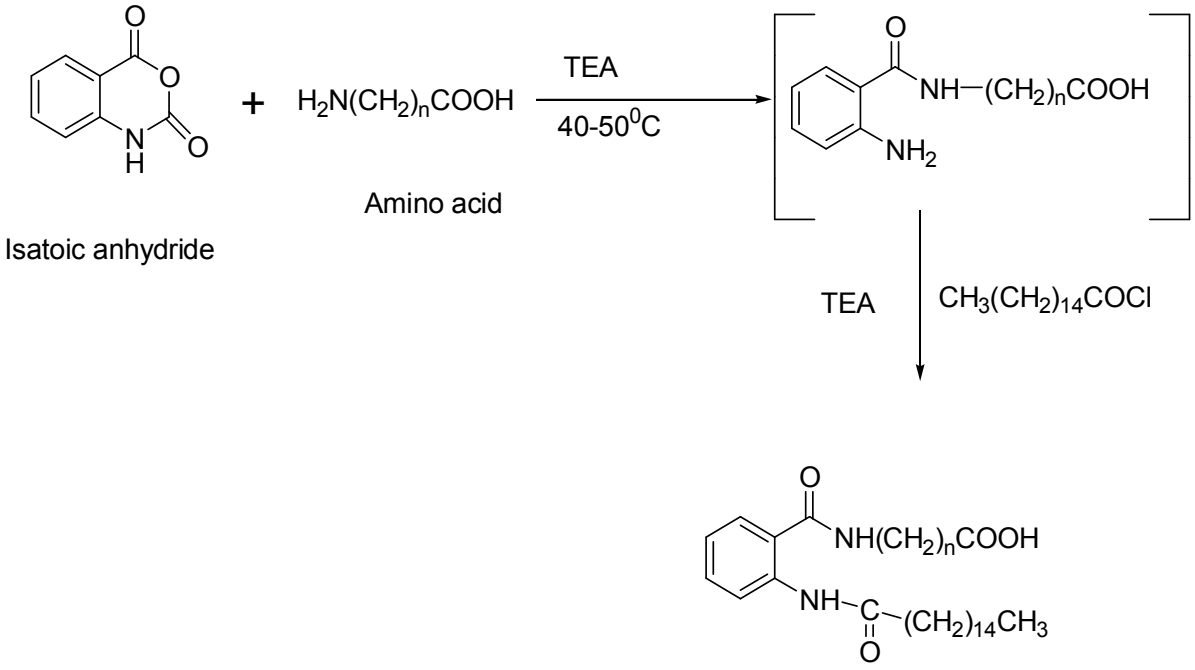
The pharmacological activity of these endogenous amides is terminated by fatty acid amide hydrolase (FAAH) [4]. This enzyme is an interesting target for drug discovery; since FAAH inhibitors have been shown to potentiate the pharmacological actions of AEA both *in vitro* and *in vivo* [5]. The study by Schmid *et al* [6] clearly indicates that the ethyl head chain is a target for structure-activity studies. Previous work in this area, usually with arachidonyl- and oleoylacyl chains, has resulted in the identification of several potent FAAH inhibitors [7]. However, this approach has an inherent disadvantage in that some of the compounds are active at cannabinoid receptors. In contrast, little is known about the effect of modification of the ethyl head group in N-palmitoylethanolamine, despite the fact that this compound, which reduces the rate of metabolism of AEA by acting as a competing substrate, is inactive at cannabinoid receptors [8]. There is thus a need to further investigate the effect of substitution of the ethyl head group of N-palmitoylethanolamine upon its ability to interact with FAAH. To this end we have synthesized three analogues of N-palmitoylethanolamine by the aromatic substitution of the ethanolamine moiety and they were screened for anti-inflammatory and anti-nociceptive activity *in vivo*.

EXPERIMENTAL

Materials

Glycine, β-alanine, γ- aminobutyric acid (GABA), palmitoylchloride and isatoic anhydride were obtained from Sigma-Aldrich, Germany. Acetylsalicylic acid, triethylamine, 1, 4-dioxan, hydrochloric acid and dichloromethane were obtained from BDH Chemicals Limited, England. Precoated thin layer chromatography (TLC), Silica Gel 60 F254 plates were obtained from Merck Darmstadt, Germany. Kieselgel 60 F254 (70-230 mesh for column chromatography, was obtained from Merck Darmstadt, Germany. Kofler electrothermal melting point apparatus CAT Number 1A 6304 was obtained from England. Buck Scientific Infra Red M500 instrument was obtained from Buck Scientific Inc, Norwalk, Connecticut, USA. Varian Gemini 200 (250MHz) for Nuclear Magnetic Resonance (NMR) characterization was obtained from Varian Inc., Palo Alto, California, USA. Varian MAT 44S mass spectrometer was obtained from Thermo Finnigan, San Jose, California, USA. Perkin-Elmer 2400 CHN elemental analyzer was obtained from Waltham, Massachusetts USA.

General reaction scheme



n= 1-3

Preparation of o-palmitoylamino N-carboxy-methylbenzamide

Isatoic anhydride (5 g, 30.67 mmol), glycine (2.3 g, 30.67 mmol) and triethylamime (TEA) (4.25 ml, 30.67 mmol) was mixed in 50 mL of water and heated at 40 - 50 ^{o}C in an oil bath for 5 h; TLC analysis revealed the disappearance of starting material at the end of the reaction. The pH of the mixture was brought to between 3 to 5 with 1 N HCl. The mixture was then partitioned with ethyl acetate (3 × 50 mL), and the solvent removed to give a product which was not isolated. The product (o-aminocarboxymethyl-benzamide; 1.793 g, 9.24 mmol) from the above procedure was treated with palmitoylchloride (2.8 mL, 9.24 mmol) in 1, 4-dioxan in the presence of TEA. At the end of the reaction, the mixture was poured into water and exhaustively extracted with ethyl acetate (3 × 50 mL). The combined organic fraction was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to leave behind a crude product which was purified on column chromatography using dichloromethane and then dichloromethane/ethyl acetate (3:1).

Preparation of o-palmitoylamino N-carboxy-ethylbenzamide

Isatoic anhydride (5 g, 30.67mmol), β-alanine (2.73 g, 30.67 mmol) and TEA (4.25 mL,30.67 mmol) was mixed in 50 mL of water and stirred at 40-50 ^{o}C in an oil bath for 5 h. TLC showed completion of reaction. The mixture was acidified with 1 N HCl (pH 3 - 5) and partitioned with ethyl acetate (3 × 50 ml). The combined organic fraction was evaporated *in vacuo* and the final product (3.134 g, 15.07 mmol) was treated with palmitoylchloride (4.56 mL, 15.07 mmol) in 1, 4-dioxan in the presence of TEA. At the end of the reaction, the mixture was poured into water and exhaustively extracted with ethyl acetate (3 × 50 mL). The combined organic fraction was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to leave behind a crude product which was purified on column chromatography (silica gel 70 - 230 mesh) eluting with dichloromethane and then dichloromethane/ ethylacetate (3:1).

Preparation of o-palmitoylamino N-carboxy-propylbenzamide

Isatoic anhydride (5 g, 30.67 mmol), GABA (3.16 g, 30.67 mmol) and TEA (4.3 mL) were mixed and stirred in 50 mL of water for 5 h at 40 - 50 ^{o}C. TLC showed completion of reaction. The mixture was acidified with 1 N HCl to pH 3-5 and extracted with ethyl acetate (3 × 50 mL). The combined organic phase was evaporated *in vacuo*. The final product (1.643 g, 7.4 mmol) was treated with palmitoyl chloride (2.4 ml, 7.4 mmol) in 1, 4-dioxan in the presence of TEA. At the end of the reaction, the mixture was poured into water and exhaustively extracted with ethyl acetate (3 × 50 mL). The combined organic fraction was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to leave behind a crude product, which was purified on column chromatography (silica gel 70 - 230 mesh) eluting with dichloromethane and dichloromethane/ ethyl acetate (3:1).

Animals

Swiss mice (17- 30 g) and Wistar rats (97- 225 g) of either sex purchased from Ambrose Alli University Animal House, Ekpoma, Nigeria were used. The animals were housed in the central animal facility of the Niger Delta University College of Health Sciences (NDUCHS) under the supervision of qualified personnel; with 12 h dark/12 h light cycles and were fed with grower feeds (Vita Feeds, Ibadan) and water *ad libitum*. Animals were fasted overnight, with free access to water, prior to experiments. The study was carried out according to the "Principles of Laboratory Animal Care" [9] and approved by the Institutional Animal Ethics Committee of NDU FPMSRCP (Protocol no. NDU CHS/SM-02/2012, Dt. 20.05.12)

Rat paw oedema assay

Anti-inflammatory activity of the synthesized compounds was evaluated using carrageenan-induced rat paw oedema assay model [10]. The animals were divided into groups (6 rats per group) of both sexes (pregnant females excluded) and were orally administered a dose (20, 50 and 100 mg/kg) of the test compounds, after an hour carrageenan suspension (0.1 mL, 1 %) in saline (0.9 % NaCl) solution was injected into the sub-plantar area of the right hind paw. The paw thickness was measured hourly over a period of 5 h with the aid of veneer caliper. Anti-inflammatory activity was evaluated by the method of Duffy *et al* [11] and the percentage inhibition of oedema level by drugs were compared to control as shown in Table 1. Acetyl salicylic acid (100 mg/kg) was administered orally as positive control while Tween 80 (10 %) used to solubilize the synthesized drugs was used as negative control. Mathematically, anti-inflammatory activity was evaluated as in Eq 1.

Activity = 100 - {100 x (Dt/C)} ……. (1)

where Dt is the mean value for drug-treated animals and C is the mean value for animals treated without drug (control).

Evaluation of analgesic activity

The method of Koster [12] and DiChiacchio [13] were employed. The animals were divided into five groups with 5 mice of both sexes, in each group (pregnant females excluded). The animals were administered a dose (20, 50 or 100 mg/kg) of the test compounds by gavage. After 1 h, the animals were injected intraperitoneally with 0.2 ml/mouse of 0.6 % v/v acetic acid solution. Acetic acid-induced writhing were counted and recorded within 20 min. Tween 80 (10 %) was used as the negative control while acetylsalicylic acid (100 mg/kg p.o) was used as reference drug. The mean of abdominal constrictions for five mice in each group which is an indication of analgesic activity was recorded. Inhibition (%) of abdominal constrictions of test compounds was compared with control group using the method of Duffy *et al* [11]. Analgesic activity was computed in terms of inhibition as in Eq 2.

Inhibition (%) = 100 - {100 x (Dr/Cr)} …….. (2)

where Dr is the mean drug response and Cr is mean control response.

Statistical analysis

Data obtained were analyzed by Student's t-test and multiple comparisons were done by one-way analysis of variance (ANOVA). A probability level of < 5 % was considered significant ($p < 0.05$).

RESULTS

Chemistry

The compounds were obtained in good yield (70 – 90 %) and high purity as shown by the melting point and elemental analysis.

o-Palmitoylamino N-carboxymethylbenzamide

Yield: 2.84 g (71 %), Melting point: 96-98 ^{o}C. IR (KBr): 3526 (OH), 3025 (NH), 2917 (C-H), 2848(CH), 1700(C=O), 1643(C=O), 1586, 1514, 1471cm^{-1}. 1H NMR (DMSOd_6) δ: 0.80-0.83(t, J = 7.5 Hz, 3H, CH_3), 1.01-1.03 (d, J = 5.0 Hz, 2H, CH_2), 1.21 (brs, 22H, $(CH_2)_{11}$), 1.58 (brs, 2H, CH_2), 2.27-2.33 (t, J = 7.3 Hz, 2H, CH_2), 7.10 (s, 1H, Ar-H), 7.44 (s, 1H, Ar-H), 7.67-7.70 (d, J = 7.0 Hz, 1H, Ar-H), 8.35-8.38 (d, J = 8.3, 1H, NH), 8.77 (s, 1H, NH), 11.23 (s,1H, COOH). 13C NMR: 14.5, 22.3, 25.4, 28.9, 29.2, 29.3, 29.5, 29.5, 29.7, 31.6, 31.7, 34.1, 37.9, 120.8 (Ar –C), 121.3 (Ar – C), 122.8 (Ar –C), 128.44 (Ar-C), 132.1 (Ar-C), 139.4 (Ar-C), 168.8 (C=O), 171.4 (C=O), 174.9 (C=O). MS: 431.4 (M+-1, 18 %), 374 (82), 309 (2), 293 (12), 255 (100). Elemental analysis: $C_{25}H_{40}N_2O_4$ (432.615) Found (C: 69.24, H: 9.14, N: 6.28), Calculated (C: 69.41, H: 9.32, N: 6.48)

o-Palmitoylamino N-carboxyethylbenzamide

Yield: 6.56 g (97.47 %) Melting point: 102-104 ^{o}C IR (KBr) 3528 (OH), 3028 (NH), 2914 (C-H), 2843(CH), 1700(C=O), 1643(C=O), 1586, 1514, 1471cm^{-1}. 1 H NMR (DMSOd6) δ: 0.80-0.83 (t, J = 6.8 Hz, 3H, CH_3), 1.02 (d, J = 6.3 Hz, 2H, CH_2), 1.21 (brs, 22H, $(CH_2)_{11}$),1.58 (brs, 2H,CH_2), 2.30 (t, J = 7.3Hz, 2H, CH_2), 7.09 (s, 1H, Ar-H),7.44 (s, 1H, Ar-H),7.67-7.70 (d, J = 7.0 Hz, 1H, Ar-H), 8.35-8.39 (d, J = 8.3 Hz, 1H, NH), 8.77 (s, 1H, NH), 11.23 (s,1H, COOH). 13C NMR: 14.5, 22.3, 25.4, 25.9, 29.0, 29.1, 29.2, 29.3, 29.5, 29.5, 31.7, 34.0, 36.0, 37.9, 120.8 (Ar-C), 121.2 (Ar-C), 122.8 (Ar-C), 128.5 (Ar-C), 132.2 (Ar-C), 139.4 (Ar-C), 168.7 (C=O), 171.5 (C=O), 173.3 (C=O). MS: 446 (M+ 6 %), 430 (6), 358 (14), 256 (18), 250 (34), 222(21), 208(100), 146 (13), 120 (21), 43 (25). Elemental analysis: $C_{26}H_{42}N_2O_4$ (446.642g); Found C: 69.80, H: 9.32, N: 6.04, Calculated C: 69.92, H: 9.48, N: 6.27

o-Palmitoylamino N-carboxypropylbenzamide

Yield: 2.50 g (73.27 %). Melting point: 98-100 ^{o}C IR (KBr) 3428 (OH), 3228 (NH), 2914 (CH), 2843 (CH), 1700 (C=O), 1643 (C=O), 1586, 1528 cm^{-1} 1H NMR (DMSOd_6) δ: 0.75-0.83(t, J = 7.0 Hz, 3H, CH_3), 1.14- 1.21 (brs, 22H $(CH_2)_{11}$), 1.57 (brs, 2H, CH_2), 1.72 – 1.77 (t, J = 7.0 Hz, 2H, CH_2), 2.24 – 2.32 (m, 2H, CH_2) 7.07-7.13 (t, J = 7.8Hz, 1H, Ar-H), 7.41-7.47 (t, J = 7.5 Hz, 1H, Ar-H), 7.67-7.71(d, J = 8.0 Hz, 1H, Ar-H), 8.72 (brt, 1H, N-H), 11.26 (s, 1H, N-H), 12.0 (brs, 1H,O-H) 13C NMR: 14.5, 22.3, 25.4, 28.9, 29.2, 29.3, 29.4, 29.5, 29.7, 31.6, 31.7, 34.1, 37.9, 120.8 (Ar –C), 121.3 (Ar – C), 122.8 (Ar –C), 128.4 (Ar-C), 132.1 (Ar-C), 139.4 (Ar-C), 168.7 (C=O), 171.4 (C=O), 174.9 (C=O). MS: 461 (M+), 264 (10 %), 256 (18), 223 (18), 222 (100), 185 (6), 174 (9), 161 (16), 146 (18), 120 (25), 104 (14). Elemental analysis: $C_{27}H_{44}N_2O_4$ (460.669); Found C: 70.24, H: 9.42, N: 6.02, Calculated C: 70.59, H: 9.63, N: 6.08)

Pharmacology properties

The results of the anti-inflammatory activity are shown in Table 1. The percentage oedema inhibition was calculated at the third hour using the formula stated under methods. It was observed that of the three compounds, o-palmitoylamino N-carboxyethyl benzamide exhibited the highest dose dependent oedema

inhibition of 42.4 - 43.8 % as the dose increased from 20- 50 mg/kg and aspirin the standard drug (100 mg/kg) produced 51.0 % inhibition of oedema when compared to control ($p < 0.05$). As can be seen in Table 2, o-palmitoylamino N-carboxymethylbenzamide (20, 50 and 100 mg/kg) exhibited the highest dose-dependent anti-nociceptive effect against acetic acid-induced writhing response in mice ($p < 0.05$). o-Palmitoylamino N-carboxymethylbenzamide at 100 mg/kg exhibited 86.2 % inhibition of writhing compared to 74.3 % inhibition of standard drug, aspirin (100 mg/kg).

DISCUSSION

The *in vivo* anti-inflammatory activity of the compounds was carried out using carrageenan induced oedema assay, which is a working model of inflammation in the search for new anti-inflammatory agents [14]. The oedema which develops in rat paw after the injection of carrageenan in the sub plantar area is a biphasic event [15]. The initial phase (within the first two and half hours) is attributed to the release of histamine and the second phase (from the third hour) is attributed to prostaglandin [16]. The results in Table 1 show that the compounds, except for o-palmitoylamino N-carboxyethyl benzamide have no anti-inflammatory activity. This indicates that aromatic substitution in this class of endogenous compounds destroys their anti-inflammatory activity.

To probe the analgesic activity of test compounds, it was investigated for inhibitory effect on acetic acid-induced abdominal constriction. It has been reported that acetic acid causes an increase in the peritoneal fluid level of prostaglandins (PGE_2 and $PGF_{2\alpha}$), involving in part peritoneal receptors [17] and inflammatory pain by inducing capillary permeability. It was proposed by some authors [18] that acetic acid acts indirectly by inducing the release of endogenous mediators, which stimulate the nociceptive neurons. Although the writhing test has poor specificity [19], it is a very sensitive method of screening the anti-nociceptive effects of compounds [18]. The acetic acid-induced writhing reaction in mice is also described as a typical model for inflammatory pain, has long been used as a screening tool for the

Table 1: Effect of palmitoylamino N-carboxybenzamide on carrageenan-induced paw oedema

Compound	Dose mg/kg(p.o.)	Change in paw diameter (Mean ± SEM)	% Oedema inhibition relative to control at the 3rd hour
o-Palmitoylamino N-	20	0.810 ± 0.029	22.8
carboxymethyl	50	0.988 ± 0.010	5.6
benzamide	100	1.123 ± 0.021	-7.1
o-Palmitoylamino N-	20	0.604 ± 0.010	42.4*
carboxyethyl	50	0.589 ± 0.013	43.8*
benzamide	100	0.894 ± 0.015	14.8
o-Palmitoylamino N-	20	1.146 ± 0.007	-9.2
carboxypropyl	50	1.038 ± 0.026	1 .0
benzamide.	100	1.270 ± 0.025	-21.1
Acetylsalicylic acid	100	0.511 ± 0.028	51.3*
Control (10 % Tween 80)	0.5 mL	1.049 ± 0.027	-

*Values are mean ± S.E.M; *p < 0.05, compared with control; paired t-test (n = 6); p.o. =per os*

Table 2: Effect of palmitoylamino N-carboxybenzamide on acetic acid induced writhing in mice

Compound	Dose (mg/kg, p.o.)	Number of writhing (Mean ± SEM)	Inhibition (%)
o-Palmitoylamino N-	20	25.8 ± 2.3	74.3*
carboxymethyl	50	15.4 ± 2.6	84.7*
benzamide	100	14.8 ± 4.3	86.2*
o-Palmitoylamino N-	20	56.8 ± 4.3	43.6
carboxyethyl	50	47.2 ± 1.2	53.5*
benzamide	100	27.4 ± 1.0	73.3*
o-Palmitoylamino N-	20	27. 8 ± 1.2	72.3*
Carboxypropyl	50	25.8 ± 4.5	74.3*
benzamide	100	26.0 ± 1.4	74.3*
Aspirin	100	25.8 ± 1.2	74.3*
Control (10% Tween 80)	0.2 mL	100 .4 ± 14.7	-

*Values are mean ± S.E.M; *p < 0.05, compared with control; paired t-test (n = 6); p.o. =per os*

assessment of analgesic or anti-inflammatory properties of new agents [19]. At the cellular level, protons depolarize sensory neurons by directly activating a non-selective cationic channel localized on cutaneous, visceral and other types of nocisponsive peripheral afferent C-fibers [20]. The results reported here indicate that oral administration of test compounds produced marked and dose-related anti-nociception when assessed in acetic acid-induced visceral nociception than anti-inflammatory response when induced by carrageenan-induced inflammation.

CONCLUSION

The findings of the present study demonstrate that the synthesized compounds, namely, palmitoyl benzamides, though showing poor anti-inflammatory activity, demonstrated promising anti-nociceptive activity. o-Palmitoylamino N-carboxymethyl benzamide exhibited the highest anti-nociceptive activity, greater than even the reference drug (aspirin). Thus, there is need for further studies on the compounds for their analgesic activity.

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