Synthesis of some long chain Fatty Acid Amide derivatives with possible Anti-inflammatory and Anti-nociceptive effect

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ABSTRACT.

Background: The discovery of anandamide as the endogenous ligand for the cannabinoid receptors and the isolation of other lipid amides such as palmitoyl ethanolamide created an avenue for further study of the biological activities of these endogenous molecules.

Objectives: The modification of the ethanolamine moiety of palmitoyl ethanolamide and the resulting biological activity was undertaken in this work.

Methods: Palmitoyl chloride was condensed with three amino acids (glycine, β-alanine and γ-aminobutyric acid). The compounds were unequivocally characterized using the combination of infra red (IR), ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). They were then screened for their anti-inflammatory and analgesic properties.

Results: The results indicate a significant (p<0.05) inhibition of rat paw oedema, with palmitoyl glycine giving the highest inhibition (49.0%) at 50 mg/kg; while the reference drug (Aspirin 100 mg/kg) gave 51.3%. Palmitoyl glycine also gave the highest antinociceptive activity with percentage inhibition of 83.2% (p<0.05) compared to 74.3% inhibition produced by Aspirin at equivalent dose of 100 mg/kg.

Conclusion: Though the exact mechanism of action of these compounds remains to be confirmed, but it may not be unrelated to the inhibition of fatty acid amide hydrolase (FAAH); the enzyme that metabolizes anandamide and palmitoyl ethanolamide in vivo.

Keywords: Palmitoylamino acids, carrageenan, anti-inflammatory, analgesic, paw oedema.
RÉSUMÉ

Contexte: La découverte de l’anandamide comme le ligand endogène des récepteurs cannabinoïdes et l’isolement d’autres amides lipidiques tels que palmitoyl éthanolamide créé une avenue pour une étude plus approfondie des activités biologiques de ces molécules endogènes.

Objectifs: La modification de la fraction de l’éthanolamine de palmitoyl éthanolamide et l’activité biologique résultant a été entrepris dans ce travail.

Méthodes: chlorure Palmitoyl a été condensé avec trois acides aminés (glycine, alanine et â-â-aminobutyrique). Les composés ont été caractérisés de manière non équivoque à l’aide de la combinaison de l’infrarouge (IR), résonance magnétique nucléaire nucléaire H et C (RMN) et la spectrométrie de masse (MS). Ils ont ensuite été criblés pour leurs propriétés anti-inflammatoires et analgésiques.

Résultats: Les résultats montrent une augmentation significative (p <0.05) l’inhibition de la patte de rat œdème, avec palmitoyl glycine donnant la plus haute inhibition (49.0%) à 50 mg / kg, alors que le médicament de référence (aspirine 100 mg / kg) a donné 51.3%. Palmitoyl glycine a également donné l’activité anti-nociceptive plus élevé avec un pourcentage d’inhibition de 83.2% (p <0.05), comparativement à 74.3% d’inhibition produite par l’aspirine à une dose équivalente de 100 mg / kg.

Conclusion: Bien que le mécanisme exact d’action de ces composés reste à confirmer, mais il peut ne pas être sans rapport avec l’inhibition de l’amide hydrolase d’acide gras (FAAH), l’enzyme qui métabolise l’anandamide et le palmitoyl éthanolamide in vivo.

Mots clés: acides Palmitoylamino, carraghénane, anti-inflammatoire, analgésique, œdème de la patte.
INTRODUCTION
Non-steroidal anti-inflammatory drugs (NSAIDs) do represent a varied and diverse family of pharmacologically active compounds used to alleviate acute and chronic inflammation, pain and fever. Historically, the first NSAID with therapeutic benefit was aspirin. After the elucidation of the molecular mechanism of action of aspirin by Vane, Samuelson and Bergstrom, other potent NSAIDS with similar mechanism of action were discovered (e.g. Ibuprofen, Diclofenac, Indomethacin) and introduced into clinical use. The use of classical NSAIDs is accompanied with some side effects that could be severe and the cost of managing such effects could be quite high. Research in the late eighties and early nineties led to the discovery of cyclooxygenase-2 (COX-2); which is an isoenzyme of COX-1. It was later revealed that COX-2 is the pro-inflamatory enzyme while COX-1 plays vital physiological role. This knowledge informed the development of selective COX-2 inhibitors (e.g celecoxib and rofecoxib) in the nineties but it was later discovered that this group of drugs are known to have influence on the rennin-angiotensin-system in the kidneys. Research in this field has led to the investigation of fatty acid amides as a potential source of anti-inflammatory agents. This followed the discovery of N-arachidonylethanolamide (anandamide) as the endogenous ligand for the cannabinoid receptors. Both unsaturated and saturated lipid amides have been isolated from different tissue preparations; and some of them have been found to have anti-nociceptive and anti-inflammatory effect. Palmitoyl ethanolamide was shown to reduce allergic reaction and inflammation. It was reported by some authors that the chain length of the amino alcohol moiety of N-oleoyl-ethanolamine dramatically affected its ability to interact with the amidohydrolase present in rat liver. This study clearly indicates that the ethyl head chain is a target for structure-activity studies. Information on the synthesis and biological activity of saturated derivatives of these endogenous lipids is scanty. We undertook the modification of the ethanolamine moiety of palmitoyl ethanolamide using different amino acids with free carboxylic functional group. The resulting compounds were screened for anti-inflammatory and anti-nociceptive effects.

MATERIALS AND METHODS
The starting materials used were purchased from commercial sources and used without any purification. Glycine, β-alanine, γ-aminobutyric acid (GABA) and palmitoylchloride were obtained from Sigma-Aldrich, Germany. Acetylsalicylic acid was obtained from BDH Chemical Ltd. England. The precoated Thin Layer Chromatography (TLC), Silica Gel 60 F plates used to monitor the reaction, was obtained from Merck (Darmstadt, Germany). Melting points were determined with an electrothermal melting point apparatus and were uncorrected. Infra red (IR) spectra were measured on a Buck scientific IR M500 instrument. 1H and 13C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 200 (250MHz). Chemical shifts are reported in part per million (ppm) relative to tetramethylsilane (TMS). Mass spectra (MS) were recorded on a Varian MAT 44S mass spectrometer operating at 70eV. Elemental analyses agreed favourably with the calculated values.

General procedure for synthesis.
To a stirring mixture of the amino acids in dichloromethane under ice, was added a stoichiometric amount of palmitoylchloride. The stirring continued for 4-6 hours. At the end of the reaction, the mixture was filtered (using Whatman No.1 filter paper) and washed in vacuo. The final product was recrystallized from methanol/water (1:1).
Preparation of palmitoyl glycine  
To a stirring mixture of glycine (0.546 g, 7.28 mmol.) in dichloromethane (10 mL) was added 1 mL triethylamine and palmitoyl chloride (2.2 mL, 7.28 mmol.); the stirring continued for 4 hrs. At the end of the reaction, the mixture was filtered and washed with 1N HCl (10 mL) and the combined organic filtrate was dried over anhydrous Na$_2$SO$_4$ and evaporated in vacuo. The resulting product was recrystallized from methanol/water (1:1). Yield: 1.03g (90%); melting point: 106-108°C. IR (KBr) 3371 (NH), 2929 (CH), 2857 (CH), 1686 (C=O), 1629 (C=O), 1543, 1457, 1400, 1200 cm$^{-1}$; $^1$H NMR (DMSO) $\delta$ppm: 0.86 (t, J = 6.0 Hz, 2H, CH$_3$), 1.22 (brs, 22H, (CH$_2$)$_{22}$), 1.55-1.64 (quint, J = 7.0 Hz, 2H, CH$_2$), 2.01 (quint, J = 7.5Hz 2H,CH$_2$), 2.13-2.21 (t, J = 4.75Hz 2H, CH$_2$), 3.0 (d, J = 6.75Hz, 2H, CH$_3$), 7.74 (brt, 1H, NH), 11.97 (brs, 1H, COOH); $^{13}$C NMR (DMSO) $\delta$ppm: 14.5, 22.53, 22.57, 29.0, 29.10, 29.2, 29.22, 29.39, 29.49, 31.50, 31.74, 34.1, 35.84, 38.22, 172.48 (C=O), 174.62 (C=O); MS: 313 (M , 12%), 256 (100), 214 (24), 185(17), 129(21), 116(26);  
**Elemental analysis:** C$_{31}$H$_{51}$NO$_3$ (313.488); Found. C: 69.10 H: 11.08 N: 4.44 Calculated. C: 68.97 H: 11.25 N: 4.47.  

Preparation of palmitoyl alanine  
To α-alanine (0.590 g, 6.6 mmol.) in dry dichloromethane (10 mL) was added 1mL triethylamine (TEA) under stirring in an ice-cold chamber (0-5°C). Palmitoylchloride was added dropwisely and finally washed down with another 10mL dichloromethane. The ice removed and the resulting syrupy mixture was stirred for 2 hours at room temperature. It was diluted with 20mL of dichloromethane. The resulting product was recrystallized from methanol/water mixture (1:1). Yield: 1.14g (92%); melting point: 110-112°C; IR (KBr) 3286 (NH), 2929 (CH), 2843 (CH), 1686 (C=O), 1628 (C=O), 1543, 1471, 1414 cm$^{-1}$; $^1$H NMR (DMSO) $\delta$ppm: 0.83 (t, J = 6 Hz, 3H,CH$_3$), 1.22 (brs, 22H, (CH$_2$)$_{22}$), 1.33 (quint, J = 6.25 Hz, 2H, CH$_2$), 1.45(brt, 2H, CH$_2$), 1.55-1.64 (quint, J = 7 Hz, 2H, CH$_2$), 1.98-2.04 (t, J = 7.5 Hz, 2H, CH$_3$), 2.13-2.21 (m, 2H, CH$_2$), 2.972- 3.05 (quint, J = 6.75 Hz, 2H, CH$_2$), 7.75 (brt, 1H, NH), 12.0 (brs, 1H, COOH); $^{13}$C NMR (DMSO) $\delta$ppm: 14.5, 23.0, 25.73, 28.99, 29.10, 29.15, 29.22, 29.39, 29.46, 29.48, 31.49,31.74,34.10, 35.84, 38.23, 38.92, 172.55 (C=O), 174.63 (C=O); MS: 341 (M`, 12%), 256 (33), 213 (14), 158 (17), 145 (100), 86(21), 55(30), 43 (54);  
**Elemental analysis:** C$_{30}$H$_{50}$NO$_3$ (341.542) Found C: 70.10 H: 11.20 N: 4.02 Calculated C: 70.33 H: 11.51 N: 4.10.  

**Animals**  
Swiss mice (17-30g) and Wistar rats (97-225g) of either sex purchased from Ambrose Alli University Animal House, Ekpoma, Edo State were used. The animals were housed in the central facility of the Niger Delta University College of Health Science (NDUCHS) Animal House under the supervision of qualified personnel; with 12 h dark/12 h light cycle. The animals were fed with grower feeds (Vita Feeds Ibadan) and water ad libitum. Animals were fasted overnight, allowing free access to water prior to experiments. The study was carried out according to the “Principles of Laboratory Animal Care” and approved by the Institutional Animal Ethics Committee of NDU FPMSRCP (Protocol No. NDU CHS/SM-01/2012, Dt. 11.04.12).
three doses (20, 50 and 100 mg/kg) of the test compounds, after an hour carrageenan suspension (0.1mL, 1%) in 0.9% NaCl was injected into the subplantar area of the right hind paw. The paw thickness was determined over a period of 5 hours with the aid of veneer caliper. The evaluation of the anti-inflammatory activity was achieved following method of Duffy et al.\textsuperscript{10} and the percentage reduction of oedema level by the test compounds were compared to control as shown in table 1 below. Aspirin (100 mg/kg) was administered orally as reference drug while Tween 80 (10%) used to solubilize the synthesized drugs was used as negative control. Mathematically, anti-inflammatory activity was evaluated as: Activity = 100- [100 × (average drug treated/average for control)].

### Analgesic activity
This was estimated using the method of Koster et al.\textsuperscript{11} and DiChiacchio et al.\textsuperscript{12} Group of five mice of both sexes excluding pregnant females were administered doses of 20, 50, 100 mg/kg of the test compounds orally. After an hour the mice were injected with 0.2ml of 0.6% acetic acid solution (intraperitoneally, i.p.). Acetic acid induced writhing were counted and recorded within 20 minutes. The reference drug was Aspirin and Tween 80 (10%) was used as the negative control. The mean of the number of abdominal constrictions per group was used as an indication of analgesic activity. The percentage inhibitions of abdominal constrictions by the test compounds were compared to control group using the method of Duffy et al.\textsuperscript{10} Mathematically, analgesic activity was estimated as:

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\text{Inhibition (\%) = 100 - [100 × (average drug response/average control response)]}
\]

### Statistical analysis
The results obtained were analyzed by student’s t-test and multiple comparisons were done by one way analysis of variance (ANOVA). A probability level of less than 5% was considered significant (p < 0.05).

### RESULTS
All the synthesized compounds were obtained in high yield and of reasonable purity as indicated by the elemental analysis. The anti-inflammatory study (Table 1) revealed a dose dependent activity as the dose was increased from 20 mg/kg to 50 mg/kg. Further increase of the dose led to decrease in activity. The compounds also demonstrated significant anti-nociceptive activity (Table 2) compared with the control.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose mg/kg (p.o)</th>
<th>Change in paw diameter Mean ± SEM in cm</th>
<th>% oedema inhibition relative to control at the 3\textsuperscript{rd} hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10% Tween 80)</td>
<td>0.5mL</td>
<td>1.049 ± 0.027</td>
<td>-</td>
</tr>
<tr>
<td>N-Palmitoyl glycine</td>
<td>20</td>
<td>0.554 ± 0.018</td>
<td>47.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.535 ± 0.035</td>
<td>49.0\textsuperscript{*}</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.745 ± 0.039</td>
<td>29.0</td>
</tr>
<tr>
<td>N-Palmitoyl alanine</td>
<td>20</td>
<td>0.814 ± 0.013</td>
<td>22.4\textsuperscript{*}</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.628 ± 0.023</td>
<td>40.1\textsuperscript{*}</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.718 ± 0.030</td>
<td>31.6\textsuperscript{*}</td>
</tr>
<tr>
<td>N- Palmitoyl GABA</td>
<td>20</td>
<td>0.671 ± 0.026</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.678 ± 0.037</td>
<td>35.4\textsuperscript{*}</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.990 ± 0.029</td>
<td>5.6</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>100</td>
<td>0.511 ± 0.027</td>
<td>51.3\textsuperscript{*}</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M * P< 0.05, significantly different from control, Paired t-test (n=5) p.o = per os
Table 2: Effect of the test compounds on acetic acid induced writhing test.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (mg/kg) p.o</th>
<th>Number of writhings (per 20mins)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10% Tween 80)</td>
<td>0.2mL</td>
<td>100.4 ± 14.71</td>
<td>-</td>
</tr>
<tr>
<td>N-Palmitoyl glycine</td>
<td>20</td>
<td>25 ± 2.71</td>
<td>75.2*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>18.6 ± 3.49</td>
<td>81.6*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>17.2 ± 1.62</td>
<td>83.2*</td>
</tr>
<tr>
<td>N-Palmitoyl alanine</td>
<td>20</td>
<td>58.6 ± 7.85</td>
<td>41.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>53.6 ± 2.79</td>
<td>46.5*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>49.8 ± 5.66</td>
<td>50.5*</td>
</tr>
<tr>
<td>N-Palmitoyl GABA</td>
<td>20</td>
<td>62.6 ± 6.07</td>
<td>37.6*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>40.6 ± 7.16</td>
<td>59.4*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>42.4 ± 5.24</td>
<td>57.4*</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>100</td>
<td>25.8 ± 1.16</td>
<td>74.3</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M  *P< 0.05, significantly different from control, Paired t-test (n=5) p.o = per os

DISCUSSION

Chemistry:
The reaction of amino acids under ice with palmitoyl chloride gave the expected compounds. These reactions were executed in dichloromethane except when γ-aminobutyric acid (GABA) was used, a slight modification was employed to optimize the yield. All the synthesized compounds were obtained in good yield and high purity as shown by the elemental analysis. The structures were unequivocally established by the combination of IR, NMR and MS. The diagnostic N-H and C=O stretches of the compounds were present in all the IR spectra. The 1H NMR revealed the amide proton as broad triplet at about 7.75ppm while the carboxylic proton was situated between 11.97 and 12.06ppm as a broad singlet. The alpha methyl group of the palmitoyl chain appeared as a triplet up field at about 0.86ppm relative to the TMS in all the spectra. The 1H NMR of the palmitoyl glycine revealed the methylene group of the glycine moiety as doublet at 3.0ppm. The methylene group of the alanine moiety of the palmitoyl alanine alpha to the amide functional group was revealed as a quartet at about 3.2 ppm, while the methylene alpha to the carboxylic group was seen as a triplet at about 2.2 ppm relative to TMS. The 13C spectra of the synthesized compounds revealed the two diagnostic carbonyl C=O peaks of the carboxylic and amide groups between 172-174 ppm relative to TMS. The carbonyl of the amide appeared generally at about 1628cm⁻¹ while that of the carboxylic acid were at about 1686 cm⁻¹. The molecular ion peak as shown by the MS corresponded with the estimated amount.

Pharmacology:
The in vivo anti-inflammatory activity of the test compounds was executed using carrageenan-induced paw oedema assay. This model has been used by a lot of researchers as a working model of inflammation in the search for new drugs with anti-inflammatory effects and indomethacin emergence as anti-inflammatory agent was discovered using this model. The development of oedema by carrageenan in the subplantar area is a biphasic event. The first phase result from the release of histamin and serotonin while the second phase is due to prostaglandin release. The results of the anti-inflammatory study is shown in Table 1 and the inhibition(%) was estimated adopting the formula earlier stated above. It was observed that of the three compounds, N-palmitoyl glycine exhibited the highest dose dependent oedema inhibition of 47-49% as the dose increased from 20-50mg/kg and aspirin the
standard drug (100 mg/kg) produced 51% inhibition of oedema. The analgesic activity of test compounds estimated by the mean reduction of abdominal constrictions induced by acetic acids is shown in Table 2. N- Palmitoyl glycine at doses of 20, 50 and 100 mg/kg elicited a dose dependent statistically significant (p<0.05) anti-nociceptive effect in mice. At 100 mg/kg, N-palmitoyl glycine exhibited 83.2 % inhibition of writhing compared to 74.3% inhibition of standard drug, aspirin (100 mg/kg). N-Palmitoyl glycine produced the most pronounced anti-nociceptive effect. Acetic acid is reported to interact with peritoneal receptors producing increase in peritoneal fluid level of prostaglandins (PGE and PGF2α); inflammatory pain is also induced by capillary permeability and induction of release of endogenous mediators which stimulate nociceptive neurons. At the cellular level sensory neurons are depolarized by activation of non-selective cationic channel located in cutaneous, visceral and other types of nociceptive peripheral afferent C-fibers.

Generally, the activity of the compounds decreases with increase in the carbon chain separating the carboxyl and the amide functional group which corroborate earlier report. The investigation reveals that increasing the dose of test compounds from 20 mg/kg to 50 mg/kg led to less than half-fold increase in inflammatory activity, more than half-fold increase in analgesic activity but this was further diminished as the dose increased to 100 mg/kg; this observation cut across all the compounds tested. This may in part suggest that the compounds have a dual action; resolution of inflammation at low doses and provocation of inflammatory response at higher dose. The test compounds were very effective as anti-nociceptive agent than anti-inflammatory. This investigation lends credence to earlier research in this field that fatty acid amides could serve as a potential source of anti-inflammatory agent. N-Palmitoyl glycine, N-palmitoyl alanine and N-palmitoyl GABA investigated demonstrate varying degree of anti-inflammatory and anti-nociceptive activities in rodents. However, both effects were more pronounced in N-palmitoyl glycine and diminished as the carbon chain separating the carboxyl and the amide functional group increases. The exact mechanism of action is yet to be established but may not be unrelated to the inhibition of fatty acid amide hydrolase (FAAH); the enzyme that metabolizes anandamide and palmitoyl ethanolamide in vivo.

CONCLUSION
The study has shown that the compounds, palmitoyl glycine, palmitoyl alanine and palmitoyl GABA do have anti-inflammatory and anti-nociceptive activity but the activity of palmitoyl glycine is more prominent in both of the assays. The exact mechanism of action of this group of compounds needs to be elucidated.

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REFERENCES