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# Fatty Acids with in Vitro Binding Affinity for Human Opioid Receptors from the Fungus *Emericella nidulans*

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**ABSTRACT:** Bioassay-guided fractionation of the EtOAc extracts of the epiphytic fungus *Emericella nidulans* resulted in the isolation of a mixture of two fatty acids. This mixture showed 98% binding affinity to human $\delta$ opioid receptor. These two fatty acids were identified as palmitic (PAM), **1**, and linoleic acids (LNA), **2**, by 1D NMR as well as by GC/MS analysis, after their methylation. We found that different ratio mixtures of **1** and **2** showed variations in selective binding activities to human $\delta$ opioid receptors. Five more fatty acids, arachidonic acid (ARA), **3**, *cis*-4,7,10,13,16,19-docosahexanoic acid (DHA), **4**, *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA), **5**, linolenic acid (ALA), **6**, and $\gamma$-linolenic acid (GLA), **7**, were evaluated for their binding affinity for opioid receptors. ARA, **3**, displayed affinity to $\delta$ and $\mu$ human opioid receptors with 68% and 80%, respectively. GLA, **7**, showed selective binding affinity to $\mu$ receptor with a value of 55%. These findings provide fascinating insight into the use of foods with high concentrations of fatty acids.

**KEYWORDS:** *Emericella nidulans, opioid receptors, fatty acids, palmitic acid, linoleic acid*

## INTRODUCTION

Fatty acids play important roles in our daily life; they serve as components of biological membranes, they aid cell membrane development, strength, and function, they are essential for organs and tissue, and they are used for metabolic fuel. They are also endogenous ligands for nuclear receptors that initiate transcription of multiple genes, for instance peroxisome proliferator-activated (PPARs) receptors.[1] In recent years, natural lipids have been identified as endogenous leads for the central cannabinoid receptors, which belong to the family of G protein coupled (GPCRs) receptors and are associated with many processes including pain, neurodegeneration, metabolic regulation, anxiety, and immune function.[2]

Some polyunsaturated fatty acids (PUFA) like docosahexaenoic acid (DHA) and arachidonic acid (ARA) play critical roles in neural development as structural components of neuronal membranes and by acting as precursors for lipid derived messengers.[3] Recent studies show that the ratio of omega-3 and omega-6 PUFA is important in the control of many brain functions.[4]

Opioid receptors are members of the superfamily of G protein-coupled receptors (GPCRs); the three major types are $\mu$ (mu), $\delta$ (delta), and $\kappa$ (kappa).[5] The three receptors mediate pain inhibition and are found throughout the nervous system including in somatic and visceral sensory neurons, spinal cord projection and interneurons, and the midbrain and cortex. Recently, opioid receptors have been identified on peripheral processes of sensory neurons.[6] In general, interaction with $\mu$ opioid receptors produces analgesia and respiratory depression and decreases gastric motility, emesis, and tolerance, while interaction with $\kappa$ agonists produces analgesia, diuresis, sedation, and dysphoria. The $\delta$ receptor antagonists have shown clinical potential as immunosuppressants, for treatment of cocaine abuse, and in the treatment of brain disorders.[7]

During the course of examining fungi for biologically active natural products,[8] we assessed the EtOAc extract from the fungus *Emericella nidulans* in a high-throughput screen employing a receptor binding assay for specific opioid receptors. The bioassay-guided fractionation and isolation resulted in the identification of a mixture of two fatty acids possessing good opioid receptor binding affinity.

## MATERIALS AND METHODS

**General Experimental Procedure.** The IR spectra were measured on a Bruker Tenso 27 instrument. $^{1}$H and $^{13}$C NMR spectra were obtained on a Varian 400 spectrometer with standard pulse sequences, operating at 400 MHz in $^{1}$H and 100 MHz in $^{13}$C. The chemical shift values were reported in parts per million units (ppm) from trimethylsilane (TMS) using $CDCl_3$ as solvent. Column chromatographic separation was performed on silica gel 60 (0.04–0.063 mm). TLC was performed on precoated TLC plates with silica gel 60 F254 (0.25 mm, EMD). The mobile phase used for TLC analyses was EtOAc:hexane (20:80). GC/MS analyses were carried out on a ThermoQuest Trace 2000 GC, equipped with a single split/splitless capillary injector, a ThermoQuest AS2000 autosampler, and a Phenomenex ZB-5 column (30 m × 0.25 mm × 0.25 $\mu$m), interfaced to a ThermoQuest-Finnigan Trace MS quadrupole ion trap detector. The injector temperature was 250 °C, and 1 $\mu$L injections were performed in splitless mode, with the splitless time set at 60 s, the split flow set at 50 mL/min, and the septum purge valve set to close 60 s

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after the injection occurred. The oven temperature was raised from 70 to 270 °C (hold 20 min) at a rate of 5 °C/min, for a total run time of 60 min; the transfer line temperature was 250 °C. Helium was used as the carrier gas at a constant pressure of 20 psi. The mass spectrometer was operated in the electron impact mode (EI+) and scanned from 40 to 800 amu at 1 scan/s, with an ionizing voltage of 70 eV and an emission current of 350 μA. Data was recorded using an IBM Netfinity 3000 Workstation with Microsoft Windows NT 4.0 operating system (Build 1381, Service pack 6) and Xcalibur data acquisition and analysis software (Version 1.2). The NIST Mass Spectral Search Program (Version 1.7, Build 11/05/1999) for the NIST/EPA/NIH Mass Spectral Library was employed to assist in the identification of the fatty acids. Standards of arachidonic, *cis*-4,7,10,13,16,19-docosahexanoic, *cis*-5,8,11,14,17-eicospentaenoic, linolenic, γ-linolenic, palmitic, and linoleic acids were purchased from Sigma Chemical Co. (Germany).

**Identification of Fungal Material.** The strain of *Emericella nidulans* (Eidam) Vuillemin used in this study was collected from a piece of orange peel in Tifton, Georgia, in 1978, and the membership of the isolate in this species was confirmed through phylogenetic, physiological, and morphological analysis. A voucher specimen (UM-032009) has been deposited in the culture collection of the Medicinal Chemistry Department, University of Mississippi.

**Phylogenetic Analysis.** Genomic DNA from the fungal strain UK-101 was extracted with DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and used as a template in PCR amplifications. The ITS1-5.8S-ITS2 genomic region (ITS) was amplified from genomic DNA using the forward primer ITS1 (5′- TCCGTAGGTGAACC-TGCGG-3′) and the reverse primer ITS4 (5′- TCCTCCGCTTA-TTGATATGC-3′).[9] PCR amplifications were carried out in 50 μL of reaction mixture containing 1× PCR reaction buffer, 0.2 mM dNTP mixture, 0.2 μM each of forward and reverse primers, 1.5 mM $MgSO_4$ and 2 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The PCR program consisted of one initial denaturation step at 94 °C for 3 min followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1:30 min, with a final extension at 72 °C for 7 min. PCR was performed in an M&J Research Gradient Cycler PTC-225. After amplification, an aliquot was analyzed by electrophoresis on a 1% TAE agarose gel and visualized under UV light, and PCR products were compared to the molecular size standard 1kb plus DNA ladder (Invitrogen, Carlsbad, CA). Successfully amplified PCR products were extracted using MinElute PCR Purification Kit (Invitrogen, Carlsbad, CA) and sequenced on an automated DNA Sequencer (model ABI 3730XL; Applied Biosystems, Foster City, CA). Consensus sequence data of the fungus was submitted to the GenBank database under the strain UK-101. The sequence obtained was submitted to phylogenetic inferences, which were estimated using MEGA Version 5.0.[10] The maximum composite likelihood model was used to estimate evolutionary distance with bootstrap values calculated from 1,000 replicate runs. The sequence of the type fungal species and some reference sequences deposited in GenBank were added to phylogenetic analysis accuracy. Information about the fungal taxonomic hierarchical levels follows the databases MycoBank (www.mycobank.org) and Index Fungorum (www.indexfungorum.org).

**Physiological and Morphological Analysis.** The physiological and morphological characteristics of the strain UK-101 were observed on CYA media (Difco, USA). The colony diameter was measured on medium, which was inoculated in three-point cultures and incubated for 7 days at 25 ± 2 °C under cool-white fluorescent lights (55 ± 5 $\mu mol/m^2/s$) with a 12 h photoperiod to sporulation. A duplicate set of CYA plates was incubated in the dark at 25 ± 2 °C to microscopic parameters determination using slide cultures mounted in methyl blue in polyvinyl-lactophenol.

**Fungal Culture.** *Emericella nidulans* was grown on potato-dextrose agar (PDA) at 24 °C until discrete fungal colonies appeared. Samples were taken from colonies and kept on PDA slants in test tubes at 24 °C, then placed in a 4 °C refrigerator until needed. The fungus was inoculated in 50 mL of potato-dextrose broth and kept for two weeks in stationary phase at 24 °C; then the fungus was seeded onto a medium in each flask consisting of 100 g of shredded wheat, 200 mL of low-pH Oxoid mycological broth, 2% yeast extract, and 20% sucrose in 2.0 L Fernbach flasks (10 flasks) followed by incubation for 22 days at 24 °C.

**Extraction and Isolation.** Following incubation, 500 mL of acetone were added to each flask, and the mycelia and the substrate were homogenized (Super Dispex, Tekmark Co., SD-45). The suspension was filtered, and then the filtrate was concentrated under vacuum at 40 °C. The residue was mixed with $H_2O$ (200 mL) and then extracted with EtOAc (500 mL × 3). The EtOAc extracts were combined, dried over anhydrous $Na_2SO_4$, and concentrated under vacuum. The EtOAc extract (23.0 g) was chromatographed on Si gel VLC with stepwise fractions from hexanes to methanol, yielding eight fractions (100% hexane, 75% hexane in EtOAc, 50% hexane in EtOAc, 25% hexane in EtOAc, 100% EtOAc, 75% EtOAc in MeOH, 50% EtOAc in MeOH, and 100% MeOH). The second fraction (75% hexane in EtOAc, 3.46 g) showed strong binding affinities for opioid receptors. This fraction was chromatographed on a silica gel column (2.0 × 40 cm) eluted with hexane−EtOAc gradient to yield eight subfractions. All fractions were submitted for opioid binding activity. Subfraction 4 (707 mg) displayed selective and strong binding affinity (98%) to δ opioid receptor. GC/MS and TLC analysis indicated that subfraction 4 was a mixture of palmitic and linoleic acids (13:87).

**Fatty Acids Methylation.** Subfraction 4 (2 mg) dissolved separately in 0.5 mL of dry ether (in a GC vial), and freshly prepared diazomethane solution was added (6 drops) twice (until effervescence ceased). The ethereal reaction mixture was evaporated in the GC vial, the residue was dissolved in 0.8 mL of dry ether, and the vial was capped and subjected to GC/MS.

**Cell Culture.** CHO-K1 cells stably transfected with opioid receptor subtypes μ, δ, and κ were used to perform the opioid receptor binding assays. These cells were maintained at 37 °C and 5% $CO_2$ in a Dulbecco's modified Eagle medium (DMEM) nutrient mixture supplemented with 2 mM L-glutamine, 10% fetal bovine serum, penicillin−streptomycin, and either G418 or hygromycin B antibiotic solutions. Membranes for the radioligand binding assays were prepared by scraping the cells in a 50 mM Tris-HCl buffer, followed by homogenization, sonication, and centrifugation for 40 min at 13650 rpm at 4 °C. These were kept at −80 °C until used for bioassays. Protein concentration was determined via Bio-Rad Protein Assay.[11]

**Radioligand Binding for Opioid Receptor Subtypes.** Saturation experiments determine the receptor number and radioligand affinity for the membrane. Opioid binding assays were performed under the following conditions: 10 μM of each mixture was incubated with [$^3$H]-DAMGO (μ), [$^3$H]-U-69,593 (κ), or [$^3$H]-enkephalin (δ) for 60 min in a 96-well plate. Percent binding was calculated as the average of the triplicate tested at 10 μM. Each sample concentration point of the compounds tested in dose response was in triplicate, and each compound showing activity was tested at least three times. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked with 0.3% bovine serum albumin (BSA) using a Perkin-Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris-HCl. Plates were read using a Perkin-Elmer Topcount. Total binding was defined as binding in the presence of 1.0% DMSO. Nonspecific binding was the binding observed in the presence of 10 μM DAMGO (μ), nor-binaltorphimine (κ), or DPDPE (δ). Specific binding was defined as the difference between total and nonspecific binding. Percent binding was calculated using the following formula:

$$100 - (\text{binding of compound} - \text{nonspecific binding}) \times 100/\text{specific binding}$$

$K_i$ and $IC_{50}$ values were calculated using Graph-Pad Prism 5; each point on the graph is representative of $n = 3$.

The binding assays of the fatty acids were performed in two independent experiments, and the results are similar for both studies (only the data for first experiment are shown).

## RESULTS AND DISCUSSION

**Identification of the Strain UK-101.** The sequence of the fungal strain UK-101 displayed 99% of identity with different

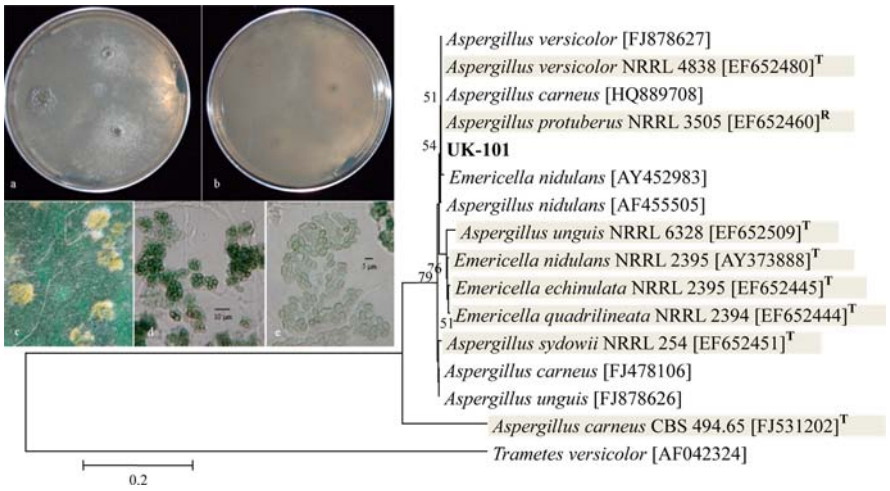


**Figure 1.** Taxonomic data of strain UK-101 morphological (a) verse and (b) reverse aspect of culture after 7 days on CYA at 7 days at 25 ± 2 °C; (c) ascomata, (d) asci, and (e) ascospores; phylogenetic analysis of the fungus (in bold) and their nearest relatives as well as $^{T}$sequence of type and $^{R}$reference species (shown in gray highlighting), the tree was constructed based on the rRNA gene sequences (ITS1-5.8S-ITS2) by using the maximum composite likelihood model; the tree was rooted with *Trametes versicolor* [AF042324] as an outgroup.

sequences of *Aspergillus* and *Emericella* species deposited in the GenBank database. However, according to Gazis et al.[12] and Ko et al.,[13] sequencing of the ITS region may fail to recognize some *Ascomycota* taxa, and for this reason there are several erroneous fungal sequences deposited in GenBank. To increase the taxonomy accuracy and avoid mistakes in the phylogenetic inferences, the ITS1-5.8S-ITS2 nuclear ribosomal gene sequence of the strain UK-101 was compared with sequence of type species of *Aspergillus* and *Emericella*. The nucleotide difference among the strain UK-101 and the sequence of the type species *Emericella nidulans* NRRL 2395 (AY373888) was 10 nucleotides (0.2%). In addition, the strain UK-101 displayed in CYA after 7 days at 25 ± 2 °C, under cool-white fluorescent lights (55 ± 5 $\mu$mol/m$^2$/s) with a 12 h photoperiod, colonies spreading rapidly, velvety, in green shades with a whitish margin; anamorph state dominating, with scattered green ascomata within and upon the conidial layer; ascomata globose, green, solitary, 200 $\mu$m diameter, maturing in 10 days, soft-walled; asci with 10 $\mu$m diameter; ascospores lenticular, 4.0 × 3.0 $\mu$m, green, maturing within 7 days, smooth-walled. Using physiological, morphological characteristics and molecular techniques, the strain UK-101 was identified as *Emericella nidulans* (Eidam) Vuillemin (Figure 1).

**Identification of Fatty Acids.** Preliminary evaluation by $^{1}$H NMR spectrum showed characteristic signals of olefinic protons (5.2−5.4 ppm), protons attached to the bis-allylic carbons (2.7 ppm), protons attached to the allylic carbons (2.0 ppm), protons $\alpha$ to an acid group (2.3 ppm), and the terminal methyl group protons (0.88 ppm). Furthermore, the methylene $(CH_2)_n$ protons at 1.2−1.4 ppm in its $^{1}$H NMR, as well as carbonyl signals at 180.3 ppm in its $^{13}$C NMR, are indicative of a fatty acid mixture. To determine which acids were present in the active fraction and their ratio, the fraction was subjected to GC/MS, and two major compounds were observed with peaks at retention times of 15 and 16 min corresponding to molecular ions at $m/z$ 256 and 280, which correspond to palmitic acid and linoleic acid, respectively, in a mixture of 13:87 ratio. To confirm the identification of the fatty acids, the methyl ester of the mixture was synthesized and molecular ions at $m/z$ 270 and 294 were observed in the GC/MS corresponding to the palmitic and linoleic methyl esters, respectively. Furthermore, the mixture was coinjected with standards of palmitic and linoleic acids, which confirmed the fatty acids produced by UK-101.

**Opioid Activity of Fatty Acid.** The active fraction proved to be PAM and LNA with 13:87 ratio. This mixture showed 98% binding affinity to human $\delta$ opioid receptors with $IC_{50}$ and $K_i$ values of 1.6 and 0.8 $\mu$M, respectively (Table 1), with no relevant binding affinity toward $\kappa$ or $\mu$ opioid receptors. In order to investigate the fatty acid concentration ratio effect on the binding affinity to $\delta$ opioid receptors, eleven different mixtures of PAM and LNA with different ratios (0% to 100%)

**Table 1. Binding Affinity Assay of the Mixture of Palmitic and Linoleic Acids, and Different Fatty Acids for Human Opioid Receptors (Subtypes $\delta$, $\kappa$, and $\mu$)**

| | opioid receptors (%) | | | binding affinity to $\delta$ opioid receptors ($\mu$M) | |
|---|---|---|---|---|---|
| sample | $\delta$ | $\kappa$ | $\mu$ | $K_i$ | $IC_{50}$ |
| active fraction, LNA:PAM (87:13) | 98 | 15 | −9 | 0.8 | 1.6 |
| LNA:PAM (100:0) | 15 | 15 | −30 | na[a] | na |
| LNA:PAM (90:10) | 76 | 28 | −27 | 16.3 | 32.0 |
| LNA:PAM (80:20) | 51 | 14 | 1 | 4.7 | 9.4 |
| LNA:PAM (70:30) | 22 | 13 | 68 | na | na |
| LNA:PAM (60:40) | 63 | 25 | −6 | 3.9 | 7.8 |
| LNA:PAM (50:50) | 103 | 15 | −3 | 4.5 | 9.0 |
| LNA:PAM (40:60) | 107 | 26 | −1 | 11.0 | 22.0 |
| LNA:PAM (30:70) | 87 | 23 | 10 | 12.5 | 25.0 |
| LNA:PAM (20:80) | 59 | 33 | −5 | 6.5 | 13.0 |
| LNA:PAM (10:90) | 42 | 16 | −13 | na | na |
| LNA:PAM (0:100) | 100 | 32 | 11 | 5.1 | 10.0 |
| ARA | 65 | 18 | 80 | 4.8 | 9.7 |
| DHA | 41 | 17 | 49 | na | na |
| EPA | 21 | −6 | 47 | na | na |
| ALA | 23 | 10 | 37 | na | na |
| GLA | 39 | −12 | 53 | na | na |
| naloxone | 99 | 103 | 99 | | |

[a]Not applicable.

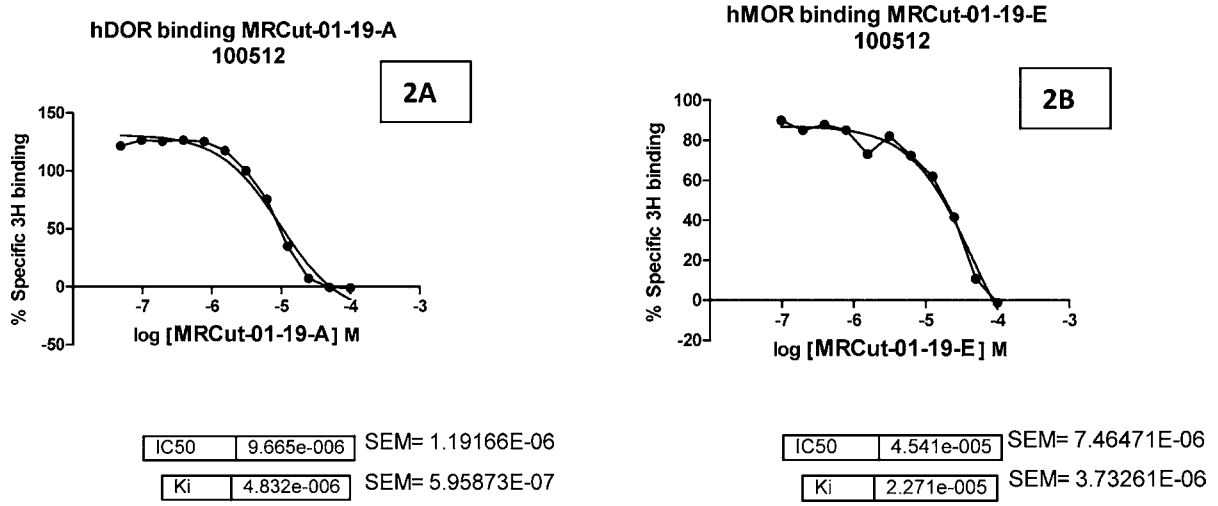


**Figure 2.** $IC_{50}$ and $K_i$ value of ARA (A) and GLA (B).

were prepared and the binding affinity for each mixture was evaluated (Table 1). In addition arachidonic, *cis*-4,7,10,13,16,19-docosahexanoic, *cis*-5,8,11,14,17-eicospentaenoic, linolenic, and γ-linolenic acids were evaluated. The results are shown in Table 1. The mixture of LNA and PAM primarily showed selective inhibition of the specific binding of [$^3$H]-enkephalin to CHO-K1 cell membranes expressing human δ-opioid receptors at a concentration of 10 μM. The ratio of LNA:PAM (40:60) completely inhibited [$^3$H]-enkephalin from binding to the δ receptor ($IC_{50}$ values of 22.0 μM).

ARA inhibited 65% of [$^3$H]-enkephalin binding to the human δ-opioid receptor (Table 1) and 80% specific binding of [$^3$H]-DAMGO to CHO-K1 cell membranes expressing human μ-opioid receptors at the concentration of 10 μM (Figure 2A); GLA inhibited 53% human μ-opioid receptors with $IC_{50}$ and $K_i$ values of 45.4 μM and 22.7 μM, respectively (Figure 2B).

The results are in accordance with those reported for lipid requirements on μ-opioid receptors binding affinity.[14] Linoleic acid has also been reported as a noncompeptitive inhibitor in some receptor binding assays.[15] Moreover, it is well-known that δ-opioid receptors could be palmitoylated, which might have a profound effect on the local conformation of this domain and possibly control interactions of GPCRs with specific regulatory proteins.[16]

The results of the binding to the human δ-opioid receptor in Table 1 shows an interesting relationship between the inhibition and the ratio of the fatty acids without statistical differences. The variations could be due to the competitive action of the fatty acids in the active site of the receptor; Hazegawa et al.[14] recognized that, even if the lipids form a simple bilayer, some lipids could take different forms such as micelles, which could impact receptor recognition. Kanicky et al. related the interaction between monolayers of one or two fatty acids with their $pK_a$, which may affect surface concentration of the fatty acid mixture and, in turn, result in poor molecular packing. They found that unequal chain length and the saturated nature of the mixture of fatty acids leads to a disruption in molecular packing and a successive decrease in the $pK_a$ values because of different intermolecular associations and/or interactions (van der Waals, ion−dipole, etc).[17,18] The difference in the $pK_a$ values could affect the binding of these fatty acids with the G-receptors. That is, the packing of the two fatty acids when in equal concentration might result in higher binding affinity than when one of the fatty acids is in greater concentration than the other fatty acid. Further studies are underway to evaluate this concept.

While the importance of the essential fatty acids is now recognized, additional studies that compare fatty acid profiles and their ratios need to be studied to understand their roles in the modulation of G-receptors.

To the best of our knowledge, this is the first time that a mixture of two fatty acids were evaluated for their binding affinity to human opioid receptors and that changing the ratio of the mixture of the two fatty acids provided a variety of selective δ-opioid receptor binding affinities. So far, the majority of clinically available opioid analgesics are μ agonists.[19] However, μ agonists, such as morphine and its derivatives, have many side effects such as tolerance and dependency.[20] In order to meet the need for an efficacious analgesic without side effects, attention has focused on other opioid receptors such as κ and δ. Currently there are only a few agents that target δ receptors. Our findings provide interesting insights on new chemical scaffolds biosynthesized by nature that may lead to the discovery of potential new selective ligands for opioid receptors.

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### Notes

The authors declare no competing financial interest.

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