Modified Akuamama Alkaloids with Increased Potency at the Mu-opioid Receptor

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Abstract. Akuammine (1) and pseudo-akuammigine (2) are indole alkaloids found in the seeds of the akuamma tree (Picralima nitida) that are used as a traditional treatment for pain in West Africa. Both alkaloids are agonists of the mu-opioid receptor (μOR); however, they produce minimal effects in animal models of antinociception, likely due to their modest potency. To further probe the interactions of 1 and 2 at the opioid receptors, we have prepared a collection of semi-synthetic derivatives with modifications to the C10, C11, C16, and N1 positions of the indole core. Evaluation of this collection at the μOR and kappa opioid receptor (κOR) revealed structural-activity relationship trends and derivatives with improved potency at the μOR. Most
notably, the introduction of a phenethyl moiety to the N1 of 2 produces a 70-fold increase in potency and a 7-fold increase in selectivity for the µOR. The *in vitro* potency of this compound was reflected *in vivo* in rodents, producing an ED$_{50}$ = 77.6 mg/kg and 77.1 mg/kg in a tail-flick antinociception assay and a hot-plate assay, respectively. The improved potency of these analogs highlights the promise of exploring natural product scaffolds that can be used to probe the opioid receptors.

**Introduction**

Amid the opioid crisis, opioid-related deaths have been on the rise with over 90 Americans dying from overdoses each day.\(^1\) Whereas in previous decades opioid deaths have been dominated by analgesics like morphine and its semi-synthetic derivatives, more recently more potent synthetic opioids like fentanyl have been the larger source of fatalities.\(^2\) Regardless of the drug class, clinically used and illicit opioids produce their powerful analgesic effects through the potent activation of the mu opioid receptor (µOR).\(^3,4\) This same activation of the µOR also produces life-threatening respiratory depression responsible for opioid-related deaths and along with other adverse effects including constipation, tolerance, and dependence that limit the clinical utility of opioid analgesics.\(^5\) Thus, while opioid analgesics remain an essential component of modern medicine, there is a pressing need to develop safer opioid analgesics.

Several promising strategies have emerged to develop safer opioid analgesics including the development of biased agonists,\(^6\) mixed pharmacology,\(^7\) partial agonism,\(^8,10\) and investigation of delta and kappa opioid receptor (δOR and κOR) agonists.\(^11\) The exploration of each of these strategies has been greatly aided by the discovery and design of novel opioid ligands, particularly those that lack the common pharmacophore shared by morphinan and fentanyl-derived analgesics. For example, the salvinorin A derivative herkinorin was among the first non-
nitrogenous μOR agonists and provided the first evidence for signaling bias with respect to β-Arrestin-2 recruitment at the μOR. More recently, of the structure-activity relationships (SAR) studies on the mitragynine alkaloids have revealed how even small changes to the scaffold allow for the fine-tuning of opioid receptor signaling. In addition to providing new chemical tools to probe the opioid receptor and its function, the development of these novel classes of opioids offers new opportunities to develop potential analgesics.

In light of the promising advantages of investigating structurally distinct μOR agonists, we began re-investigating the alkaloids of the akuamma plant (Picralima nitida), which has been used in West Africa as a traditional treatment for opioid withdrawal, fever, and pain. We recently developed an extraction protocol that allows for the isolation of six monoterpenoid indole alkaloids known as the akuamma alkaloids directly from commercial P. nitida seeds (Figure 1). As a class, these alkaloids are structurally distinct from traditional opioid ligands represented by morphine and fentanyl. Akuammine (1), pseudo-akuammigine (2), and akuammidine (3) act as moderately potent μOR agonists with potencies ranging from 2.6 – 5.2 μM. Due to their agonist activity at the μOR, 1-3 were evaluated in thermal antinociception assays in mice. However, contrary to previous studies and the traditional use of P. nitida as an analgesic, 1-3 produced minimal pain-relieving effects. Given the modest potency of these alkaloids at the μOR, we reasoned that a derivative with increased potency at the μOR would produce more effective antinociceptive effects. To test this hypothesis, here we report the first structure-activity relationship (SAR) study of the akuamma alkaloids 1 and 2. We employed strategic, chemoselective, late-stage functionalization of the indole nucleus, to introduce modifications at the C10, C11, and N1 positions of 1 and 2. Ultimately, these SAR studies resulted in the discovery of a μOR agonist with a 70-fold greater potency than the parent
compound 2. This dramatic increase in agonistic potency highlights the utility of semi-synthesis to discover new ligand classes that can serve as both suitable chemical probes to further understand opioid receptor signaling and promising drug leads to develop much-needed safer opioid analgesics.

![Figure 1. The six major alkaloids (1-6) isolated from akuamma seeds.](image)

**Results**

The structural complexity of 1 and 2 presents several challenges for investigating their activity at the opioid receptors. Although functional groups like the C10 phenol serve as apparent synthetic handles to alter the scaffold, the complexity of the molecules could also confound the selectivity and reactivity of certain chemical transformations. Therefore, to gain initial insight into the SAR of 1 and 2, we employed a series of highly chemoselective transformations to diversify their indole rings.

**Aromatic modifications (C10 and C11)**

The C10 phenol of 1 represents a logical starting point for our SAR studies; however, we found that the aliphatic N4 tertiary amine is generally more nucleophilic than the corresponding phenoxide anion. Our initial attempts to functionalize the phenol via alkylation with
iodomethane resulted in the methylation of the aliphatic nitrogen to produce the corresponding quaternary ammonium ion. However, methylation of 1 with trimethylsilyl diazomethane exclusively produced the methyl ether 7 (Scheme 1). Similarly, the acetylation of 1 with acetic anhydride, but not acetyl chloride, favored O-acylation to produced ester 8.

Scheme 1. (A) Semi-synthesis of C10 analogues of 2 and C11 analogues of 1. Reagents and conditions: (a) (CH$_3$)$_2$SiCHN$_2$, THF, MeOH, rt, 48 h (b) Ac$_2$O, DMAP, TEA, DCM, rt, 4 h (c) Tf$_2$NPh, DMAP, DCM, rt, 1 h (d) Zn(CN)$_2$, Pd(dppf)Cl$_2$, TEA, DMF, 120 °C, 24 h (e) boronic acid, K$_2$CO$_3$, Pd(PPh$_3$)$_4$, PhMe, MeOH, 80 °C, 4 – 30 h (f) NBS, TFA, DCM, 0°C to rt, 5 h (g) NIS, TFA, DCM, 0°C to rt, 5 h. Numbers in parentheses represent isolated percent yields. All Suzuki coupling analogues (11 – 15 and 21 – 26) consist of para substituted aryl rings except for 3-furanyl analogues 16 and 26.

After successful substitution of the phenol, the corresponding aryl triflate was prepared by reacting the phenol with N-phenyl-bis-(trifluoromethane sulfonimide) and 4-dimethylaminopyridine. The resulting aryl triflate, 9, was leveraged as a pseudohalide intermediate in palladium-catalyzed cross-coupling reactions to compounds 10-16. Nitrile analog 10 was synthesized from 9 in a palladium-catalyzed cyanation with Zn(CN)$_2$ as the nitrile source. Suzuki-Miyaura cross-couplings were used to append aromatic and heterocyclic rings (11-16). In addition to introducing substitutions at C10, reduction of 9 with formic acid as the hydride source provides 2. Given the relatively low isolation yields of 2 from P. nitida, this
two-step process provides a convenient way to access 2 for additional SAR studies from the more abundant 1.

To further investigate the impact of substitutions on the aromatic ring, halides were introduced at C10 of 2 and C11 of 1 employing an acid-mediated halogenation with N-bromosuccinimide and N-iodosuccinimide (17-20). Intriguingly, the use of trifluoroacetic acid was necessary for the reaction to progress to completion. In addition to accelerating the reaction, the acidic conditions likely also serve as an in situ protection of the alkyl amines. The aryl iodide 20 was also shown to be a competent coupling partner for Suzuki-Miyaura reactions allowing for the synthesis of a series of C11-substituted derivatives of 1 (21-26).

**Modifications to the esters**

The C16 methyl ester of 1 and 2 were targeted as we anticipated they could be converted into useful points of diversification through reduction to the primary alcohol (Scheme 2, 27-28) or hydrolysis to the carboxylic acid. However, the ester proved remarkably resistant to chemical modifications, presumably due to its attachment to a sterically encumbered quaternary carbon. For example, no reaction is observed when subjecting 1 to lithium aluminum hydride in refluxing THF. Surprisingly, the methyl ester of 2 is readily reduced to the primary alcohol 28 with lithium aluminum hydride at room temperature. At the moment, it is unclear how the phenol of 1 has such a profound impact on the reactivity of the remote ester. Further highlighting the chemical stability of the methyl ester, treatment of 5 with KOH effectively hydrolyzes the acetyl ester to the primary alcohol 29, while leaving the adjacent methyl ester intact.
Scheme 2. Semi-synthesis of C16 akuamma alkaloid analogues. Reagents and conditions: (a) LAH, TFA, 0 °C to rt, 4 h (b) KOH, MeOH, rt, 1 h. Number in parentheses represent isolated yields.

Indole nitrogen modifications

After modifying the C10 and C11 positions of 1 and 2, we shifted our efforts to substituting the N1 position of the indole ring (Scheme 3). Direct substitution of 1 and 2 are not feasible as the N1 position is substituted with methyl group which, despite multiple attempts, proved difficult to remove.\textsuperscript{25-28} An alternative to directly substituting 1 or 2 is to alkylate the structurally similar alkaloid 5, which conveniently lacks an N1 methyl group. Notably, the deacetylated analog of 5 (29) exists in equilibrium with the hemiaminal ether 30, which is favored under acidic conditions.\textsuperscript{29} Recently Zhang et. al. exploited this equilibrium in the total synthesis of 2 utilizing a trioxane, TFA, and triethylsilane in a modified Eschweiler-Clarke reaction to methylate 29.\textsuperscript{29,30} Inspired by this finding, we adopted a similar strategy by replacing trioxane with various dimethyl acetals to produce derivatives of 2 bearing alkyl substituents to indolic nitrogen (31-33, Scheme 3).\textsuperscript{31,32} The requisite dimethyl acetals are generated from reacting aldehydes with methanol in the presence of catalytic aqueous HCl.\textsuperscript{33}
Scheme 3. Semi-synthesis of N1 pseudoakuammigine analogues. Reagents and conditions: (a) dimethyl acetal, TFA, TES, DCM, rt, 18 h. Number in parentheses represent isolated yields.

**In vitro pharmacology**

Having generated a series of 22 novel “akuammalogs”, we looked to study what effects these modifications to the aromatic core of 1 and 2 would have on their activity at the opioid receptors. Since our previous study had determined that 1 and 2 have moderate affinities for the µOR and κOR, with minimal affinity for the δOR, we first examined the derivatives in competitive radioligand binding assays at these two receptors. To streamline our process and more efficiently identify SAR trends, we began by assessing all compounds at 1 µM for their ability to displace \[^{3}H\]-DAMGO or \[^{3}H\]-U69,593 from at the µOR and κOR, respectively (Figure 2A). Compounds that displaced >50% of radioligand at 1 µM in these initial screening assays were then further evaluated in full dose-response experiments to determine the binding affinity and cAMP inhibition functional activity assays to determine opioid receptor activation (Figure 2B-E).
Figure 2. Pharmacological characterization of the akuammalogs at the µOR and κOR. The akuammalogs were screened at 1 µM in a competitive radioligand binding assay with [³H]DAMGO and [³H]U69,593, using DAMGO and U50,488 as controls (A). Compounds 19-20 and 31-33 were further screened in a full-dose response radioligand binding assay at the µOR using [³H]DAMGO and akuammine (1) and pseudoakuammigine (2) as controls, respectively (B, D). Inhibition of forskolin-induced cAMP in a GloSensor assay in transfected HEK-293 cells at the µOR (C, E). All curves are representative of the averaged values from a minimum of three independent assays.

When the binding affinity was assessed, compounds bearing modifications at the C10 position (7, 8, and 10-18) displaced <50% of the radioligands and possess considerably less affinity than 1 or 2 (Figure 2A). These results indicate that C10 substitutions of the akuamma alkaloids are not well tolerated and suggest that the C10 phenol of 1 may make important ligand-receptor interactions with the opioid receptor. This notion was supported by the binding affinity for the C11 substituted derivatives of 1 that retain the C10 phenol. Although the Suzuki-Miyaura coupling products 21-26 do not displace >50% of the radioligand at either the µOR or κOR at 1 µM, they do induce more displacement than the C10 modified analogs (Figure 2A). Moreover, at 10 µM, 21-26 displace nearly 100% of [³H]DAMGO from the µOR, whereas 10-18 still
induce little displacement at this higher concentration (Supplementary Figure S1). This higher concentration also revealed that most of the C11 Suzuki-Miyaura analogs do not retain a preference for the µOR that we observed in 1 and 2 and show similar affinities for the κOR.

In contrast to the other C11-substituted derivatives, halogenation of 1 at C11 (19-20) results in slightly improved affinity for the opioid receptors. Both 19 and 20 induced >50% displacement at 1 µM and possess an increased affinity to the µOR and κOR compared to 1 (Figure 2A, Table 1). This improved affinity for the receptor is consistent with the phenol acting as hydrogen bond donor with the electronegative halogens lowering the pKa of the phenol and increasing its hydrogen bond donating ability. Although 19 and 20 retain a preference for the µOR, their selectivity over the κOR is diminished relative to 1 (Supplementary Figure S2). We next used the GloSensor cAMP assay to measure G-protein activation; 19 and 20 possess increased potency (EC₅₀ = 0.93 µM and 1.2 µM, respectively) at the µOR relative to 1 (Table 1). In addition to increasing the potency of 1, these modifications also decrease the efficacy relative to the parent compound with compounds 19 and 20 producing partial agonism at the µOR with E₉₅ values of 40% and 32%, respectively (Figure 2C, Table 1).
Table 1. In vitro pharmacology of akuammalogs using radioligand binding and cAMP inhibition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>μOR Affinity a</th>
<th>κOR Affinity a</th>
<th>κOR/μOR selectivity</th>
<th>cAMP inhibition at μOR b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢ ± SEM c</td>
<td>(Kᵢ, µM)</td>
<td>pKᵢ ± SEM c</td>
<td>(Kᵢ, µM)</td>
</tr>
<tr>
<td>DAMGO</td>
<td>9.1 ± 0.1</td>
<td>0.0009</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U-50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>6.5 ± 0.1</td>
<td>0.30</td>
<td>9.5 ± 0.1</td>
<td>0.0005</td>
</tr>
<tr>
<td>2</td>
<td>6.2 ± 0.1</td>
<td>0.33</td>
<td>5.6 ± 0.1</td>
<td>2.25</td>
</tr>
<tr>
<td>19</td>
<td>6.9 ± 0.1</td>
<td>0.12</td>
<td>6.6 ± 0.1</td>
<td>0.33</td>
</tr>
<tr>
<td>20</td>
<td>6.8 ± 0.1</td>
<td>0.22</td>
<td>6.2 ± 0.1</td>
<td>0.58</td>
</tr>
<tr>
<td>31</td>
<td>7.0 ± 0.1</td>
<td>0.10</td>
<td>5.9 ± 0.1</td>
<td>1.17</td>
</tr>
<tr>
<td>32</td>
<td>7.3 ± 0.1</td>
<td>0.054</td>
<td>7.3 ± 0.1</td>
<td>0.049</td>
</tr>
<tr>
<td>33</td>
<td>7.9 ± 0.1</td>
<td>0.012</td>
<td>6.2 ± 0.1</td>
<td>0.58</td>
</tr>
</tbody>
</table>

a Affinity at the μOR and κOR was determined by radioligand displacement using [³H]U69,593 and [³H]DAMGO; b cAMP inhibition at the μOR was determined through GloSensor assay; c Mean ± standard error on the mean.

Having assessed the effects of C10 and C11 modifications of the aromatic ring, we moved to investigate the modifications to the indolic nitrogen of 2. Upon the initial binding screen, we saw a significant increase in radioligand displacement at both the μOR and κOR for compounds 31 – 33 (Figure 2A). Extending the length of the alkyl group from methyl in 2 to an ethyl group in 31 increased binding affinity at the μOR (Kᵢ = 0.10 µM) while retaining selectivity over the κOR (Kᵢ = 1.2 µM) (Figure 2D, Table 1). Appending a phenyl ring to the methyl group of 2 (32) dramatically increased opioid receptor affinity; however, it also resulted in a loss of μOR selectivity with a μOR Kᵢ = 0.054 µM and a κOR Kᵢ = 0.049 µM. Unexpectedly, extending the linker by a single methylene unit as in 33 produced a 27-fold increase in μOR binding affinity relative to 2. This modification also produces a decreased affinity for the κOR relative to 32, resulting in 33 possessing 48-fold selectivity for the μOR (Kᵢ = 0.012 µM) over the κOR (Kᵢ = 0.58 µM). Using the GloSensor cAMP inhibition assay to measure μOR activation, derivatives 31-33 activated the μOR with potencies following a similar
trend to that observed in the radioligand binding assays. Most notably, compound 33, bearing the phenethyl substitution on the N1 position was again identified as being considerably more potent than the parent natural product 2 with similar levels of efficacy (EC50 = 0.075 µM, Emax = 76%) (Figure 2E, Table 1).

Given the potency of 33 in the GloSensor assay, we sought to further interrogate its in vitro activity by measuring β-arrestin (βArr2) recruitment. It is now well-appreciated that the recruitment of β-arrestins to GPCR is capable of initiating G-protein-independent signaling cascades. In the case of the µOR, βArr2-recruitment has been associated in a number of studies with the adverse effects of opioid analgesics including respiratory depression and tolerance. So-called G-protein biased agonists that preferentially activate the G-protein pathway have been proposed as a strategy to develop safer opioid analgesics. We had previously observed that activation of the µOR by the akuamma alkaloids 1-3 does not result in recruitment of βArr2 to the receptor. Due to the low potency of 1-3 at the µOR, it is also possible these ligands are capable of inducing βArr2 recruitment but at concentrations higher than were tested. However, 33, which is considerably more potent in the GloSensor assay, yet still does not produce any significant recruitment of βArr2 to the µOR (<10% at 10 µM; Figure 3A), indicating 33 is a G-protein biased agonist. The limited recruitment complicates calculating a reliable bias factor for the compound.

To evaluate the G-protein signaling properties of 33, we employed the recently developed TRUPATH assay (Figure 3B-C). The GloSensor assay measures the inhibition of cAMP production by adenylate cyclase as a downstream measure of activation of the Gi/o -signaling cascade induced by agonists activating a GPCR. By relying on the measurement of downstream events, these assays can overestimate the efficacy of ligands at the opioid receptors.
Conversely, the TRUPATH assay system employs bioluminescence resonance energy transfer (BRET) biosensors to measure the dissociation of the G-protein heterotrimer that initiates the signaling cascade. Importantly, the TRUPATH system can interrogate activation of specific $G_{\alpha_{i/o}}$ transducers, which in turn, could reveal signaling bias for different ligands.

The novel derivative $33$, the natural product $2$, and the positive control DAMGO were evaluated using the BRET-based TRUPATH assay to determine their abilities to activate the $\mu$OR coupled to the $G_{\alpha_{i/o}}$ subtypes $G_{\alpha_{i-1}}$, $G_{\alpha_{i-2}}$, $G_{\alpha_{i-3}}$, $G_{\alpha_{oA}}$, $G_{\alpha_{oB}}$, and $G_{\alpha_{z}}$. Using this assay, both $2$ and $33$ appear as balanced agonists, producing similar potencies and efficacies in all $G_{\alpha_{i/o}}$ subtypes. Consistent with the GloSensor assay, natural product $2$ is a low potency, partial agonist producing 40-86% of the maximal effect produced by DAMGO (Figure 3B-C). Similarly, compound $33$ possessed increased potency compared to $2$ with all $G_{\alpha_{i/o}}$ subtypes, although produced considerably higher levels of efficacy.

**Figure 3.** $\mu$OR signaling properties of compound $33$. The ability of $33$ to recruit $\beta$-arrestin-2 in a PathHunter assay at $\mu$OR with DAMGO as a control (A); Recruitment $G_{\alpha_{i/o}}$- subtype screening of $2$, $33$, and DAMGO in $h\mu$OR. TRUPATH heatmaps demonstrate $33$ and $\mu$OR agonists activate the $G_{\alpha_{i/o}}$-class of transducers with varying levels of potency (A) and efficacy (B). Akuammalog $33$ appears to exhibit increased potency and efficacy of $G_{z}$ as relative to other G-protein transducers as compared to the parent compound $2$. Heatmap colors refer to mean log($EC_{50}$) and normalized efficacy values. All curves are representative of the averaged values from a minimum of three independent assays.
In vivo activity

Given the increased potency of 33 relative to the natural products 1 and 2, we elected to evaluate its antinociceptive effects in mice. In the first series of experiments, mice received different doses of 33, and were subjected to the tail-flick and hot-plate antinociception assays. For the positive control, we used another naturally occurring compound morphine (10 mg/kg) in these assays. Compound 33 at high doses (80 and 100 mg/kg) exhibited antinociceptive effect in both warm-water tail-flick assay and hot-plate assay. At 100 mg/kg, 33’s antinociception was comparable to that of the morphine (10 mg/kg) in the tail-flick assay with respect to peak effect and the duration of action, whereas the peak effect time was slightly delayed (Figure 4A). In the subsequent dose response assay, ED$_{50}$ was estimated to be 77.6 mg/kg (Figure 4B). Similar antinociception effect was observed in the hot-plate assay, although the effects at 80 or 100 mg/kg were not as robust as in the tail-flick assay in comparison to the positive control (Figure 4C), with an estimated ED$_{50}$ of 77.1 mg/kg (Figure 4D). The small differences in efficacy are likely due to differences in stimulus intensity and nociceptive pathways. The tail-flick assay is largely a spinal reflex assay whereas hotplate involves more processing from the supraspinal regions. These results from tail-flick and hot plate assays clearly demonstrate that the increased potency of 33 relative to the naturally occurring akuamma alkaloids is translated into improved in vivo efficacy. However, compound 33 at the high doses (80, 100 mg/kg) also impaired motor coordination in the accelerating rotarod test (Supplementary Figure S3). Although this impaired locomotor activity is significant, other µOR agonists, including morphine, also produce motor incoordination. As such, this effect is likely produced through via µOR activation and further supports that compound 33 is active in vivo.
Figure 4. Antinociceptive effect of compound 33. (A) Tail-flick assay. (B) Dose-response curve of compound 33 based on the tail-flick assay. (C) Hot-plate assay. (D) Dose-response curve of compound 33 based on the hot-plate assay. Mice received different doses of compound 33 (10–100 mg/kg subcutaneously) or morphine (10 mg/kg subcutaneously) or vehicle (control group). Time course of the antinociception response after dosing was measured and plotted over 4 h. The ED50 was estimated to be 77.6 mg/kg for the tail-flick assay and 77.1 mg/kg for the hot-plate assay. Data are expressed in mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs the control group.

Discussion and Conclusion

Natural products have been used for millennia for the treatment of pain.46 Within the last century, the isolation of bioactive components from these ethnomedicines has provided numerous chemical probes to elucidate many signaling mechanisms in the central nervous system.47 For example, the investigation of the pharmacology of morphine, isolated from *Papaver somniferum*, resulted in the identification of the opioid receptors.48 In more recent instances, by exploring the SAR of other naturally occurring opioid ligands like salvinorin A and mitragynine, medicinal chemists have unearthed novel compounds with unique pharmacology
that have revealed further insight into the molecular mechanisms of analgesia and opioid-induced side effects.12-14, 49

The results from this SAR study add to this tradition by providing new chemical scaffolds that can engage the µOR binding pocket in unique ways. To rationalize the SAR trends we observed, we used induced-fit docking (Maestro) to identify potential binding modes and interactions made between two of the more potent derivatives (19 and 33) and the µOR (Figure 5). When docked into the nanobody-stabilized structure of the active-state µOR, both 19 and 33 adopted similar top-scoring docking poses that share considerable overlap with the co-crystallized morphinan ligand BU72. Notably, the alkyl tertiary amines of 19 and 33 form salt-bridge with Asp147, which have been previously observed to anchor other cationic nitrogens within the opioid binding pocket.50, 51 Similarly, the aromatic rings of 19 and 33 occupy similar space to the phenolic ring of BU72.52 In the case of 19, the phenol moiety engages His297 via a network of water-mediated hydrogen bonds (Figure 5A). This hydrogen-bond interaction between a phenol oxygen and this network of water appears to be a highly conserved interaction and has been observed in both the morphinans BU72 and β-FNA and a tyrosine residue of DAMGO bound to the µOR.53-55 This proposed binding mode of 19 is consistent with the SAR we observed that indicated replacing or substituting the C10 phenol of 1 led to significantly reduced affinity for the µOR. Furthermore, while the binding pocket adjacent to C11 can accommodate the bromine of 19, the steric bulk from the aryl rings of 21-26 prevents the ligands from binding tightly to the µOR.
Figure 5. Binding modes and interactions of 19 (cyan) and 33 (green) as compared to BU72 (orange) (A – B, respectively). Crystal structure of μOR co-crystallized with agonist BU-72 (PDB: 5C1M) was used as a starting point for all molecular docking simulation studies. Ligand receptor interactions are depicted as dashed lines: hydrogen bonds (magenta), salt-bridge (yellow), pi-pi stacking (blue). For clarity, ligand-receptor interactions are omitted for BU-72.

In our SAR studies, we observed the installation of a phenethyl moiety to the N1 position of 2 dramatically increased its affinity and potency at the μOR. This improved activity likely indicates that the additional phenethyl moiety in 33 extends into a subpocket of the binding site, allowing it to make additional ligand-receptor interactions. This notion is supported by the docking pose of 33 that suggests the phenethyl group extends into a cavity toward the bottom of the binding site. Interestingly, in the co-crystal structures, this subpocket is occupied by the N-methyl group of BU72 and the cyclopropylmethyl group of β-FNA. As exemplified by β-FNA, in the classic “message-address” concept of opioid ligands, longer alkyl at this position generally possesses antagonist activity. However, the relatively high efficacy of 33 (E_{max} = 76%) suggests that extending further into this pocket or making additional ligand interactions allows for receptor activation. Notably, the binding pose does indicate a possible pi-pi stacking interactions with Tyr326, which has been previously implicated in μOR activation. By offering a new
scaffold to probe this subpocket by engaging these and other potential ligand-receptor
interactions, the akuamma alkaloids may reveal new ways to modulate µOR activation and
signaling properties.

In summary, we have conducted the first SAR of the akuamma alkaloids by leveraging
highly chemoselective, late-stage functionalization of the indole nucleus of 1 and 2. Through
these studies we identified that replacement of the N1-methyl group of 2 with larger alkyl groups
engages an allosteric pocket of the µOR, leading to increased affinity. Most notably, we
discovered lead compound 33 that possess considerably improved potency and selectivity
compared to the parent natural product 2. Compound 33 also exhibited antinociceptive effect in
tail-flick and hot-plate assays in mice. Taken together, these *in vitro* and *in vivo* results provide
further support that the continued investigation of opioid natural products can lead to new classes
of potent opioid ligands. Moreover, these initial discoveries provide a foundation for future
studies to probe how the akuamma alkaloids interact with the µOR binding pocket.

**Experimental**

**Chemistry**

*General Experimental Procedures.* All solvents and reagents were purchased from commercial
sources and used directly without further purification. Akuamma seed powder was purchased from
Relax Remedy and alkaloids were isolated and purified as previously described.\(^{18}\) Bruker 400
MHz, Bruker 400 MHz HD, and Bruker 600 MHz spectrometers were used to record \(^1\)H and \(^{13}\)C
NMR spectra and they were referenced to the residual solvent peaks (CHCl\(_3\): \(^1\)H δ=7.26, \(^{13}\)C
δ=77.16 ppm). High-resolution mass spectra were obtained on a Shimadzu LCMS-IT-TOF and
observed values are within 5.0 ppm of calculated exact masses of the indicated ions. High-
performance liquid chromatography (HPLC) was conducted on an Agilent 1260 Infinity II fitted
with a DAD detector and a Phenomenex Luna Omega PS-C18 column (100 x 4.6 mm). A gradient of acetonitrile/water (20-45%) each containing 0.1% formic acid with a flow rate of 1 mL/min was used. The purity of all compounds was found to be >95% as determined by HPLC.

*O*-methylakuammine (7). In an oven-dried round bottom flask under nitrogen, 1 (150.5 mg, 0.393 mmol) was dissolved in 10 mL of a 4:1 mixture of THF/MeOH. A solution of TMS-diazomethane in hexanes (2 M, 1.9 mL, 3.8 mmol) was added dropwise and the reaction stirred for 21 hours at room temperature. After 21 hours an additional 5 equivalents of TMS-diazomethane were added. After 48 hours, the reaction was quenched with 1M acetic acid and basified with sodium bicarbonate. The reaction solvent was removed on the rotary evaporator and the aqueous layer was extracted with ethyl acetate (3x50 mL). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with 2-4% methanol/dichloromethane to afford 126 mg of 7 (81% yield) as a white solid.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.74 (d, $J = 2.6$ Hz, 1H), 6.66 (dd, $J = 8.4, 2.6$ Hz, 1H), 6.54 (d, $J = 8.4$ Hz, 1H), 5.41 (q, $J = 6.6$ Hz, 1H), 4.17 (s, 1H), 3.96 (s, 1H), 3.94 (d, $J = 7.72$ Hz, 1H), 3.81 (s, 3H), 3.73 (s, 3H), 3.66 (dd, $J = 13.9, 4.3$ Hz, 1H), 3.61 (d, $J = 7.5$ Hz, 1H), 3.48 (s, 1H), 3.36 – 3.25 (m, 1H), 2.85 (d, $J = 16.6$ Hz, 1H), 2.78 (s, 3H), 2.58 (dd, $J = 13.8, 5.9$ Hz, 1H), 2.32 (d, $J = 13.8$ Hz, 1H), 2.05 (d, $J = 14.1$ Hz, 1H), 1.52 (d, $J = 7.0$ Hz, 3H), 1.47 (d, $J = 15.0$ Hz, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 172.17, 154.03, 145.93, 140.59, 118.69, 111.02, 110.98, 110.02, 104.25, 74.17, 58.46, 56.00, 54.65, 53.84, 52.85, 52.09, 50.58, 41.06, 31.10, 29.84, 29.50, 28.04, 13.07. HRMS calculated for C$_{23}$H$_{29}$N$_2$O$_4$: [M + H]$^+$: 397.2124 (found); 397.2127 (calcd).

*O*-acetylakuammine (8). In an oven-dried round bottom flask under nitrogen, 1 (50.0 mg, 0.131 mmol) was dissolved in 5 mL dichloromethane. DMAP (16.0 mg, 0.131 mmol), TEA (55 µL, 0.39 mmol), and acetic anhydride (25 µL, 0.26 mmol) were added and the reaction stirred for 4 hours
at room temperature. The reaction was diluted with water and basified with sodium bicarbonate. The aqueous layer was extracted with dichloromethane (3x10 mL). The combined organic layers were dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography with 1-6% methanol/dichloromethane to afford 39 mg of 8 (71% yield) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 6.87 (dd, $J$ = 8.4, 2.4 Hz, 1H), 6.82 (d, $J$ = 2.3 Hz, 1H), 6.63 (d, $J$ = 8.5 Hz, 1H), 5.60 (q, $J$ = 7.2 Hz, 1H), 4.54 – 4.49 (s, 1H), 4.16 (d, $J$ = 16.4 Hz, 1H), 3.98 (d, $J$ = 7.9 Hz, 1H), 3.91 – 3.83 (m, 1H), 3.82 (s, 3H), 3.66 (d, $J$ = 7.9 Hz, 1H), 3.60 (s, 1H), 3.38 (ddd, $J$ = 16.1, 14.4, 5.8 Hz, 1H), 3.13 (d, $J$ = 16.4 Hz, 1H), 2.85 (d, $J$ = 5.8 Hz, 1H), 2.83 (s, 3H), 2.47 (ddd, $J$ = 14.5, 4.3, 2.0 Hz, 1H), 2.25 (s, 3H), 2.10 (ddd, $J$ = 14.6, 3.7, 1.9 Hz, 1H), 1.72 (dd, $J$ = 15.9, 4.6 Hz, 1H), 1.57 (dd, $J$ = 7.1, 2.4 Hz, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.17, 170.02, 148.86, 144.58, 138.60, 134.99, 123.06, 121.06, 116.73, 110.83, 102.59, 74.40, 57.98, 53.94, 53.38, 53.07, 52.50, 50.04, 39.98, 29.58, 28.65, 26.95, 21.24, 13.20. HRMS calculated for C$_{24}$H$_{29}$N$_2$O$_5$: [M + H]$^+$: 425.2071 (found); 425.2076 (calcd).

O-trifluoromethansulfonylakuammine (9). In an oven dried round bottom flask under nitrogen 1 (100 mg, 0.2618 mmol) was dissolved in 10 mL anhydrous dichloromethane at room temperature. To the solution was added DMAP (96 mg, 0.7854 mmol) and N-phenyl-bis(trifluoromethanesulfonimid) (187 mg, 0.524 mmol). The reaction was stirred at room temperature for 1 hour. The reaction was concentrated via rotary evaporation. The crude mixture was diluted into 30 mL water and extracted with ethyl acetate (3x30 mL). The combine organic layers were dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified via column chromatography (0-4% MeOH/DCM) to yield 130 mg of the desired product (97% yield) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.03 (d, $J$ = 7.2 Hz, 2H), 6.61 (d, $J$ = 8.5 Hz, 1H), 5.46 (q, $J$ = 7.2 Hz, 1H), 4.25 (s, 1H), 4.01 – 3.93 (m, 2H), 3.82 (s, 3H), 3.66 – 3.52
(m, 3H), 3.34 (td, J = 14.6, 5.7 Hz, 1H), 2.89 (d, J = 16.8 Hz, 1H), 2.85 (s, 3H), 2.67 (dd, J = 13.5, 5.7 Hz, 1H), 2.37 – 2.29 (m, 1H), 2.14 – 2.04 (m, 1H), 1.54 (dd, J = 7.1, 2.4 Hz, 3H), 1.48 (dd, J = 15.3, 4.4 Hz, 1H). 13C NMR (151 MHz, CDCl3) δ 170.80, 150.77, 143.42, 139.03, 124.79, 121.45, 117.07, 116.85, 111.66, 111.31, 102.19, 74.66, 57.81, 53.99, 53.47, 53.27, 52.75, 50.21, 39.55, 29.57, 27.96, 26.60, 13.29. HRMS calculated for C23H26N3O3: [M + H]+: 392.1970 (found); 392.1974 (calcd).

**Pseudoakuummigine (2).** In an oven-dried round bottom flask under nitrogen, 9 (200 mg, 0.3891 mmol) was dissolved in 3 mL of dry DMF. To the solution at room temperature was added TEA (1.08 mL, 7.7821 mmol), formic acid (0.74 mL, 1.945 mmol), and dpppPd(II)Cl2 (46 mg, 0.07782 mmol). Following the additions, the temperature of the reaction was increased to 80°C and allowed to stir under N2 for 4 hours. Upon completion, the reaction was cooled to room temperature and diluted with 300 mL H2O. The solution was extracted with 50 mL EtOAc 3X. The combined organic layers were washed with 30 mL H2O 2X and 30 mL brine 1X. The combined organic layers were dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography eluting with 0–20 % methanol/dichloromethane to afford 97 mg of 7 (69% yield) as a white solid whose 1H and 13C NMR spectra were identical to 2 isolated from *P. nitida*.18

**10-cyanopseudoakuummigine (10).** In an oven-dried round bottom flask under nitrogen, 9 (200.0 mg, 0.3891 mmol) was dissolved in 10 mL anhydrous DMF at room temperature. To the solution was added anhydrous TEA (5.3 mL, 40 mmol) followed by Pd(dppf)Cl2 (22.9 mg, 0.0389 mmol) and zinc cyanide (23.0 mg, 0.194 mmol). The reaction was stirred at 120 °C for 24 hours. The reaction was diluted with 400 mL water and extracted with ethyl acetate (3x75 mL). The combined organic layers were washed with fresh water (150 mL) three times and 200 mL of
brane once. The organic layer was dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by recrystallization in ether and ethanol to afford 8.2 mg of the desired product (6 % yield) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.44 (dd, $J = 8.2$, 1.7 Hz, 1H), 7.35 (d, $J = 1.7$ Hz, 1H), 6.64 (d, $J = 8.2$ Hz, 1H), 5.42 (q, $J = 6.9$ Hz, 1H), 4.18 (s, 1H), 3.96 (d, $J = 7.6$ Hz, 1H), 3.91 (d, $J = 17.0$ Hz, 1H), 3.84 (s, 3H), 3.55 (d, $J = 7.6$ Hz, 1H), 3.52 (s, 1H), 3.46 – 3.27 (m, 2H), 2.89 (s, 3H), 2.82 (d, $J = 17.4$ Hz, 1H), 2.61 (dd, $J = 12.7$, 4.6 Hz, 1H), 2.28 (d, $J = 12.6$ Hz, 1H), 2.08 (ddd, $J = 13.9$, 3.7, 2.0 Hz, 1H), 1.53 (dd, $J = 7.1$, 2.6 Hz, 3H), 1.39 (dd, $J = 15.0$, 4.0 Hz, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 171.77, 155.55, 141.89, 140.30, 133.29, 126.50, 120.54, 118.92, 109.83, 103.74, 102.05, 74.83, 58.52, 54.60, 54.07, 52.69, 52.38, 51.18, 41.09, 31.30, 28.81, 28.03, 13.03. HRMS calculated for C$_{23}$H$_{26}$N$_3$O$_3$: [M + H]$^+$: 392.1970 (found); 392.1974 (calcd).

General Procedure A: Suzuki-Miyaura Coupling. In an oven-dried round bottom flask under nitrogen, 9 or 20 (1.0 equiv) was dissolved in 3 mL anhydrous toluene and 2 mL anhydrous methanol at room temperature. To the solution was added K$_2$CO$_3$ (2.0 equiv) followed by boronic acid (1.1 equiv) and Pd(PPh$_3$)$_4$ (5 mol%). The reaction was stirred at 80 °C for until consumption of the starting material as indicated by TLC. The reaction was cooled to room temperature concentrated in vacuo. The residue was purified by column chromatography (0 - 4% MeOH/DCM) afford the desired product as a white solid.

10-phenylseudoakuammigine (11). Prepared from 9 (45.0 mg, 0.0875 mmol) according to General Procedure A to yield 16.6 mg of 11 (43% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.49 (d, $J = 7.1$ Hz, 2H), 7.39 (t, $J = 7.7$ Hz, 3H), 7.35 (s, 1H), 7.28 (d, $J = 7.2$ Hz, 1H), 6.73 (d, $J = 8.1$ Hz, 1H), 5.55 (t, $J = 7.5$ Hz, 1H), 4.41 (s, 1H), 4.09 (d, $J = 16.3$ Hz, 1H), 3.99 (d, $J = 7.8$ Hz, 1H), 3.83 (s, 3H), 3.75 (dd, $J = 13.8$, 5.1 Hz, 1H), 3.68 (d, $J = 7.7$ Hz, 1H), 3.57 (s, 1H), 3.49 –
3.34 (m, 1H), 3.03 (d, $J = 16.5$ Hz, 1H), 2.89 (s, 3H), 2.76 (dd, $J = 13.5$, 5.8 Hz, 1H), 2.43 (d, $J = 13.7$ Hz, 1H), 2.11 (d, $J = 14.2$ Hz, 1H), 1.68 (dd, $J = 15.6$, 4.6 Hz, 1H), 1.57 (d, $J = 4.8$ Hz, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 171.75, 150.94, 141.64, 139.24, 138.08, 133.80, 128.86, 126.92, 126.75, 126.55, 121.52, 121.15, 110.58, 103.25, 74.52, 58.20, 54.27, 53.57, 52.95, 52.32, 50.44, 40.50, 29.96, 29.37, 27.48, 13.19. HRMS calculated for C$_{28}$H$_{31}$N$_2$O$_3$: [M + H]$^+$: 443.2330 (found); 443.2335 (calcd).

10-(p-tolyl)-psuedoakuammigine (I2). Prepared from 9 (50.0 mg, 0.0972 mmol) according to General Procedure A to yield 34 mg of 12 (77% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.38 (d, $J = 8.1$ Hz, 3H), 7.32 (d, $J = 1.8$ Hz, 1H), 7.21 (d, $J = 7.9$ Hz, 2H), 6.73 (d, $J = 8.1$ Hz, 1H), 5.58 (q, $J = 7.2$ Hz, 1H), 4.46 (s, 1H), 4.13 (d, $J = 16.4$ Hz, 1H), 4.00 (d, $J = 7.8$ Hz, 1H), 3.83 (s, 3H), 3.69 (d, $J = 7.8$ Hz, 1H), 3.60 (s, 1H), 3.44 (td, $J = 15.0$, 5.8 Hz, 2H), 3.10 (d, $J = 16.4$ Hz, 1H), 2.88 (s, 3H), 2.81 (dd, $J = 13.2$, 5.7 Hz, 1H), 2.48 (d, $J = 4.0$ Hz, 1H), 2.37 (s, 3H), 2.12 (ddd, $J = 14.5$, 3.8, 1.9 Hz, 1H), 1.72 (dd, $J = 15.7$, 4.6 Hz, 1H), 1.58 (dd, $J = 7.1$, 2.4 Hz, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 171.58, 150.54, 138.88, 138.72, 136.74, 136.34, 134.00, 129.60, 126.82, 126.63, 122.01, 121.35, 110.74, 102.92, 74.49, 58.07, 54.26, 53.43, 53.23, 52.37, 50.43, 40.27, 29.84, 29.42, 27.26, 21.15, 13.23. HRMS calculated for C$_{29}$H$_{33}$N$_2$O$_3$: [M + H]$^+$: 457.2479 (found); 257.2491 (calcd).

10-(p-methoxyphenyl)-psuedoakuammigine (I3). Prepared from 9 (45.0 mg, 0.0875 mmol) according to General Procedure A to yield 7.0 mg of 13 (17%). $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.44 – 7.36 (m, 2H), 7.34 (dt, $J = 8.1$, 2.0 Hz, 1H), 7.29 (t, $J = 2.0$ Hz, 1H), 6.97 – 6.91 (m, 2H), 6.71 (dd, $J = 8.1$, 2.7 Hz, 1H), 5.53 (q, $J = 7.5$ Hz, 1H), 4.38 (s, 1H), 4.08 (d, $J = 16.7$ Hz, 1H), 3.99 (dd, $J = 7.8$, 2.7 Hz, 1H), 3.83 (s, 6H), 3.75 (d, $J = 13.9$ Hz, 1H), 3.67 (d, $J = 2.7$ Hz, 1H), 3.57 (s, 1H), 3.46 – 3.37 (m, 1H), 3.02 (d, $J = 16.5$ Hz, 1H), 2.87 (s, 3H), 2.78 – 2.72 (m, 1H),
2.42 (d, J = 14.3 Hz, 1H), 2.14 – 2.08 (m, 1H), 1.66 (dt, J = 15.7, 3.6 Hz, 1H), 1.57 (d, J = 7.4 Hz, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 171.80, 158.66, 150.47, 139.24, 138.38, 134.33, 133.50, 127.74, 126.41, 121.16, 120.95, 114.31, 110.55, 103.29, 74.47, 58.21, 55.49, 54.36, 53.57, 53.05, 52.30, 50.52, 40.52, 30.05, 29.36, 27.51, 13.18. HRMS calculated for C₂₉H₃₃N₂O₄: [M + H]⁺: 473.2439 (found); 473.2440 (calcd).

10- (p-cyanophenyl)-psuedoakuammigine (14). Prepared from 9 (50.0 mg, 0.0972 mmol) according to General Procedure A to yield 9.8 mg of 14 (22% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.67 – 7.55 (m, 4H), 7.42 – 7.35 (m, 2H), 6.73 (d, J = 8.1 Hz, 1H), 5.50 – 5.42 (q, 1H), 4.23 (s, 1H), 3.98 (d, J = 7.6 Hz, 1H), 3.93 (s, 1H), 3.83 (s, 3H), 3.63 (d, J = 7.6 Hz, 1H), 3.61 – 3.55 (m, 1H), 3.54 (s, 1H), 3.44 – 3.31 (m, 1H), 2.89 (s, 3H), 2.63 (dd, J = 13.4, 5.6 Hz, 1H), 2.33 (d, J = 13.8 Hz, 1H), 2.14 – 2.06 (m, 2H), 1.54 (dd, J = 7.0, 2.4 Hz, 3H), 1.51 (d, J = 4.2 Hz, 1H). ¹³C NMR (151 MHz, CDCl₃) δ 170.99, 151.51, 145.65, 138.54, 132.79, 132.24, 127.94, 127.33, 127.16, 125.18, 121.62, 119.19, 111.49, 110.24, 101.89, 74.59, 57.77, 53.81, 53.31, 52.74, 49.98, 39.62, 29.58, 27.92, 26.58, 13.36. HRMS calculated for C₂₉H₃₀N₃O₃: [M + H]⁺: 468.2280 (found); 468.2287 (calcd).

10- (p-fluorophenyl)-psuedoakuammigine (15). Prepared from 9 (36.0 mg, 0.0760 mmol) according to General Procedure A to yield 14.1 mg of 15 (44% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.47 – 7.38 (m, 2H), 7.34 (dd, J = 8.1, 1.9 Hz, 1H), 7.28 (d, J = 1.9 Hz, 1H), 7.07 (t, J = 8.7 Hz, 2H), 6.73 (d, J = 8.1 Hz, 1H), 5.57 (q, J = 7.1 Hz, 1H), 4.45 (s, 1H), 4.12 (d, J = 16.5 Hz, 1H), 4.00 (d, J = 7.8 Hz, 1H), 3.83 (s, 3H), 3.78 (dd, J = 13.6, 4.6 Hz, 1H), 3.68 (d, J = 7.8 Hz, 1H), 3.62 – 3.57 (bs, 1H), 3.43 (td, J = 15.0, 5.8 Hz, 1H), 3.07 (d, J = 16.4 Hz, 1H), 2.88 (s, 3H), 2.79 (dd, J = 13.2, 5.6 Hz, 1H), 2.45 (d, J = 14.8 Hz, 1H), 2.11 (d, J = 12.6 Hz, 1H), 1.70 (dd, J = 15.6, 4.5 Hz, 1H), 1.58 (dd, J = 7.1, 2.4 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 171.84,
HRMS calculated for C\textsubscript{28}H\textsubscript{30}N\textsubscript{2}O\textsubscript{3}F: [M + H]\(^+\): 461.2237 (found); 461.2240 (calcd).

10-(3'-furanyl)-pseudoakuammigine (16). Prepared from \textbf{9} (50.0 mg, 0.0972 mmol) according to General Procedure A to yield 28.3 mg of \textbf{16} (67% yield).\(^1\)H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.59 (t, \(J = 1.2\) Hz, 1H), 7.42 (t, \(J = 1.7\) Hz, 1H), 7.28 (d, \(J = 1.8\) Hz, 1H), 7.22 (d, \(J = 1.8\) Hz, 1H), 6.65 (d, \(J = 8.1\) Hz, 1H), 6.60 (t, \(J = 1.3\) Hz, 1H), 5.47 (q, \(J = 7.2\) Hz, 1H), 4.28 (s, 1H), 4.02 (s, 1H), 3.97 (d, \(J = 7.7\) Hz, 1H), 3.84 (s, 3H), 3.72 – 3.61 (m, 2H), 3.53 (d, \(J = 3.3\) Hz, 1H), 3.43 – 3.30 (m, 1H), 2.92 (d, \(J = 16.7\) Hz, 2H), 2.85 (s, 3H), 2.65 (dd, \(J = 13.4, 5.8\) Hz, 1H), 2.42 – 2.32 (m, 1H), 2.09 (ddd, \(J = 14.2, 3.7, 1.9\) Hz, 1H), 1.59 (d, \(J = 4.4\) Hz, 1H), 1.55 (dd, \(J = 7.1, 2.4\) Hz, 3H). \(^{13}\)C NMR (101 MHz, CDCl\textsubscript{3}) \(\delta\) 172.05, 150.90, 143.53, 139.64, 137.48, 126.95, 125.43, 124.59, 120.51, 119.82, 110.36, 109.03, 103.62, 74.47, 58.36, 54.37, 52.70, 52.18, 50.50, 40.81, 30.62, 29.84, 29.25, 27.79, 13.13. HRMS calculated for C\textsubscript{26}H\textsubscript{29}N\textsubscript{2}O\textsubscript{4}: [M + H]\(^+\): 433.2118 (found); 433.2127 (calcd).

\textit{General Procedure B:} In an oven-dried round bottom flask under nitrogen, \textbf{1} or \textbf{2} (1.0 equiv) was dissolved in 1:1 anhydrous dichloromethane and trifluoroacetic acid at 0 °C. Over 1 hour, a cold solution of NBS or NIS (1.1 equiv) dissolved in a 1mL 1:1 DCM/TFA was added to the solution. After the addition of NBS or NIS, the reaction was allowed to stir at 0°C for 5 hours. The reaction was cooled poured into ice water and basified with NaHCO\textsubscript{3}. The solution was washed with sodium thiosulfate and extracted DCM (3x10 mL). The organic layers were combined, dried over sodium sulfate, filtered, and concentrated \textit{in vacuo}. The residue was then purified by column chromatography (0.5-10% MeOH/DCM) to yield the desired product.
10-bromopseudoakuammigine (17). Prepared from 2 (70 mg, 0.1913 mmol) and NBS according to general procedure B to yield 32 mg of 17 (38% yield) as a white solid. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.22 (dd, J = 8.3, 2.1 \text{ Hz}, 1H), 7.17 (d, J = 2.0 \text{ Hz}, 1H), 6.51 (d, J = 8.3 \text{ Hz}, 1H), 5.43 (q, J = 7.2 \text{ Hz}, 1H), 4.21 (s, 1H), 3.95 (d, J = 7.7 \text{ Hz}, 1H), 3.93 (s, 1H), 3.83 (s, 3H), 3.60 (d, J = 7.6 \text{ Hz}, 1H), 3.55 (dd, J = 13.8, 4.5 \text{ Hz}, 1H), 3.50 (d, J = 3.5 \text{ Hz}, 1H), 3.30 (dd, J = 15.3, 14.0, 5.8 \text{ Hz}, 1H), 2.86 (d, J = 16.7 \text{ Hz}, 1H), 2.81 (s, 3H), 2.61 (dd, J = 13.4, 5.7 \text{ Hz}, 1H), 2.31 (d, J = 14.1 \text{ Hz}, 1H), 2.07 (dd, J = 14.1, 3.7, 1.9 \text{ Hz}, 1H), 1.53 (dd, J = 7.1, 2.5 \text{ Hz}, 3H), 1.47 (dd, J = 15.1, 4.3 \text{ Hz}, 1H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta 171.90, 150.97, 141.32, 139.45, 130.33, 126.01, 119.16, 111.84, 111.53, 103.77, 74.51, 58.50, 54.57, 54.07, 52.82, 52.24, 50.81, 41.02, 31.02, 29.15, 27.93, 13.07. HRMS calculated for C\(_{22}\)H\(_{26}\)N\(_2\)O\(_3\)Br: [M + H]\(^+\): 445.1125 (found); 445.1127 (calcd).

10-iodopseudoakuammigine (18). Prepared from 2 (150 mg, 0.4098 mmol) and NIS according to general procedure B to afford 150.3 mg of 18 (75% yield) as a white solid. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.44 (dd, J = 8.2, 1.8 \text{ Hz}, 1H), 7.34 (d, J = 1.8 \text{ Hz}, 1H), 6.45 (d, J = 8.2 \text{ Hz}, 1H), 5.52 (q, J = 7.4 \text{ Hz}, 1H), 4.37 (s, 1H), 4.05 (d, J = 16.7 \text{ Hz}, 1H), 3.97 (d, J = 7.8 \text{ Hz}, 1H), 3.84 (s, 3H), 3.73 – 3.64 (m, 1H), 3.62 (d, J = 7.7 \text{ Hz}, 1H), 3.55 (s, 1H), 3.34 (td, J = 14.9, 5.8 \text{ Hz}, 1H), 2.98 (d, J = 16.5 \text{ Hz}, 1H), 2.81 (s, 3H), 2.72 (dd, J = 13.2, 5.7 \text{ Hz}, 1H), 2.38 (d, J = 14.2 \text{ Hz}, 1H), 2.08 (ddd, J = 14.4, 3.7, 1.9 \text{ Hz}, 1H), 1.59 (d, J = 3.9 \text{ Hz}, 1H), 1.55 (dd, J = 7.1, 2.5 \text{ Hz}, 3H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta 171.48, 151.27, 141.11, 136.75, 131.57, 121.25, 112.68, 102.88, 81.89, 74.53, 58.20, 54.22, 53.72, 52.87, 52.39, 50.44, 40.51, 29.21, 27.41, 22.82, 14.25, 13.16. HRMS calculated for C\(_{22}\)H\(_{26}\)N\(_2\)O\(_3\)I: [M + H]\(^+\): 493.0987 (found); 493.0988 (calcd).

11-bromoakuammine (19). Prepared from 1 (30.0 mg, 0.0785 mmol) and NBS according to general procedure B to afford 11.9 mg of 19 (34% yield) as a white solid. \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta 7.22 (dd, J = 8.3, 2.1 \text{ Hz}, 1H), 7.17 (d, J = 2.0 \text{ Hz}, 1H), 6.51 (d, J = 8.3 Hz, 1H), 5.43 (q, J = 7.2 Hz, 1H), 4.21 (s, 1H), 3.95 (d, J = 7.7 Hz, 1H), 3.93 (s, 1H), 3.83 (s, 3H), 3.60 (d, J = 7.6 Hz, 1H), 3.55 (dd, J = 13.8, 4.5 Hz, 1H), 3.50 (d, J = 3.5 Hz, 1H), 3.30 (dd, J = 15.3, 14.0, 5.8 Hz, 1H), 2.86 (d, J = 16.7 Hz, 1H), 2.81 (s, 3H), 2.61 (dd, J = 13.4, 5.7 Hz, 1H), 2.31 (d, J = 14.1 Hz, 1H), 2.07 (dd, J = 14.1, 3.7, 1.9 Hz, 1H), 1.53 (dd, J = 7.1, 2.5 Hz, 3H), 1.47 (dd, J = 15.1, 4.3 Hz, 1H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta 171.90, 150.97, 141.32, 139.45, 130.33, 126.01, 119.16, 111.84, 111.53, 103.77, 74.51, 58.50, 54.57, 54.07, 52.82, 52.24, 50.81, 41.02, 31.02, 29.15, 27.93, 13.07. HRMS calculated for C\(_{22}\)H\(_{26}\)N\(_2\)O\(_3\)Br: [M + H]\(^+\): 445.1125 (found); 445.1127 (calcd).
6.84 (d, J = 2.3 Hz, 1H), 6.65 (d, J = 2.4 Hz, 1H), 5.59 (q, J = 7.2 Hz, 1H), 4.46 (s, 1H), 4.14 (d, J = 16.5 Hz, 1H), 3.98 (d, J = 8.0 Hz, 1H), 3.88 (d, J = 7.8 Hz, 1H), 3.83 (s, 3H), 3.73 (d, J = 7.9 Hz, 1H), 3.56 (s, 1H), 3.35 (td, J = 15.1, 5.8 Hz, 1H), 3.15 – 3.08 (m, 1H), 3.08 (s, 3H), 2.83 – 2.78 (m, 1H), 2.45 (d, J = 14.9 Hz, 1H), 2.11 – 2.06 (m, 1H), 1.56 (dd, J = 7.2, 2.6 Hz, 4H).

13C NMR (151 MHz, CDCl3) δ 171.12, 151.65, 142.01, 141.44, 125.28, 119.80, 110.71, 110.12, 105.40, 73.72, 57.96, 53.85, 53.07, 52.58, 49.79, 40.00, 32.62, 29.85, 28.72, 26.99, 14.27, 13.28.

HRMS calculated for C22H26N2O4Br: [M + H]+: 461.1078 (found); 461.1076 (calcd).

11-iodoakuammine (20). Prepared from 2 (86.0 mg, 0.225 mmol) and NIS according to general procedure B afford 20.1 mg of 19 (22% yield) as a white solid. 1H NMR (600 MHz, CDCl3) δ 7.12 (d, J = 2.5 Hz, 1H), 6.66 (d, J = 2.5 Hz, 1H), 5.59 (q, J = 7.3 Hz, 1H), 4.49 (s, 1H), 4.16 (d, J = 16.6 Hz, 1H), 3.96 (d, J = 8.0 Hz, 1H), 3.90 (s, 1H), 3.82 (s, 3H), 3.72 (d, J = 7.9 Hz, 1H), 3.56 (s, 1H), 3.35 (td, J = 15.3, 5.7 Hz, 1H), 3.14 (d, J = 15.3 Hz, 1H), 3.06 (s, 3H), 2.83 (d, J = 12.5 Hz, 1H), 2.46 (d, J = 14.8 Hz, 1H), 2.08 (d, J = 15.2 Hz, 1H), 1.58 (s, 1H), 1.55 (dd, J = 7.1, 2.5 Hz, 3H). 13C NMR (151 MHz, CDCl3) δ 171.02, 151.35, 144.90, 141.35, 128.74, 126.48, 114.39, 111.35, 103.10, 75.09, 73.69, 57.81, 53.84, 53.42, 52.63, 52.36, 49.80, 39.91, 33.48, 29.85, 28.72, 26.99, 14.27, 13.28. HRMS calculated for C22H26N2O4I: [M + H]+: 509.0938 (found); 509.0937 (calcd).

11-phenyl-akuammine (21). Prepared from 20 (30.0 mg, 0.0590 mmol) according to General Procedure A to yield 14.9 mg of 21 (55% yield). 1H NMR (400 MHz, CDCl3) δ 7.35 (d, J = 4.4 Hz, 4H), 7.30 (m, 1H), 6.64 (d, J = 2.6 Hz, 1H), 6.50 (d, J = 2.6 Hz, 1H), 5.45 (q, J = 7.3 Hz, 1H), 4.13 (s, 1H), 4.00 (s, 1H), 3.94 (d, J = 7.7 Hz, 1H), 3.85 (d J = 4.3 Hz, 1H), 3.82 (s, 3H), 3.75 (d, J = 7.6 Hz, 1H), 3.48 (d, J = 3.6 Hz, 1H), 3.40 – 3.28 (m, 1H), 2.92 (d, J = 16.7 Hz, 1H), 2.68 (dd, J = 13.2, 5.6 Hz, 1H), 2.33 (s, 1H), 2.28 (s, 3H), 2.08 – 1.99 (m, 1H), 1.63 – 1.54 (m, 1H), 1.54 – 1.47 (m, 3H). 13C NMR (151 MHz, CDCl3) δ 170.78, 150.80, 141.19, 139.61, 139.13, 130.60,
HRMS calculated for C_{28}H_{31}N_{2}O_{4}: [M + H]^+: 459.2273 (found); 459.2284 (calcd).

11-(p-tolyl)-akuammine (22). Prepared from 20 (30.0 mg, 0.0590 mmol) according to General Procedure A to yield 17.3 mg of 22 (62% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.21 (d, \(J = 7.5\) Hz, 2H), 7.16 (d, \(J = 7.9\) Hz, 2H), 6.64 (d, \(J = 2.6\) Hz, 1H), 6.55 (d, \(J = 2.6\) Hz, 1H), 5.58 (q, \(J = 7.4\) Hz, 1H), 4.38 (s, 1H), 4.16 (d, \(J = 16.5\) Hz, 1H), 4.09 – 3.98 (m, 1H), 3.96 (d, \(J = 7.8\) Hz, 1H), 3.85 (s, 3H), 3.78 (d, \(J = 7.8\) Hz, 1H), 3.54 (s, 1H), 3.46 – 3.32 (m, 1H), 3.13 (d, \(J = 16.4\) Hz, 1H), 2.42 (d, \(J = 15.2\) Hz, 1H), 2.37 (s, 3H), 2.29 (s, 3H), 2.04 (dd, \(J = 14.7, 3.1\) Hz, 1H), 1.72 (dd, \(J = 15.6, 4.5\) Hz, 1H), 1.57 (dd, \(J = 7.1, 2.3\) Hz, 3H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta\) 170.98, 150.81, 141.32, 139.81, 137.35, 136.33, 129.09, 124.74, 117.83, 109.37, 102.75, 73.54, 57.67, 53.73, 53.43, 52.70, 52.64, 49.57, 39.42, 33.01, 27.80, 26.55, 21.38, 13.36. HRMS calculated for C_{29}H_{33}N_{2}O_{4}: [M + H]^+: 473.2438 (found); 473.2440 (calcd).

11-(p-methoxyphenyl)-akuammine (23). Prepared from 20 (30.0 mg, 0.0590 mmol) according to General Procedure A to yield 10.6 mg of 23 (62% yield). \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\) 7.27 (s, 2H), 6.89 (d, \(J = 8.2\) Hz, 2H), 6.61 (s, 1H), 6.50 (s, 1H), 5.47 (q, \(J = 7.4\) Hz, 1H), 4.18 (s, 1H), 4.01 (d, \(J = 16.4\) Hz, 1H), 3.94 (d, \(J = 7.5\) Hz, 1H), 3.83 (s, 3H), 3.83 (s, 3H), 3.76 (d, \(J = 2.5\) Hz, 1H), 3.75 (d, \(J = 1.4\) Hz, 1H), 3.50 – 3.48 (m, 1H), 3.35 (s, 1H), 2.94 (d, \(J = 16.8\) Hz, 1H), 2.70 (bs, 1H), 2.34 (s, 1H), 2.31 (s, 3H), 2.03 (d, \(J = 13.2\) Hz, 1H), 1.57 (s, 1H), 1.54 (dd, \(J = 7.1, 2.4\) Hz, 3H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta\) 171.93, 158.81, 150.45, 142.23, 141.42, 132.37, 130.21, 127.87, 120.03, 116.58, 114.28, 113.49, 111.73, 109.49, 73.46, 58.34, 55.41, 54.32, 53.27, 52.88, 52.26, 50.22, 40.61, 32.83, 31.74, 27.68, 13.15. HRMS calculated for C_{29}H_{33}N_{2}O_{5}: [M + H]^+: 489.2389 (found); 489.2389 (calcd).
**11-\(p\)-cyanophenyl)-akuammine (24).** Prepared from \(20\) (39.5 mg, 0.0778 mmol) according to General Procedure A to yield 14.4 mg of \(24\) (38% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.66\) (d, \(J = 8.4\) Hz, 2H), \(7.47\) (d, \(J = 7.7\) Hz, 2H), \(6.72\) (d, \(J = 2.5\) Hz, 1H), \(6.52\) (d, \(J = 2.6\) Hz, 1H), \(5.56\) (q, \(J = 7.4\) Hz, 1H), \(4.29\) (s, 1H), \(4.09\) (d, \(J = 16.4\) Hz, 1H), \(3.96\) (d, \(J = 7.8\) Hz, 1H), \(3.92\) (s, 1H), \(3.85\) (s, 3H), \(3.75\) (d, \(J = 7.7\) Hz, 1H), \(3.54\) (s, 1H), \(3.40\) (ddd, \(J = 21.7, 13.8, 6.3\) Hz, 1H), \(3.06\) (d, \(J = 17.2\) Hz, 1H), \(2.82\) (dd, \(J = 13.2, 5.5\) Hz, 1H), \(2.38\) (d, \(J = 14.4\) Hz, 1H), \(2.28\) (s, 3H), \(2.05\) (dt, \(J = 14.1, 3.3\) Hz, 1H), \(1.69 - 1.60\) (m, 1H), \(1.56\) (dd, \(J = 7.2, 2.3\) Hz, 3H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta 171.34, 151.48, 144.77, 141.66, 132.01, 126.20, 122.67, 118.91, 116.38, 111.01, 103.58, 73.52, 57.98, 53.91, 53.16, 53.00, 52.57, 49.86, 39.94, 33.58, 29.08, 27.07, 13.26. HRMS calculated for C\(_{29}\)H\(_{30}\)N\(_3\)O\(_4\): [M + H]\(^+\): 484.2235 (found); 484.2236 (calcd).

11-(\(p\)-fluorophenyl)-akuammine (25). Prepared from \(20\) (44 mg, 0.0868 mmol) according to General Procedure A to yield 18.8 mg of \(25\) (46% yield). \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta 7.32\) (s, 2H), \(7.08 - 7.02\) (m, 2H), \(6.64\) (d, \(J = 2.8\) Hz, 1H), \(6.48\) (d, \(J = 2.9\) Hz, 1H), \(5.46\) (q, \(J = 7.8\) Hz, 1H), \(4.15\) (s, 1H), \(3.98\) (d, \(J = 19.2\) Hz, 1H), \(3.94\) (d, \(J = 7.6\) Hz, 1H), \(3.83\) (s, 3H), \(3.38 - 3.77\) (m, 1H), \(3.74\) (d, \(J = 7.6\) Hz, 1H), \(3.48\) (s, 1H), \(3.38 - 3.30\) (m, 1H), \(2.91\) (d, \(J = 16.9\) Hz, 1H), \(2.67\) (d, \(J = 13.9\) Hz, 1H), \(2.32\) (s, 1H), \(2.29\) (s, 3H), \(2.04\) (dd, \(J = 14.7, 4.4\) Hz, 1H), \(1.57\) (d, \(J = 14.0\) Hz, 1H), \(1.53\) (d, \(J = 4.6\) Hz, 3H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta 171.99, 162.94, 161.30, 150.23, 142.52, 141.80, 136.03, 131.00, 126.84, 119.61, 116.39, 115.03, 110.01, 104.63, 73.50, 58.43, 54.39, 53.38, 52.85, 52.25, 50.31, 40.73, 32.96, 30.73, 27.79, 13.13. HRMS calculated for C\(_{28}\)H\(_{30}\)N\(_2\)O\(_4\)F: [M + H]\(^+\): 477.2190 (found); 477.2190 (calcd).

11-(3'-furanyl)-akuammine (26). Prepared from \(20\) (80.0 mg, 0.158 mmol) according to General Procedure A to yield 8.0 mg of \(26\) (11% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.42\) (d, \(J = 3.2\) Hz, 2H), \(6.58\) (dd, \(J = 44.2, 2.5\) Hz, 2H), \(6.47\) (s, 1H), \(5.56\) (q, \(J = 7.5\) Hz, 1H), \(4.37\) (s, 1H), \(4.12\) (d,
16-hydroxymethyl pseudoakuammigine (28). In an oven dried round bottom flask under nitrogen 5 mL of anhydrous THF was added. At 0°C, LAH (11 mg, 2 equiv) was added to the reaction flask. A solution of 2 (50 mg, 1 equiv) in 2 mL of anhydrous THF was added dropwise to the reaction at 0 °C. The reaction was stirred for 3 hours at room temperature. LiAlH₄ (11 mg, 2 equiv) was added at 0 °C. After 1 hour the reaction was halted over ice by the dropwise addition of 300 uL H₂O, 300 uL 15% NaOH, and 1 mL H₂O. 3 mL of H₂O was added to the reaction and the solution was filtered over celite. The filtrate was extracted with 30 mL EtOAc three times. The organic layers were combined, dried over sodium sulfate, filtered, and concentrated in vacuo. The crude product was purified via column chromatography (0-5% MeOH/DCM) to yield 13 mg (67% yield) of the desired product. 

\[ J = 16.5 \text{ Hz}, 1H \], 3.96 (d, \( J = 7.9 \text{ Hz}, 1H \)), 3.83 (s, 3H), 3.76 (d, \( J = 7.7 \text{ Hz}, 1H \)), 3.54 (s, 1H), 3.40 (dd, \( J = 15.2, 5.7 \text{ Hz}, 1H \)), 3.09 (d, \( J = 16.4 \text{ Hz}, 1H \)), 2.81 (dd, \( J = 13.2, 5.5 \text{ Hz}, 1H \)), 2.53 (s, 3H), 2.42 (d, \( J = 14.7 \text{ Hz}, 1H \)), 2.06 (d, \( J = 14.4 \text{ Hz}, 1H \)), 1.64 (dd, \( J = 15.9, 4.6 \text{ Hz}, 1H \)), 1.56 (d, \( J = 6.2 \text{ Hz}, 3H \)). \( ^{13} \text{C} \) NMR (151 MHz, CDCl₃) δ 171.32, 150.42, 142.66, 142.28, 140.19, 123.69, 119.14, 117.04, 112.47, 110.03, 73.51, 57.95, 53.92, 53.11, 52.82, 52.48, 49.83, 39.93, 32.39, 28.81, 27.02, 13.23. HRMS calculated for C₂₆H₂₉N₂O₅: [M + H]⁺: 449.2070 (found); 449.2076 (calcd).
55.07, 54.74, 53.18, 50.85, 36.30, 30.57, 29.86, 29.20, 28.17, 13.43. HRMS calculated for C_{21}H_{27}N_{2}O_{2}: [M + H]^+: 339.2069 (found); 339.2073 (calcd).

Desacetyl-akuammiline (29). In a round bottom flask, 5 (115 mg, 0.2919 mmol) was solubilized in 10 mL of MeOH at room temperature. To the solution was added 8.2 mL of a 10% w/v KOH solution (50 equiv). After 2 hours, the MeOH was removed by rotary evaporation. The resultant solution was diluted with 10 mL of H_{2}O and extracted with 20 mL DCM 3X. The organic layers were combined, dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was then purified by column chromatography (0-5% MeOH/DCM) to yield 103 mg the desired product (99% yield) whose spectra were consistent with the literature.\(^{59}\)

General Procedure C: Reductive Alkylation. In an oven-dried round bottom flask under nitrogen, 29 (1.0 equiv) was dissolved in 5 mL anhydrous dichloromethane. The solution was treated with trifluoroacetic acid (9.0 equiv), triethylsilane (30 equiv), and appropriate dimethyl acetal (10 equiv). The reaction was stirred at room temperature for 18 hours. The solution was neutralized with saturated sodium bicarbonate and extracted DCM (3x20 mL). The organic layers were combined, dried over sodium sulfate, and concentrated in vacuo. The crude product was purified via column chromatography (2% MeOH/DCM) to yield the desired product.

N-1-ethyl psuedoakuammigine (31). Prepared from 29 (40 mg, 0.1135 mmol) according to General Procedure C and acetaldehyde dimethyl acetal to yield 13 mg of 31 (30%). \(^{1}\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.17 (t, J = 7.8 \text{ Hz}, 1\text{H}), 7.08 (d, J = 7.6 \text{ Hz}, 1\text{H}), 6.78 (t, J = 7.4 \text{ Hz}, 1\text{H}), 6.71 (d, J = 8.0 \text{ Hz}, 1\text{H}), 5.68 (q, J = 7.2 \text{ Hz}, 1\text{H}), 4.70 (s, 1\text{H}), 4.28 (d, J = 15.9 \text{ Hz}, 1\text{H}), 4.00 (d, J = 7.7 \text{ Hz}, 1\text{H}), 3.85 (s, 3\text{H}), 3.71 (d, J = 7.9 \text{ Hz}, 1\text{H}), 3.64 (s, 1\text{H}), 3.53 – 3.39 (m, 2\text{H}), 3.28 (s, 1\text{H}), 3.24 (t, J = 7.5 \text{ Hz}, 1\text{H}), 2.95 (dd, J = 12.7, 5.4 \text{ Hz}, 1\text{H}), 2.59 (d, J = 14.9 \text{ Hz}, 1\text{H}), 2.13 (d, J = 14.8 \text{ Hz}, 1\text{H}), 1.83 – 1.74 (m, 1\text{H}), 1.61 (d, J = 7.3 \text{ Hz}, 4\text{H}), 1.31 (t, J = 7.3 \text{ Hz}, 3\text{H}). \(^{13}\)C NMR (151 MHz,
CDCl$_3$ δ 170.82, 149.41, 136.93, 128.68, 128.42, 125.18, 122.50, 120.54, 110.21, 101.48, 74.50, 57.16, 53.63, 52.45, 49.78, 39.25, 38.55, 29.70, 27.42, 26.30, 22.69, 14.71, 13.23. HRMS calculated for C$_{23}$H$_{29}$N$_2$O$_3$: [M + H]$^+$: 381.2172 (found); 381.2178 (calcd).

*N*-1-benzyl psuedoakuammigine (32). Prepared from 29 (40 mg, 0.1135 mmol) according to General Procedure C and benzaldehyde dimethyl acetal to yield 32 mg of 32 (64%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.42 (d, $J = 7.1$ Hz, 2H), 7.34 (dd, $J = 8.4$, 6.8 Hz, 2H), 7.30 – 7.21 (m, 1H), 7.10 (dd, $J = 7.5$, 1.3 Hz, 1H), 7.00 (td, $J = 7.7$, 1.3 Hz, 1H), 6.75 (td, $J = 7.5$, 1.0 Hz, 1H), 6.49 (d, $J = 7.8$ Hz, 1H), 5.43 (q, $J = 7.1$ Hz, 1H), 4.68 (d, $J = 16.1$ Hz, 1H), 4.23 (dt, $J = 3.5$, 1.7 Hz, 1H), 4.07 (d, $J = 16.2$ Hz, 1H), 3.98 (d, $J = 7.5$ Hz, 1H), 3.94 (d, $J = 17.7$ Hz, 1H), 3.88 (dd, $J = 13.9$, 4.6 Hz, 1H), 3.83 (s, 3H), 3.67 (d, $J = 7.5$ Hz, 1H), 3.50 (d, $J = 3.7$ Hz, 1H), 3.46 – 3.32 (m, 1H), 2.86 (d, $J = 16.8$ Hz, 1H), 2.66 (ddd, $J = 13.6$, 5.8 Hz, 1H), 2.37 (ddd, $J = 14.1$, 4.3, 2.0 Hz, 1H), 2.08 (ddd, $J = 14.0$, 3.7, 1.9 Hz, 1H), 1.58 (d, $J = 4.2$ Hz, 1H), 1.54 (dd, $J = 7.0$, 2.4 Hz, 3H) $^{13}$C NMR (101 MHz, CDCl$_3$) δ 172.24, 152.02, 142.17, 139.42, 128.75, 127.52, 127.41, 127.09, 122.53, 120.63, 118.56, 111.37, 104.50, 74.56, 58.71, 54.74, 53.91, 53.60, 52.08, 51.03, 49.62, 41.04, 31.47, 28.28, 13.09. HRMS calculated for C$_{28}$H$_{31}$N$_2$O$_3$: [M + H]$^+$: 443.2332 (found); 443.2335 (calcd).

*N*-1-phenethyl psuedoakuammigine (33). Prepared from 29 (30 mg, 0.0851 mmol) according to General Procedure C and phenylacetaldehyde dimethyl acetal to yield 25 mg mg of 33 (63%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.35 – 7.27 (m, 4H), 7.25 – 7.17 (m, 1H), 7.16 – 7.06 (m, 2H), 6.75 (t, $J = 7.5$ Hz, 1H), 6.68 (d, $J = 7.8$ Hz, 1H), 5.50 (q, $J = 7.4$ Hz, 1H), 4.42 (s, 1H), 4.07 (d, $J = 16.4$ Hz, 1H), 3.96 (d, $J = 7.7$ Hz, 1H), 3.83 (s, 3H), 3.72 – 3.55 (m, 3H), 3.53 (d, $J = 3.4$ Hz, 1H), 3.43 – 3.29 (m, 2H), 3.09 – 2.93 (m, 3H), 2.69 (dd, $J = 13.4$, 5.7 Hz, 1H), 2.11 (d, $J = 14.2$ Hz, 1H), 1.55 (d, $J = 7.2$ Hz, 4H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ172.27, 150.75, 141.84, 139.86, 128.83,
128.70, 127.55, 126.40, 122.70, 120.45, 119.79, 118.72, 109.38, 103.93, 74.57, 58.39, 54.67, 53.79, 53.53, 52.06, 50.98, 46.06, 41.00, 36.28, 31.20, 28.13, 13.08. HRMS calculated for C_{29}H_{33}N_{2}O_{3}: [M + H]^+: 457.2486 (found); 457.2491 (calcd).

**In Vitro Pharmacology**

*Drugs.* Forskolin and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, United States). (2S)-2-[[2-[(2R)-2-[(2S)-2-Amino-3-(4-hydroxyphenyl)propanoyl]amino]propanoyl]amino]acetyl]-methylamino]-N-(2hydroxyethyl)-3-phenylpropanamide (DAMGO), and 2-(3,4-dichlorophenyl)-Nmethyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl] acetamide (U50,488) were purchased from Tocris Bioscience (Bio-Techne Corporation, Minneapolis, MN, United States).[^3]H]DAMGO (53.7 Ci/mmol, lot#2376538; 51.7 Ci/mmol, lot#2815607), [^3]H]U69,593 (60 Ci/mmol, lot#2367921 and lot#2644168; 49.2 Ci/mmol, lot#2791786), were purchased from PerkinElmer (Waltham, MA, United States).

*Competitive radioligand binding.* Competitive radioligand binding experiments with full dose-response curves were completed as previously described[^18,60] using[^3]H]-DAMGO and[^3]H]-U69,593 at μOR and κOR, respectively, with one exception. Instead of incubating the reaction mix for 90 minutes, an incubation time of 180 minutes was used to capture slower binding kinetics of some of the analogs. The same competitive radioligand binding method was used for the binding screens except instead of full dose-response curves, single concentrations of 10 μM and 1 μM were used. In these experiments, the single concentration points were run in duplicate and a full-dose response curve of a positive control was included in each assay for data normalization purposes (DAMGO for μOR, U50,488 for κOR). Binding screen composites are made up of three individual replicates.
**GloSensor cAMP inhibition assay.** GloSensor assays were completed as previously described using HEK cells transiently expressing pGloSensor22F, and either HA-mouse µOR or FLAGmouse κOR. For assays shown in Figure 2E, DMSO was added to the buffer for positive controls to control for any solvent-related effects.

**PathHunter β-Arrestin-2 assay.** β-Arrestin-2 recruitment assays were completed as previously described using PathHunter cell lines stably expressing μOR and β-arrestin-2.

**TRUPATH assay.** The TRUPATH assay was performed as previously described, using DAMGO as a positive control. Cells were plated either in 6-well dishes at a density of 700,000–800,000 cells per well, or 10 cm dishes at 7–8 million cells per dish. Cells were transfected 2–4 h later, using a 1:1:1:1 DNA ratio of receptor:Gα-RLuc8:Gβ:Gγ-GFP2 (100 ng per construct for 6-well dishes, 750 ng per construct for 10 cm dishes), except for the Gγ-GFP2 screen, where an ethanol coprecipitated mixture of Gβ1–4 was used at twice its normal ratio (1:1:2:1). Transit 2020 (Mirus Biosciences) was used to complex the DNA at a ratio of 3 µl Transit per µg DNA, in OptiMEM (Gibco-ThermoFisher) at a concentration of 10 ng DNA per µl OptiMEM. The next day, cells were harvested from the plate using Versene (0.1 M PBS + 0.5 mM EDTA, pH 7.4) and plated in polyD-lysine-coated white, clear-bottom 96-well assay plates (Greiner Bio-One) at a density of 30,000–50,000 cells per well. One day after plating in 96-well assay plates, white backings (PerkinElmer) were applied to the plate bottoms, and growth medium was carefully aspirated and replaced immediately with 60 µl of assay buffer (1× Hank’s balanced salt solution (HBSS) + 20 mM HEPES, pH 7.4), followed by a 10 µl addition of freshly prepared 50 µM coelenterazine 400a (Nanolight Technologies). After a 5 min equilibration period, cells were treated with 30 µl of drug for an additional 5 min. Plates were then read in an LB940 Mithras plate reader (Berthold Technologies) with 395 nm (RLuc8-coelenterazine 400a) and 510 nm (GFP2)
emission filters, at integration times of 1 s per well. Plates were read serially six times, and measurements from the sixth read were used in all analyses. BRET2 ratios were computed as the ratio of the GFP2 emission to RLuc8 emission.

Statistics. Cellular pharmacological data was analyzed using GraphPad 9 (GraphPad Prism software, La Jolla, CA, United States) and is shown as mean ± SEM. For binding, GloSensor and PathHunter assays, composite figures consist of a curve averaged from a minimum of three independent assays that were each normalized to a positive control.

Animal Studies.

Animals. C57BL/6 mice (20-25 g, mixed sexes, Jackson laboratory) were used in the study. The mice were housed in a 14/10-hour light/dark cycle (5:00 AM on/7:00 PM off) with access to food and water ad libitum. All animal experiments were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals after getting approval from the University of Illinois Institutional Animal Care and Use Committee.

Tail-Flick Assay. To determine basal nociception and antinociception, tail-flick test was performed. Distal one-third of the mouse tail was immersed in a water bath (VWR Model 1130S) that was maintained at 52°C. The latency of the mouse to a rapid tail-flick response was recorded. Antinociceptive effects of morphine (10 mg/kg s.c) and different doses of 33 (mg/kg s.c) were evaluated at 15, 30, 60, 120, 180 and 240 min after drug administration. The antinociceptive effect was expressed as percentage of maximal possible effect (MPE) and the cut-off time was set at 12 seconds to prevent damage to the tail.

Hot-Plate Assay. Hot-plate test was performed to test basal nociception and antinociception. Mice were placed in a glass cylinder on a heated plate (Ugo Basil Hot/Cold Plate 35100) maintained at 55 ± 0.1°C. The latency to hind-paw licking, flinching, withdrawal or jumping was
recorded as a response. Antinociceptive effects of morphine (10 mg/kg s.c) and different doses of 33 (mg/kg s.c) were evaluated at 15, 30, 60, 120, 180 and 240 min after drug administration. Compound 33 was formulated using 5.4:6.5:0.1 ratio of DMSO, PBS, and 20% aqueous tartaric acid. The antinociceptive effect was expressed as percentage of maximal possible effect (MPE). Cut-off time was set at 45 seconds to prevent damage to the paws.

**Rotarod Assay.** The locomotor activity of the mice after the treatment with 33 was tested using the rotarod test. On Day 1, mice were placed on the rotarod (Model series 8, IITC Life Science, Woodland Hills, CA) and trained on a fixed speed (4 rpm) for 60 s. The training session from Day 1 was repeated on the second day and the mice that failed to stay on the rotarod for 60 s were excluded from further studies. On test day (Day 3), the mice were placed on an accelerating rotarod (4 - 40 rpm over 300 seconds) and the latencies to fall were recorded 15 min after the administration of 33 (50, 65, 80 mg/kg s.c).

**Statistics.** All data are presented as Mean ± S.E.M. MPE% = 100% *(postdrug latency - predrug latency) / (cutoff - predrug latency). Comparisons between groups were analyzed using a one-way analysis of variance followed by post hoc analyses using Dunnett’s t test (multiple groups). For comparison between groups for antinociceptive effect at different time points a two-way repeated measure analysis of variance followed by post hoc analyses using Dunnett’s t test (multiple groups) was used. Statistical significance was established at 95% confidence limit.

**Molecular Docking.** The crystal structure of the µOR bound to the agonist BU72 (PDB: 5C1M) was imported and prepared using the Protein Preparation module in Schrödinger Maestro (version 12.9.137). Compounds 19 and 33 were imported as SMILES and lowest energy conformations generated using the LigPrep module. The Induced Fit Docking Module was used to generate possible posses with the extended sampling protocol and without any constraints. The docking
pose with the highest docking score for each ligand was exported to PyMOL for visualization and figure generation.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge.

Additional supplementary figures (S1-3)

$^1$H NMR and $^{13}$C NMR spectra for compounds 7-33.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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REFERENCES


akuammine (1)  pseudo-akuammigine (2)

Picralima nitida synthesis

Analogue synthesis

G-protein activation

μOR

Antinociception

DAMGO

\(2\)  \(33\) (mg/kg)

\(33\) (mg/kg)

\(\text{CAMP Production} \) (normalized \%DAMGO-DMSO)

\(\% \text{ MPE} \)

Control  Manzamine  Aku  Aku +

10 mg/kg  33 mg/kg

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TOC Figure