New Analgesics Synthetically Derived from the Paracetamol Metabolite
N-(4-Hydroxyphenyl)-(5Z,8Z,11Z,14Z)-icosatetra-5,8,11,14-enamide

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N-(4-Hydroxyphenyl)-(5Z,8Z,11Z,14Z)-icosatetra-5,8,11,14-enamide (AM404) is a metabolite of the well-known analgesic paracetamol. AM404 inhibits endocannabinoid cellular uptake, binds weakly to CB1 and CB2 cannabinoid receptors, and is formed by fatty acid amide hydrolase (FAAH) in vivo. We prepared three derivatives of this new (endo)cannabinoind using bioisosteric replacement (1), homology (2), and derivationization (3) of the 4-aminophenol moiety in AM404 and tested them against CB1, CB2, and FAAH. We found affinities toward both cannabinoid receptors equal to or greater than that of AM404. Shortening the acyl chain from C20 to C2 led to three new paracetamol analogues: N-(1H-indazol-5-yl)acetamide (5), N-(4-hydroxybenzyl)acetamide (6), and N-(4-hydroxy-3-methoxyphenyl)acetamide (7). Again, 5, 6, and 7 were tested against CB1, CB2, and FAAH without significant activity. However, 5 and 7 behaved like inhibitors of cyclooxygenases in whole blood assays. Finally, 5 (50 mg/kg) and 6 (275 mg/kg) displayed analgesic activities comparable to paracetamol (200 mg/kg) in the mouse formalin test.

Introduction

The proteins of the endocannabinoid system, including CB1, CB2, and FAAH, represent excellent targets for the development of new therapeutic drugs to be employed in acute or neuropathic pain states. As a consequence, a drug containing Δ²-THC and cannabidiol has been approved as a pain killer in Canada for half of a century. They identified the arachidonic acid derivative AM404 in the CNS of rats that were treated with a common ip dose of paracetamol. Intriguingly, AM404 had been established as a compound capable to enhance CB1 receptor activity either directly (as an agonist) or indirectly (by prolonging endocannabinoid life-span) some time before its bioanalytical identification. Hoegestaett et al. elegantly showed that paracetamol undergoes a primary deacetylation step in liver, brain, and spinal cord that is followed by the N-acetylation of the resulting 4-aminophenol with arachidonic acid by FAAH to form AM404 in the CNS. The biological significance of this route of biotransformation was confirmed by two publications that reported on the abolition of paracetamol’s efficacy in pain models when cannabinoid receptor antagonists were given concomitantly.

In view of this background, we reasoned that a possible and novel strategy for the development of new paracetamol analogues might be the design of AM404 derivatives using (a) bioisosteric replacement (1), (b) homology (2) and (c) derivatization (3) of the 4-aminophenol moiety in AM404 (Scheme 1). Our first aim was to determine the affinities of the prepared AM404 derivatives against CB1, CB2, and FAAH in comparison to AM404. The promising results from these primary assays were fundamental for the next step of our study. Shortening the C20 acyl chain to C2 led to three paracetamol analogues that are, in the form of their respective arachidonic acid conjugates, equipotent or even better modulators of the endocannabinoid system. The paracetamol analogues were again tested against CB1, CB2, FAAH, and against both cyclooxygenases in whole blood assays as these proteins are promising targets for analgesic agents. Finally, we determined the analgesic potential of 5, 6, and 7 in the mice formalin test to evaluate their activity in vivo.

Chemistry. The derivatives 1, 2, 3, and AM404 were prepared as detailed in the Experimental Section or the Supporting Information of this article. Briefly, arachidonic acid was dissolved in suitable organic solvents and activated with oxalyl chloride or DCC, followed by the addition of the appropriate amine components. All reactions were carried out under an argon atmosphere. The crude products were purified by silica gel chromatography.

The synthesis of the acetamides 5, 6, and 7 started with the appropriate amine components that were dissolved in suitable organic solvents and N-acetylated by acetic anhydride or acetyl chloride. Silica gel chromatography and crystallization were used to purify the paracetamol analogues.

Biological Evaluation. All compounds were tested against CB1 and CB2 in radioligand ([³H]CP-55,940) competition assays with membranes prepared from HEK-293 cells overexpressing CB1 and CB2 cannabinoid receptors 1 or 2; CNS, central nervous system; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; ip, intraperitoneal; PBS, phosphate buffered saline; PGF2α, PGF2β, PGH2, prostaglandins E2, F2α, H2; TBME, tert-butyl methyl ether; Δ²-THC, Δ²-tetrahydrocannabinol; TXB2, thromboxane B2.
blood assays by detecting serum or plasma levels of TXB\textsubscript{2} or compared to the paracetamol metabolite AM404, whereas displayed higher affinity toward both cannabinoid receptors measuring the effect on [14C]anandamide hydrolysis. Inhibition activity of all compounds on FAAH was determined using rat.

**Results**

The AM404 derivatives 1, 2, and 3 were tested against CB\textsubscript{1}, CB\textsubscript{2}, and FAAH. The results are summarized in Table 1. 1 and 2 displayed higher affinity toward both cannabinoid receptors compared to the paracetamol metabolite AM404, whereas 3 was roughly equipotent to AM404 at both CB\textsubscript{1} and CB\textsubscript{2}. Only 2 behaved as a FAAH inhibitor in the rat brain preparation showing 56% inhibition at 10 μM. These promising affinities of 1, 2, and 3 prompted us to reverse the biosynthetic route of AM404 described by Hoegestaett et al., i.e., to obtain ethanoyl compounds’ efficacy on both acute peripheral pain (the first phase of the nocifensive response to formalin) and inflammatory-like pain (the second phase of the nocifensive response to formalin).

**Scheme 1. Design of AM404 Derivatives by Using (a) Bioisosteric Replacement, (b) Homology, and (c) Derivatization of the 4-Aminophenol moiety in AM404**

![Scheme 1](image)

"Shortening the acyl chain led to the paracetamol analogues 5, 6, and 7.

**Table 1. Kᵢ Values [μM] ± SEM at CB\textsubscript{1} and CB\textsubscript{2} and Inhibition [%] ± SD of FAAH in Rat Brain Preparations (Concentration of Compounds in Squared Brackets)***

<table>
<thead>
<tr>
<th>no.</th>
<th>CB\textsubscript{1}</th>
<th>CB\textsubscript{2}</th>
<th>FAAH [50 μM]</th>
<th>FAAH [10 μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3 ± 0.03</td>
<td>1.0 ± 0.07</td>
<td>2 ± 0.1</td>
<td>1 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>0.2 ± 0.02</td>
<td>0.1 ± 0.01</td>
<td>88 ± 1</td>
<td>56 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>1.4 ± 0.07</td>
<td>1.0 ± 0.08</td>
<td>62 ± 3</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>AM404</td>
<td>1.5 ± 0.09</td>
<td>1.3 ± 0.08</td>
<td>55 ± 0.4</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

**Table 2. Displacement [%] ± SD of [3H]CP-55,940 from CB\textsubscript{1} and CB\textsubscript{2} Receptors and Inhibition [%] ± SD of FAAH in Rat Brain Preparations (Concentration of Compounds in Squared Brackets)***

<table>
<thead>
<tr>
<th>no.</th>
<th>CB\textsubscript{1} [10 μM]</th>
<th>CB\textsubscript{2} [10 μM]</th>
<th>FAAH [50 μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1 ± 4</td>
<td>12 ± 3</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>6</td>
<td>5 ± 5</td>
<td>7 ± 4</td>
<td>4 ± 5</td>
</tr>
<tr>
<td>7</td>
<td>16 ± 15</td>
<td>22 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>13 ± 0.9</td>
<td>15 ± 1</td>
<td>22 ± 3</td>
</tr>
</tbody>
</table>

**Table 3. pIC\textsubscript{50} Values (+95% Confidence Interval) of 5, 6, 7, and Paracetamol at COX-1 and COX-2 in Whole Blood Assays***

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>a</td>
<td>3.63 ± 0.57</td>
</tr>
<tr>
<td>6</td>
<td>3.97 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>3.86 ± 0.15</td>
</tr>
</tbody>
</table>

"No inhibition. a inhibition but no pIC\textsubscript{50} value determinable.

inhibition of FAAH at the concentrations tested (10 μM for receptor binding, 50 μM for enzyme inhibition). Next, we evaluated the acetamides 5, 6, and 7 in comparison to paracetamol for COX inhibition in whole blood assays. The results are shown in Table 3. 5 behaved like a selective COX-2 inhibitor with an IC\textsubscript{50} of about 107 μM. 6 did not show inhibition of either isoform, whereas 7 behaved as a dual inhibitor at both isoforms (IC\textsubscript{50}: 234 μM (COX-1); IC\textsubscript{50}: 135 μM (COX-2)). Paracetamol behaved as a dual COX inhibitor as well (IC\textsubscript{50}: 141 μM (COX-2)) roughly matching data from literature. As whole blood assays measure the sum of several biochemical events, no statement can be made how much inhibition of cyclooxygenases accounts for the reduced production of TXB\textsubscript{2} or PGE\textsubscript{2}. Therefore 5 was tested against isolated COX-1 and COX-2 and displayed a tendency toward an inhibition of COX-2 that was consistent with the results of the whole blood assays. We abstained from testing the very similar paracetamol analogue 7 against isolated cyclooxygenases, as paracetamol is already established as an inhibitor of the isolated isoforms. Figure 1 includes the results obtained with the isolated cyclooxygenases.

Having established that the paracetamol analogues act partially as COX inhibitors and that their potential biotransformation products 1, 2, and 3 act as cannabinoid receptor ligands, we were encouraged to examine the analgesic activity of the acetamides 5, 6, and 7 in comparison to paracetamol using the formalin test in mice. Compound 7 was excluded from further experiments due to its overt toxicity to mice, which was observed when the first five animals received the highest dose of 325 mg/kg. The animals were almost inactive after receiving 7. They did not show the usual locomotor behavior and stayed where they were placed in the
Figure 1. Effects of 5 on the production of PGH₂ by COX-1 and COX-2 at concentrations of 1 and 10 µM compared to Ketoprofen at a concentration of 10 µM. The generated PGH₂ was reduced to PGE₂α by stannous chloride to quantify the assay. Reflected are means of four experiments ± standard error of the mean.

Figure 2. Time course of the licking behavior in mice after ip administration of 5, 6, paracetamol (P), or solvent (C) and injection of formalin into the left hind paw. Each point represents the mean + standard error of the mean of 6–8 animals per group. The first phase of the formalin test lasts from 0–10 min, the second phase from 15–45 min.

Discussion

At the highest doses tested, 5 (50 mg/kg) and 6 (275 mg/kg) were equipotent to paracetamol (200 mg/kg) in the second phase of the formalin test (no differences at p ≤ 0.05). In addition, both compounds elicited analgesic activity in the first phase of the formalin test, whereas paracetamol was inactive in our hands. We are, however, not able to explain the apparent, albeit not statistically significant, pro-algesic effect of 6 at the lowest examined dose of 27.5 mg/kg. The mechanism of action of 5 and 6, responsible for the analgesic effect remains speculative after this study, but two explanations for the observed analgesic potential can be deduced from the Results Section.

First, what can be gleaned from behavior toward cyclooxygenases? Paracetamol is a well-established inhibitor of both cyclooxygenases 1 and 2, of which only inhibition of COX-2 seems to be relevant under common pharmacological conditions. 6 did not show any inhibition of cyclooxygenases, whereas 5 shared paracetamol’s ability to inhibit COX-2 even with a slightly better efficacy as compared to paracetamol in the whole blood assays. Thus, COX-2 inhibition might be suggested to be at the basis of the analgesic activity of 5. However, it is known from the literature that both dual COX inhibitors as well as selective COX-2 inhibitors show significant efficacy only in the second phase of the formalin test after ip administration. 11,17,18 Actually, we found a statistically significant (p ≤ 0.05) efficacy of 5 in both phases pointing to another or an additive mode of action. Even 6 that did not show any inhibition of cyclooxygenases displayed a statistically significant (p ≤ 0.05) efficacy, so we briefly suggest a second hint that is possibly involved in the analgesic activity of 5 and 6.

In view of the activity of cannabinoid receptor agonists against both phases of the formalin nociceptive response 19–22 and with regard to the good affinities of 1 and 2 for both CB₁ and CB₂ receptors, we tend to speculate that these arachidonic acid derivatives might participate in the observed analgesic activity of 5 and 6 after biotransformation of the latter. Clearly, these speculations need to be supported by further experiments like in vivo data about the formation of 1 and 2 after ip administration of 5 and 6 to mice and studies with cannabinoid receptor antagonists that would indeed prove an involvement of these receptors.

Conclusion

Compound 5 represents an attractive lead for medicinal chemistry purposes. It is a small molecule that is synthesized with ease and at low cost. Furthermore, the heteroaromatic scaffold provides some opportunities for chemical modifications that might lead to improved pharmacological activities. We qualified 5 as an inhibitor of COX-2 in whole blood assays and in assays with isolated cyclooxygenases. This compound showed analgesic activity comparable to that of paracetamol in the second phase of the formalin test. In addition, 5 influenced the first phase of the formalin test, in which paracetamol was ineffective in our hands. We were able to achieve these effects with 5 at one-quarter of the paracetamol dose. 5, as well as 6, should be devoid of one of paracetamol’s main drawbacks, i.e., its oxidation to a hepatotoxic benzoquinonimine. In our hands, deacetylated 5 and 6, more precisely 5-aminooizazole and 4-aminomethylphenol, were not oxidized by a 0.1 M cerium(IV) sulfate solution under conditions where deacetylated paracetamol (= 4-aminophenol) was oxidized.23 However, comprehensive toxicity data of the new paracetamol analogues described here are not yet available.

Experimental Section

Chemistry. All chemical reagents were purchased commercially unless otherwise stated. Chemical yields were not optimized.
Melting points were determined using a Boëtius hot-stage microscope and are uncorrected. Routine NMR spectra were recorded using a Varian Gemini 2000 or a Varian Inova Unity 500. Mass spectra were recorded using an AMD Intectra DP 10 mass spectrometer. Combustion analyses were performed with a Leco CHNS-932 analyzer and were within 0.4% of theoretical values unless otherwise indicated. For combustion analysis data exceeding this range, the compound’s purity was confirmed via high-resolution mass spectrometry with a Waters Micromass Q-TOF-2 using positive mode.

Synthetic routes and characterization of key compounds 1, 2, 5, and 6 are presented below and can be found for compounds 3, 4, 7, and AM404 in the Supporting Information of this article.

**N-(1H-Indazol-5-yl)-(5Z,8Z,11Z,14Z)-icosatetra-5,8,11,14-ename (1).** First, 152.2 mg (0.5 mmol) arachidonic acid and 36.5 mg (0.5 mmol) DMF were dissolved in 10 mL of dry THF and stirred under ice cooling for 10 min. Then 63.5 mg (0.5 mmol) oxalyl chloride was added and the mixture was stirred under ice cooling for 45 min. After the addition of 266.3 mg (2 mmol) 5-aminoindazole, the reagents were allowed to react 45 min at room temperature. The reaction mixture was diluted with 20 mL MeOH, decanted into a round-bottom flask, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (40–63 µm) using TBME as eluent and then precipitated from TBME/hexane to afford 34.5 mg (15%) of a brownish-red solid; mp: 135–138°C (TBME/hexane). 1H NMR (400 MHz, DMSO-d6), δ (ppm): 12.90 (br s, 1H, NH), 9.81 (s, 1H, CO-NH), 8.11–7.96 (m, 2H, 2 × Ar-H), 7.43 (d, J = 9.1 Hz, 1H, Ar-H), 7.39 (dd, J = 9.1 Hz, 4J = 1.7 Hz, 1H, Ar-), 5.42–5.25 (m, 8H, 4 × CH=CH), 2.82–2.73 (m, 6H, 3 × CH=CH–CH–CH=CH), 2.31 (t, J = 7.5 Hz, 2H, CH2–CH=C–CO-NH), 2.13–1.96 (m, 4H, 2 × CH=CH–CH3), 1.66 (tt, J = 7.5 Hz, 2H, CH2–CH–CH2–CO-NH), 1.33–1.19 (m, 6H, CH3–CH2–CH–CH2–CH3), 0.83 (t, J = 7.1 Hz, 3H, CH3). 13C NMR (125 MHz, CD3OD), δ (ppm): 174.4, 139.0, 134.9, 133.3, 131.2, 130.2, 129.9, 129.4, 129.2, 129.1, 128.9, 128.7, 124.3, 123.0, 112.7, 111.2, 37.2, 32.6, 30.4, 28.2, 27.7, 26.8, 26.6, 26.55, 26.53, 23.6, 14.4. EI-MS (70 eV), m/z (%): 133 (100), 175 (57.9), 134 (34.3), 419 (2.9, M+). Anal. (C27H37N3O) C, H, N.

**N-(4-Hydroxybenzyl)-(5Z,8Z,11Z,14Z)-icosatetra-5,8,11,14-ename (2).** First, 121.8 mg (0.4 mmol) arachidonic acid and 29.2 mg (0.4 mmol) DMF were dissolved in 10 mL of dry THF and stirred under ice cooling for 10 min. Then 63.5 mg (0.5 mmol) oxalyl chloride was added and the mixture was stirred under ice cooling for 45 min. After the addition of 266.3 mg (2 mmol) 5-aminobenzaldehyde, the reagents were allowed to react 45 min at room temperature. The reaction mixture was diluted with 20 mL MeOH, decanted into a round-bottom flask, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (40–63 µm) using TBME as eluent and then precipitated from TBME/hexane to afford 34.5 mg (15%) of a brownish-red solid; mp: 135–138°C (TBME/hexane). 1H NMR (400 MHz, DMSO-d6), δ (ppm): 12.90 (br s, 1H, NH), 9.81 (s, 1H, CO-NH), 8.11–7.96 (m, 2H, 2 × Ar-H), 7.43 (d, J = 9.1 Hz, 1H, Ar-H), 7.39 (dd, J = 9.1 Hz, 4J = 1.7 Hz, 1H, Ar-), 5.42–5.25 (m, 8H, 4 × CH=CH), 2.82–2.73 (m, 6H, 3 × CH=CH–CH–CH=CH), 2.31 (t, J = 7.5 Hz, 2H, CH2–CH=C–CO-NH), 2.13–1.96 (m, 4H, 2 × CH=CH–CH3), 1.66 (tt, J = 7.5 Hz, 2H, CH2–CH–CH2–CO-NH), 1.33–1.19 (m, 6H, CH3–CH2–CH–CH2–CH3), 0.83 (t, J = 7.1 Hz, 3H, CH3). 13C NMR (125 MHz, CD3OD), δ (ppm): 174.4, 139.0, 134.9, 133.3, 131.2, 130.2, 129.9, 129.4, 129.2, 129.1, 128.9, 128.7, 124.3, 123.0, 112.7, 111.2, 37.2, 32.6, 30.4, 28.2, 27.7, 26.8, 26.6, 26.55, 26.53, 23.6, 14.4. EI-MS (70 eV), m/z (%): 133 (100), 175 (57.9), 134 (34.3), 419 (2.9, M+). Anal. (C27H37N3O) C, H, N.

Figure 3. Effects of 5, 6, and paracetamol (P) administered ip on the formalin response manifested during the first phase. The cumulative response time of licking the injected paw was measured during the period of 0–10 min. The vertical bars denote the standard error of the mean. The number of animals used for each group was 6–8 (except fourth row; n = 4). *p ≤ 0.05 compared to the control group of mice (two-tailed t test). Licking time of the control group = 100%.

Figure 4. Effects of 5, 6, and paracetamol (P) administered ip on the formalin response manifested during the second phase. The cumulative response time of licking the injected paw was measured during the period of 15–45 min. The vertical bars denote the standard error of the mean. The number of animals used for each group was 6–8 (except fourth row; n = 4). *P < 0.05 compared to the control group of mice (one-tailed t test). Licking time of the control group = 100%.
stirred under ice cooling for 10 min. Then 50.8 mg (0.4 mmol)
oxalyl chloride was added, and the mixture was stirred under ice
cooling for 45 min. After the addition of 246.3 mg (2 mmol) 4, the
reagents were allowed to react 30 min at room temperature. The
reaction mixture was diluted with 25 mL TBMDE, decanted into a
round-bottom flask, and the solvent was removed by rotary
evaporation. The crude product was purified by column chroma-
tography on silica gel (40–63 μm) using TBME + 3% NEt3 as
eluent to afford 34.0 mg (20%) of a yellowish oil. 1H NMR (400
MHz, DMSO-d6), δ (ppm): 9.21 (s, 1H, OH), 8.12 (br s, 1H, CO-
NH), 7.01 (dd, 3J = 6.6 Hz, 4J = 2.1 Hz, 2H, 2 × Ar-H), 6.67 (dd,
3J = 6.6 Hz, 4J = 2.1 Hz, 2H, 2 × Ar-H), 5.39–5.27 (m, 8H, 4 ×
CH=CH), 4.11 (d, 1J = 5.8 Hz, 2H, NH-CH2), 2.82–2.74 (m,
6H, 3 × CH=CH-CH2-CH=CH2), 2.10 (t, 1J = 7.5 Hz, 2H,
CH2-CH2-CONH), 2.05–1.98 (m, 4H, 2 × CH=CH2), 1.55
(t, 1J = 7.5 Hz, 2H, CH2-CH2-CONH-), 1.33–1.20 (m, 6H,
CH2-CH2-CH2-CH3), 0.84 (t, 1J = 6.8 Hz, 3H, –CH3). 13C NMR
(100 MHz, CDCl3), δ (ppm): 172.7, 25.7, 23.7, 43.9, 22.6). EI-MS (70 eV),
EI-MS (70 eV), m/z (ppm): 171.5, 138.9, 134.8, 133.0, 124.1, 122.9, 112.7, 111.1, 23.7.

Ar-C=C-N2 (46%) of slightly violet crystals; mp: 203
°C (MeOH/TBME), 1H NMR (400 MHz, DMSO-d6), δ (ppm): 12.88 (br s, 1H, NH), 9.85 (s, 1H, CO-NH), 8.10–7.97 (m, 2H, 2 × Ar-H), 7.42 (d, 1J = 8.7 Hz, 1H, Ar-H), 7.35 (dd, 3J = 8.7 Hz, 4J = 1.7 Hz, 1H, Ar-H), 2.01 (s, 3H, CO-CH3). 13C NMR (100 MHz, CD2OD), δ
(ppm): 171.5, 138.9, 134.8, 133.0, 124.1, 122.9, 112.7, 111.1, 23.7. El-MS (70 eV), m/z (%): 133 (100), 175 (40, M+).

N-(1H-Indazol-5-yl)acetamide (5). First, 266.4 mg (2 mmol)
5-aminooindazole were dissolved in 10 mL of dry THF and stirred
under ice cooling for 10 min. After the addition of 78.5 mg (1
mmol) acetyl chloride, the reagents were allowed to react 30 min
at room temperature. The reaction mixture was diluted with MeOH,
decanted into a round-bottom flask, and the solvent removed by rotary
evaporation. The crude product was purified by column chroma-
tography on silica gel (40–63 μm) using EtOAc/NEt3 (97/3
) as eluent. Crystallization from MeOH/TBME afforded 238.8 mg
(20%) of a yellowish oil. 1H NMR (400 MHz, DMSO-d6), δ
(ppm): 9.22 (s, 1H, O–), 8.70 (d, 3J = 8.7 Hz, 2H, 2 × Ar-H), 7.57 (d, 4J
= 7.5 Hz, 2H, 2 × Ar-H), 7.18 (d, 3J = 8.7 Hz, 2H, 2 × Ar-H), 6.20 (d,
3J = 8.7 Hz, 2H, 2 × Ar-H), 5.27 (m, 8H, 4 × CH=CH), 4.11 (d, 1J = 5.8 Hz, 2H, NH-CH2), 2.82–2.74 (m,
6H, 3 × CH=CH-CH2-CH=CH2), 2.10 (t, 1J = 7.5 Hz, 2H,
CH2-CH2-CONH), 2.05–1.98 (m, 4H, 2 × CH=CH2), 1.55
(t, 1J = 7.5 Hz, 2H, CH2-CH2-CONH-), 1.33–1.20 (m, 6H,
CH2-CH2-CH2-CH3), 0.84 (t, 1J = 6.8 Hz, 3H, –CH3). Anal. (C27H30N2O) C, H, N.

N-(4-Hydroxybenzyl)acetamide (6). First, 369.5 mg (3 mmol)
4 were dissolved in 10 mL of dry THF and stirred under ice cooling
for 10 min. After the addition of 117.8 mg (1.5 mmol) acetyl
chloride, the reagents were allowed to react for 30 min at room
temperature. The reaction mixture was diluted with MeOH,
decanted into a round-bottom flask, and the solvent was removed by rotary
evaporation. The crude product was purified by column chroma-
tography on silica gel (40–63 μm) using TBME/MeOH (95/5
) as eluent. Hydrolysis of [14C]anandamide was studied by using membranes
from Escherichia coli 026:B2, 10 μg aspirin, plus 2
µL of the test agent dissolved in DMSO. Blood was allowed to clot for 1 h at 37 °C. Serum was separated by centrifugation, and TXB2 levels were determined per GC-MS/MS as described previously.24 All data points were determined in quadruplicate.

Whole Blood Assay for COX-1 Inhibition. Blood was drawn from a healthy 50 year old volunteer who had not taken any nonsteroidal anti-inflammatory drug 2 weeks prior to blood sampling. One mL aliquots of whole blood were immediately transferred to plastic tubes containing 2 µL of the test
agent dissolved in DMSO. Blood was allowed to clot for 1 h at 37 °C. Lipopolysaccharides were used as stimulants for COX-2 and the contribution of platelet COX-1 activity was inhibited by aspirin. Plasma was separated by centrifugation, and PGE2 levels were determined per GC-MS/MS as described previously.24 All data points were determined in quadruplicate.

Inhibition of Isolated Cyclooxygenases. A COX inhibitor screening assay was used to determine the activity of isolated ovine COX-1 and human recombinant COX-2 as described by the manufacturer (Cayman Chemical Company, USA). Briefly, COX-1 or COX-2 was incubated with 5 at 37 °C for 10 min in a Tris-HCl buffer (0.1 M, pH 8.0 containing 5 mM EDTA and 2 mM phenol). Then, the COX reaction was initiated by addition of arachidonic acid. The reaction was stopped 2 min later by 50 µL of 1 M HCl, followed by addition of 100 µL saturated SnCl2 to reduce the COX-
produced PGH2 into PGF2α, which was further quantified by EIA using PGE2 as standard. Blank was previously subtracted from each value. Ketoprofen was used as a reference COX-inhibitor. Results are means of four experiments.

Test of Antinociceptive Activity in Mice Treated with Formalin. Formalin injection induces a biphasic stereotypic nociceptive behavior. Nociceptive responses are divided into an early, short-lasting first phase (0–10 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period and then a second, prolonged phase (15–45 min) of tonic pain. Mice (C57BL/6) were randomly assigned to one of the experimental groups (n = 6–8). Each mouse was placed in the observation chamber and allowed to move freely for 15–20 min before the start of the experiment. Mirrors were placed in order to allow full view of the hind paws and licking of the injected paw was recorded as nociceptive response. Four animals each received an ip administration (6 µL/g) of PBS/DMSO (9/1) or PBS/DMSO (99/1). Licking times between the two groups did not vary statistically significantly (p ≥ 0.05), and both groups were combined as the control group. 5 (50, 10, and 5 mg/kg) dissolved in PBS/ DMSO (DMSO proportions 10, 2, and 1%), 6 (27.5, 5, and 27.5
mg/kg) dissolved in PBS/DMSO (DMSO proportions 10, 2, and 1%), and paracetamol (200 mg/kg) dissolved in PBS/DMSO (9/1) were administered ip in a volume of 6 µL/g. Mice received formalin (5%, 20 µL) in the dorsal surface of the left hind paw 15 min after
the administration of the test compounds. Recording of licking the injected paw as the nociceptive behavior commenced immediately after formalin injection and was continued for 45 min. To compare the nociceptive behavior with the control group, the sums of licking times during the first and the second phase were submitted to the Student’s t test.

Data Analysis. PRISM Version 3.0 (GraphPad, San Diego, CA) was used to fit all dose response curves and for the statistical analysis of the animal experiments.

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Supporting Information Available: Synthetic routes and characterization of compounds 3, 4, 7, and AM404 as well as combustion analysis data for all compounds presented in this article. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(23) Sinning, C. (Data not shown.) The amines were tested for oxidation according to the quantitative analysis of paracetamol described in the European Pharmacopoeia. 4-Aminophenol was quantitatively oxidized after titration with the stoichiometric amount of 0.1 M cerium(IV) sulphate, whereas only the indicator (ferroin) was oxidized in case of 5-aminooxazole and 4-aminomethylphenol, 2008.

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