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

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N-(4-Hydroxyphenyl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide (AM404) is a metabolite of the well-known analgesic paracetamol. AM404 inhibits endocannabinoid cellular uptake, binds weakly to CB_1 and CB_2 cannabinoid receptors, and is formed by fatty acid amide hydrolase (FAAH) in vivo. We prepared three derivatives of this new (endo)cannabinoid using bioisosteric replacement (**1**), homology (**2**), and derivatization (**3**) of the 4-aminophenol moiety in AM404 and tested them against CB_1, CB_2, and FAAH. We found affinities toward both cannabinoid receptors equal to or greater than that of AM404. Shortening the acyl chain from C_{20} to C_2 led to three new paracetamol analogues: *N*-(1*H*-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 CB_1, CB_2, 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 CB_1, CB_2, and FAAH, represent excellent targets for the development of new therapeutic drugs to be employed in acute or neuropathic pain states.[1−4] As a consequence, a drug containing Δ^9-THC and cannabidiol has been approved as a pain killer in Canada since 2005. In 2003 Hoegestaett et al. reported data suggesting that we might have already been using, without knowing it, a cannabinoid to treat pain for half of a century.[5] They identified the arachidonic acid derivative AM404[a] in the CNS of rats that were treated with a common ip dose of paracetamol.[6] Intriguingly, AM404 had been established as a compound capable to enhance CB_1 receptor activity either directly (as an agonist) or indirectly (by prolonging endocannabinoid life-span) some time before its bioanalytical identification.[7] Hoegestaett et al. elegantly showed that paracetamol undergoes a primary deacetylation step in liver, brain, and spinal cord that is followed by the N-acylation 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.[8,9]

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 CB_1, CB_2, and FAAH in comparison to AM404. The promising results from these primary assays were fundamental for the next step of our study. Shortening the C_{20} acyl chain to C_2 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 CB_1, CB_2, 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 CB_1 and CB_2 in radioligand ([^{3}H]CP-55,940) competition assays with membranes prepared from HEK-293 cells overexpressing

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[a] Abbreviations: AM404, *N*-(4-hydroxyphenyl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide; CB_1, CB_2, cannabinoid receptors 1 or 2; CNS, central nervous system; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; ip, intraperitoneal; PBS, phosphate buffered saline; PGE_2, $PGF_{2\alpha}$, PGH_2, prostaglandins E_2, $F_{2\alpha}$, H_2; TBME, *tert*-butyl methyl ether; Δ^9-THC, Δ^9-tetrahydrocannabinol; TXB_2, thromboxane B_2

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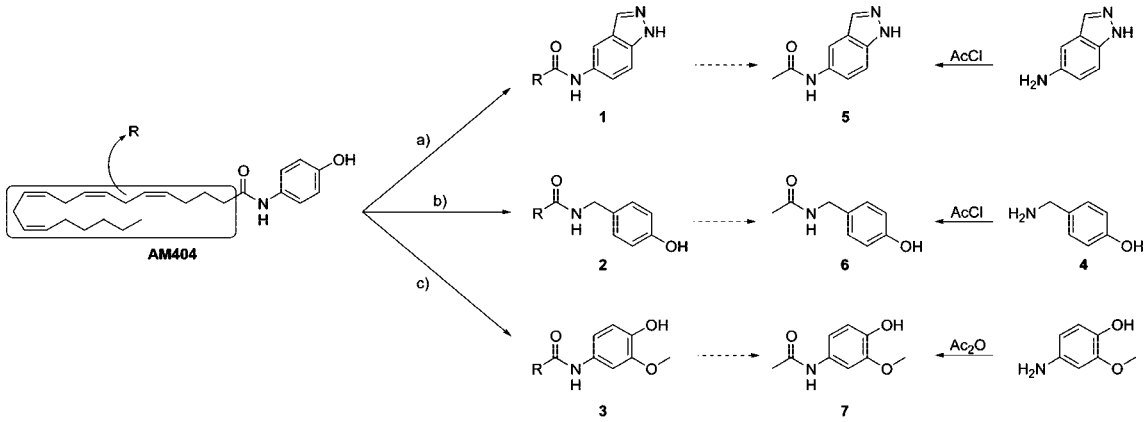
Scheme 1. Design of AM404 Derivatives by Using (a) Bioisosteric Replacement, (b) Homology, and (c) Derivatization of the 4-Aminophenol moiety in AM404[a]



[a] Shortening the acyl chain led to the paracetamol analogues **5**, **6**, and **7**.

Table 1. K_i Values [μM] ± SEM at CB_1 and CB_2 and Inhibition [%] ± SD of FAAH in Rat Brain Preparations (Concentration of Compounds in Squared Brackets)

no.	CB_1	CB_2	FAAH [50 μM]	FAAH [10 μM]
1	0.3 ± 0.03	1.0 ± 0.07	2 ± 0.1	1 ± 4
2	0.2 ± 0.02	0.1 ± 0.01	88 ± 1	56 ± 0.3
3	1.4 ± 0.07	1.0 ± 0.08	62 ± 3	23 ± 3
AM404	1.5 ± 0.09	1.3 ± 0.08	55 ± 0.4	15 ± 2

Table 2. Displacement [%] ± SD of [^{3}H]CP-55,940 from CB_1 and CB_2 Receptors and Inhibition [%] ± SD of FAAH in Rat Brain Preparations (Concentration of Compounds in Squared Brackets)

no.	CB_1 [10 μM]	CB_2[10 μM]	FAAH [50 μM]
5	1 ± 4	12 ± 3	4 ± 3
6	5 ± 5	7 ± 4	4 ± 5
7	16 ± 15	22 ± 2	8 ± 2
Paracetamol	13 ± 0.9	15 ± 1	22 ± 3

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

	5	**6**	**7**	Paracetamol
COX-1	*a*	*a*	3.63 ± 0.57	*b*
COX-2	3.97 ± 0.06	*a*	3.86 ± 0.15	3.85 ± 0.21

[a] No inhibition. [b] inhibition but no pIC_{50} value determinable.

the human recombinant CB_1 or CB_2 receptors. The inhibitory activity of all compounds on FAAH was determined using rat brain membranes as an abundant source of the enzyme and by measuring the effect on [^{14}C]anandamide hydrolysis. Inhibition of COX-1 or COX-2 by **5**, **6**, and **7** was evaluated in whole blood assays by detecting serum or plasma levels of TXB_2 or PGE_2. In addition, **5** was tested against both isolated cyclooxygenases. Lastly, we also evaluated the effect of different doses of **5**, **6**, and **7** on the biphasic nociceptive response induced by an intrapaw injection of formalin in mice. This test was used for three reasons: (1) it allowed the determination of the 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),[10] (2) it was already employed to determine the analgesic effect of paracetamol on multiple occasions,[11−13] and (3) it allowed extrapolations regarding the mechanism of action of analgesic compounds depending on the affected nocifensive phase.[11] The time spent on licking the injected paw was measured as the marker of nociceptive behavior.

Results

The AM404 derivatives **1**, **2**, and **3** were tested against CB_1, CB_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_1 and CB_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 derivatives from the appropriate arachidonoyl congeners in the laboratory. The paracetamol analogues **5**, **6**, and **7** were again tested against CB_1, CB_2, and FAAH along with their "parent compound" paracetamol. Table 2 integrates the results of these experiments. None of the compounds displayed pronounced affinity for either the CB_1 or CB_2 receptor, nor any significant 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_{50} of about 107 μM. **6** did not show inhibition of either isoform, whereas **7** behaved as a dual inhibitor at both isoforms (IC_{50}: 234 μM (COX-1); IC_{50}: 138 μM (COX-2)). Paracetamol behaved as a dual COX inhibitor as well (IC_{50}: 141 μM (COX-2)) roughly matching data from literature.[14] 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_2 or PGE_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.[15] 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

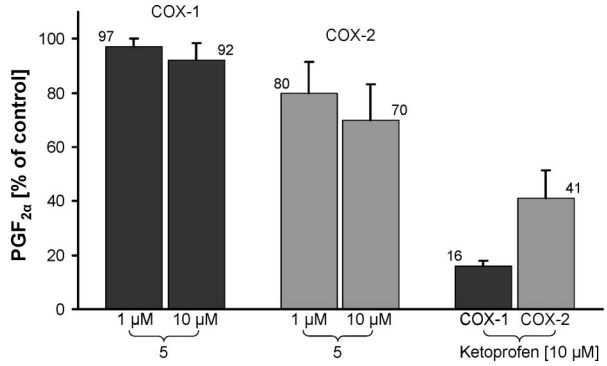


Figure 1. Effects of **5** on the production of PGH_2 by COX-1 and COX-2 at concentrations of 1 and 10 μM compared to Ketoprofen at a concentration of 10 μM. The generated PGH_2 was reduced to $PGF_{2\alpha}$ by stannous chloride to quantify the assay. Reflected are means of four experiments + standard error of the mean.

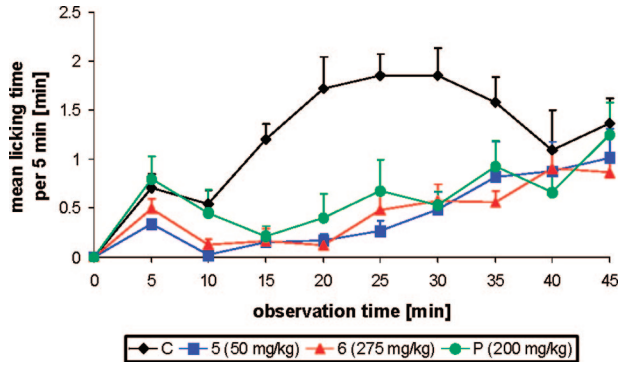


Figure 2. Time course of the licking behavior in mice after ip administration of **5**, **6**, paracetamol (P), or solvent (C) and injection of formalin into the left hind paw. Each point represents the mean + standard error of the mean of 6−8 animals per group. The first phase of the formalin test lasts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

observation chamber. In contrast, **5** and **6** did not influence locomotor behavior of the animals at any examined dose. However, they did reduce nociception: **5** reduced the nociceptive behavior in a statistically significant manner ($p \leq 0.05$) at a dose of 50 mg/kg in both phases of the formalin test,10 mg/kg of **5** diminished the nociceptive behavior ($p \leq 0.05$) only in the second phase of the formalin test, 5 mg/kg of **5** were without effect at either phase, **6** reduced the nociceptive behavior in a statistically significant manner ($p \leq 0.05$) at 275 mg/kg in both phases of the formalin test, 55 mg/kg of **6** shortened the nociceptive behavior ($p \leq 0.05$) only in the second phase of the formalin test, and 27.5 mg/kg did not display analgesic efficacy at any phase. Paracetamol was chosen as an analgesic reference substance as it has already proven its efficacy in the formalin test.[11−13] We employed a standard dose (200 mg/kg) of paracetamol and were surprisingly unable to detect efficacy in the first phase of the formalin test. In the second phase, 200 mg/kg of paracetamol displayed the expected and validated analgesic effect. Figure 2 displays the time course of the licking behavior for **5** (50 mg/kg), **6** (275 mg/kg), and paracetamol (200 mg/kg) compared to the control group within the 45 min observation period. Figures 3 and 4 show the dose−response relationships of **5** and **6** compared to paracetamol and the control group for the first and second phase of the formalin test.

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 $p \leq 0.05$). 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**,[15,16] of which only inhibition of COX-2 seems to be relevant under common pharmacological conditions.[14] **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 ($p \leq 0.05$) 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 ($p \leq 0.05$) 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 CB_1 and CB_2 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-aminoindazole 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.

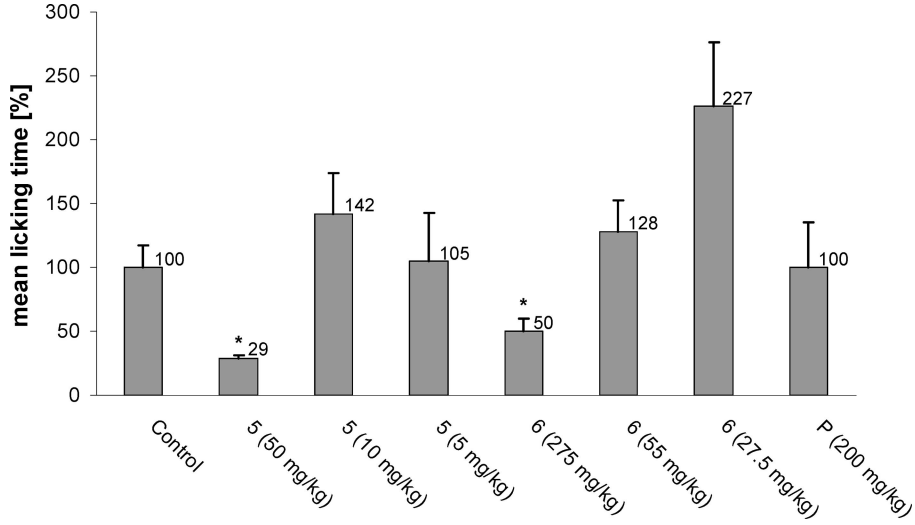


Figure 3. Effects of **5**, **6**, and paracetamol (P) administered ip on the formalin response manifested during the first phase. The cumulative response time of licking the injected paw was measured during the period of 0−10 min. The vertical bars denote the standard error of the mean. The number of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

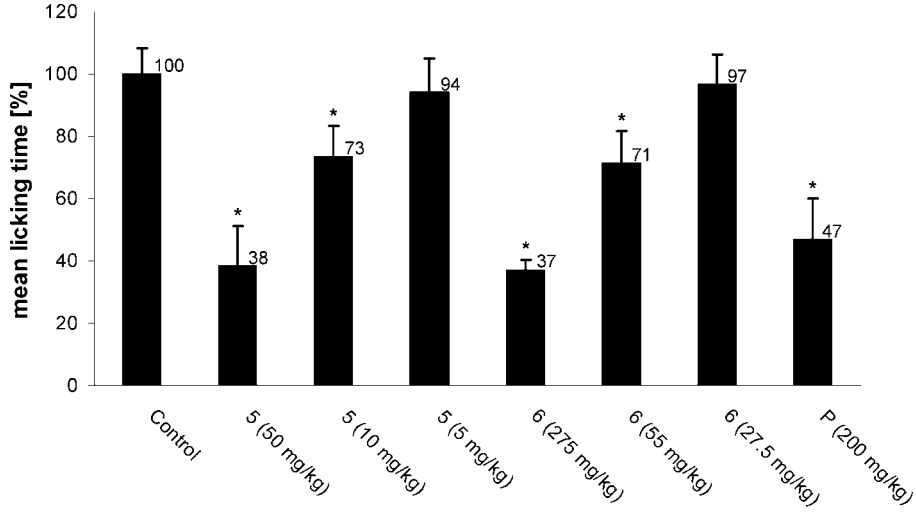


Figure 4. Effects of **5**, **6**, and paracetamol (P) administered ip on the formalin response manifested during the second phase. The cumulative response time of licking the injected paw was measured during the period of 15−45 min. The vertical bars denote the standard error of the mean. The number 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%.

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*-(1*H*-Indazol-5-yl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide (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). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 12.90 (br s, 1H, N**H**), 9.81 (s, 1H, CO-N**H**), 8.11−7.96 (m, 2H, 2 × Ar-**H**), 7.43 (d, $^3J = 9.1$ Hz, 1H, Ar-**H**), 7.39 (dd, $^3J = 9.1$ Hz, $^4J = 1.7$ Hz, 1H, Ar-), 5.42−5.25 (m, 8H, 4 × C**H**═C**H**), 2.82−2.73 (m, 6H, 3 × CH═CH-C**H**$_2$−CH═CH), 2.31 (t, $^3J = 7.5$ Hz, 2H, CH$_2$−C**H**$_2$−CO-NH), 2.13−1.96 (m, 4H, 2 × CH═CH-C**H**$_2$), 1.66 (tt, $^3J = {}^3J = 7.5$ Hz, 2H, C**H**$_2$−CH$_2$−CO-NH), 1.33−1.19 (m, 6H, C**H**$_2$−C**H**$_2$−C**H**$_2$−CH$_3$), 0.83 (t, $^3J = 7.1$ Hz, 3H,... −C**H**$_3$). ^{13}C NMR (125 MHz, CD_3OD), 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, 26.61, 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. ($C_{27}H_{37}N_3O$) C, H, N.

***N*-(4-Hydroxybenzyl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide (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 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 TBME, 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 + 3% NEt_3 as eluent to afford 34.0 mg (20%) of a yellowish oil. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 9.21 (s, 1H, O**H**), 8.12 (br s, 1H, CO-N**H**), 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 × C**H**=C**H**), 4.11 (d, ^3J = 5.8 Hz, 2H, NH-C$\mathbf{H}_2$), 2.82−2.74 (m, 6H, 3 × CH=CH-C$\mathbf{H}_2$-CH=CH), 2.10 (t, ^3J = 7.5 Hz, 2H, CH_2−C$\mathbf{H}_2$-CO-NH), 2.05−1.98 (m, 4H, 2 × CH=CH-C$\mathbf{H}_2$), 1.55 (tt, ^3J = ^3J = 7.5 Hz, 2H, C$\mathbf{H}_2$-CH_2−CO-NH), 1.33−1.20 (m, 6H, C$\mathbf{H}_2$-C$\mathbf{H}_2$-C$\mathbf{H}_2$-CH_3), 0.84 (t, ^3J = 6.8 Hz, 3H,... −C$\mathbf{H}_3$). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 173.1, 155.9, 130.5, 129.6, 129.2, 129.0, 128.9, 128.6, 128.3, 128.1, 127.9, 127.5, 115.7, 43.4, 36.2, 31.6, 29.4, 27.3, 26.7, 25.7, 25.6, 22.6, 14.1. EI-MS (70 eV), *m*/*z* (%): 107 (100), 122 (28.6), 165 (21.4), 409 (3.6, M^+). Anal. ($C_{27}H_{39}NO_2$) C, H, N.

***N*-(1*H*-Indazol-5-yl)acetamide (5).** First, 266.4 mg (2 mmol) 5-aminoindazole 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 chromatography on silica gel (40−63 μm) using EtOAc/NEt_3 (97/3) as eluent. Crystallization from MeOH/TBME afforded 238.8 mg (46%) of slightly violet crystals; mp: 203−206 °C (MeOH/TBME). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 12.88 (br s, 1H, N**H**), 9.85 (s, 1H, CO-N**H**), 8.10−7.97 (m, 2H, 2 × Ar-**H**), 7.42 (d, ^3J = 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-C$\mathbf{H}_3$). ^{13}C NMR (100 MHz, CD_3OD), δ (ppm): 171.5, 138.9, 134.8, 133.0, 124.1, 122.9, 112.7, 111.1, 23.7. EI-MS (70 eV), *m*/*z* (%): 133 (100), 175 (40, M^+), 106 (20.7). Anal. ($C_9H_9N_3O$) 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 chromatography on silica gel (40−63 μm) using TBME/MeOH (95/5) as eluent. Crystallization from EtOAc afforded 155.2 mg (63%) of white crystals; mp: 124−126 °C (EtOAc)). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 9.22 (s, 1H, O**H**), 8.16 (br s, 1H, CO-N**H**), 7.03 (dd, ^3J = 6.2 Hz, ^4J = 2.1 Hz, 2H, 2 × Ar-**H**), 6.68 (dd, ^3J = 6.2 Hz, ^4J = 2.1 Hz, 2H, 2 × Ar-**H**), 4.10 (d, ^3J = 5.8 Hz, 2H, NH-C$\mathbf{H}_2$), 1.82 (s, 3H, CO-C$\mathbf{H}_3$). ^{13}C NMR (100 MHz, CD_3OD), δ (ppm): 172.7, 157.6, 130.5, 129.9, 116.1, 43.9, 22.6. EI-MS (70 eV), *m*/*z* (%): 165 (100, M^+), 122 (72.1), 107 (47.9). Anal. ($C_9H_{11}NO_2$) C, H, N.

Biological Evaluation. CB_1 and CB_2 Assay. For CB_1 and CB_2 receptor binding assays, the compounds were tested using membranes from HEK-293 cells overexpressing either the human recombinant CB_1 or CB_2 receptor and [^{3}H]CP-55,940 (K_d = 0.18 nM for CB_1 and 0.31 nM for CB_2 receptors) as the high affinity ligand as described by the manufacturer (Perkin-Elmer, Italy). Membranes were incubated with increasing concentrations (1 nM to 10 μM) of test compounds and [^{3}H]CP-55,940 at 30 °C for 90 min, and the amount of displaced radioligand was determined by scintillation counting. K_i values were calculated by applying the Cheng−Prusoff equation to the IC_{50} values for the displacement of the bound radioligand by increasing concentrations of the test compounds. Data points for preliminary results were determined in duplicate. Data points for the determination of K_i values were measured in triplicate.

FAAH Assay. The effect of the compounds on the enzymatic hydrolysis of [^{14}C]anandamide was studied by using membranes prepared from rat brain. In brief, a half-rat brain was homogenized in Tris-HCl (50 mM, pH 7.0) by using an Ultraturrax (16000 rpm, 3 min) and a Dounce homogenizer at 4 °C. Homogenates were first centrifuged at 3000 rpm (4 °C, 6 min) to rid the debris, and the supernatant was centrifuged at 11000 rpm (4 °C, 25 min). The pellet from this latter centrifugation was resuspended in Tris-HCl (50 mM, pH 9.0) and used for the assay. Tissue suspensions (containing 70 μg of protein) were incubated with two concentrations of the test compounds (10 and 50 μM) and [^{14}C]anandamide (10000 cpm) in Tris-HCl (50 mM, pH 9) for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C]anandamide hydrolysis was used to calculate FAAH activity and was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3$/MeOH (1:1 by volume). All data points were determined in duplicate.

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. Serum was separated by centrifugation, and TXB_2 levels were determined per GC-MS/MS as described previously.[24] All data points were determined in quadruplicate.

Whole Blood Assay for COX-2 Inhibition. One mL aliquots of heparinized whole blood from a healthy 50 year old volunteer who had not taken any nonsteroidal anti-inflammatory drug 2 weeks prior to blood sampling were incubated with 10 μg of lipopolysaccharides (from *Escherichia coli* 026:B26), 10 μg aspirin, plus 2 μL of the test agent dissolved in DMSO for 24 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 PGE_2 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 $SnCl_2$ to reduce the COX-produced PGH_2 into $PGF_{2\alpha}$, which was further quantified by EIA using PGE_2 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 stereotypical nocifensive 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 ($p \leq 0.05$), 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** (275, 55, 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.

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