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Effects of synthetic alkamides on Arabidopsis fatty acid amide hydrolase activity and plant development

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ABSTRACT

Alkamides and *N*-acylethanolamines (NAEs) are bioactive, amide-linked lipids that influence plant development. Alkamides are restricted to several families of higher plants and some fungi, whereas NAEs are widespread signaling molecules in both plants and animals. Fatty acid amide hydrolase (FAAH) has been described as a key contributor to NAE hydrolysis; however, no enzyme has been associated with alkamide degradation in plants. Herein reported is synthesis of 12 compounds structurally similar to a naturally occurring alkamide (*N*-isobutyl-(2*E*,6*Z*,8*E*)decatrienamide or affinin) with different acyl compositions more similar to plant NAEs and various amino alkyl head groups. These "hybrid" synthetic alkamides were tested for activity toward recombinant Arabidopsis FAAH and for their effects on plant development (*i.e.*, cotyledon expansion and primary root length). A substantial increase in FAAH activity was discovered toward NAEs *in vitro* in the presence of some of these synthetic alkamides, such as *N*-ethyllauroylamide (**4**). This "enhancement" effect was found to be due, at least in part, to relief from product inhibition of FAAH by ethanolamine, and not due to an alteration in the oligomerization state of the FAAH enzyme. For several of these alkamides, an inhibition of seedling growth was observed with greater results in FAAH knockouts and less in FAAH over-expressing plants, suggesting that these alkamides could be hydrolyzed by FAAH *in planta*. The tight regulation of NAE levels *in vivo* appears to be important for proper seedling establishment, and as such, some of these synthetic alkamides may be useful pharmacological tools to manipulate the effects of NAEs *in situ*.

1. Introduction

Alkamides and *N*-acylethanolamines (NAEs) are small, amide-containing lipids (Chapman et al., 1998; López-Bucio et al., 2006). The compositions of alkamides and NAEs vary in chain length and the number of double bonds in their acyl groups (Boonen et al., 2012; Chapman, 2004; Chapman et al., 1998, 1999). The structural diversity of alkamides is greater than NAEs due to the composition of the amino alkyl head group, which can include but are not limited to butyl, isopropyl, or isobutyl moieties (Boonen et al., 2012; López-Bucio et al., 2006).

NAEs occur widely and are used as signaling substances in animals, plants and some microorganisms (Blancaflor et al., 2014; Coulon et al., 2012; Okamoto et al., 2007). In plants, *N*-palmitoylethanolamine (NAE 16:0), *N*-oleoylethanolamine (NAE 18:1), *N*-linoleoylethanolamine (NAE 18:2) and *N*-linolenoylethanolamine (NAE 18:3) are generally the most abundant NAE types (Blancaflor et al., 2014; Chapman et al., 1999) reflecting both the common fatty acids found in membranes and their biosynthetic origin. Alkamides, on the other hand, appear to be more restricted in their distribution; they have been found in about 10 plant families and some fungi (Boonen et al., 2012; López-Bucio et al., 2006; Ramírez-Chávez et al., 2004) but have not been reported in animal systems. While NAEs are derived from membrane phospholipid precursors, alkamides in plants are likely made from amino acid

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precursor (Cortez-Espinosa et al., 2011). Despite these structural and biosynthetic differences, NAEs and alkamides exhibit some similar biological effects when applied to plants suggesting there may be some overlap in metabolism and/or targets of both groups of compounds.

Different alkamide moieties have been reported in plants (Boonen et al., 2012; López-Bucio et al., 2006; Ramírez-Chávez et al., 2004; Hajdu et al., 2014). The most common naturally-occurring alkamide has an acyl group containing a 2*E* double bond and an isobutyl head group, identified as *N*-isobutyl-(2*E*,6*Z*,8*E*)decatrienamide, also called affinin (Ramírez-Chávez et al., 2004). Different plants such as *Echinacea purpurea* or *Echinacea angustifolia* can accumulate high levels of alkamides in certain organs, and extracts have been used for many years for their purported therapeutic benefits (López-Bucio et al., 2006). Recently several alkamide compounds such as *N*-benzyl-(9*Z*,12*Z*)octadecadienamide were shown to inhibit FAAH activity (IC_{50} = 4 μM) and/or to interfere with the cellular uptake of *N*-(2-hydroxyethyl)-(5*Z*,8*Z*,11*Z*,14*Z*)eicosatetraenamide (also named anandamide) the most widely recognized NAE in the mammalian endocannabinoid system (Hajdu et al., 2014).

NAEs have been associated with several functions in plants such as seedling establishment (Blancaflor et al., 2003; Chapman, 2004; Chapman et al., 1999; Kilaru et al., 2007; Wang et al., 2006), flowering (Teaster et al., 2012), responses to pathogens (Kang et al., 2008; Tripathy et al., 1999), inhibition of phospholipase (Austin-Brown and Chapman, 2002) and lipoxygenase (Keereetaweep et al., 2010). *N*-(2-Hydroxyethyl)dodecanamide (also referred to as *N*-lauroylethanolamine or NAE 12:0) has been widely used to study the effects of NAEs especially on plant root development (Chapman, 2004; Coulon et al., 2012). Although the precise mechanism remains unknown, NAEs, in part, appear to interact with the abscisic acid signaling pathway to mediate their negative effects on seedling growth (Keereetaweep et al., 2013; Teaster et al., 2007).

Besides their medicinal properties, alkamides are also known for their insecticidal effects (Ramírez-Chávez et al., 2004). Recently, a new function for alkamides has been ascribed to affinin, and a reduced form of this lipid (*N*-isobutyl-(2*E*)decanamide and *N*-isobutyldecanamide), for the modulation of Arabidopsis seedling growth (Ramírez-Chávez et al., 2004). Application of exogenous affinin at levels below 28 μM increased the primary root length, whereas affinin concentrations above 28 μM inhibited root development (Ramírez-Chávez et al., 2004). The effects of alkamides on plant development may operate through the cytokinin-signaling pathway to control the activity of the plant meristem and differentiation processes (López-Bucio et al., 2007). In addition, genetic evidence suggests that alkamides modulate lateral root formation via interaction with the jasmonic acid signaling pathway (Méndez-Bravo et al., 2011).

Thus, NAEs and alkamides share some similarities and differences in structure, distribution and their influence on plant development (López-Bucio et al., 2006, 2007). The studies of the effects of *N*-decanoylethanolamide (NAE 10:0) on root development

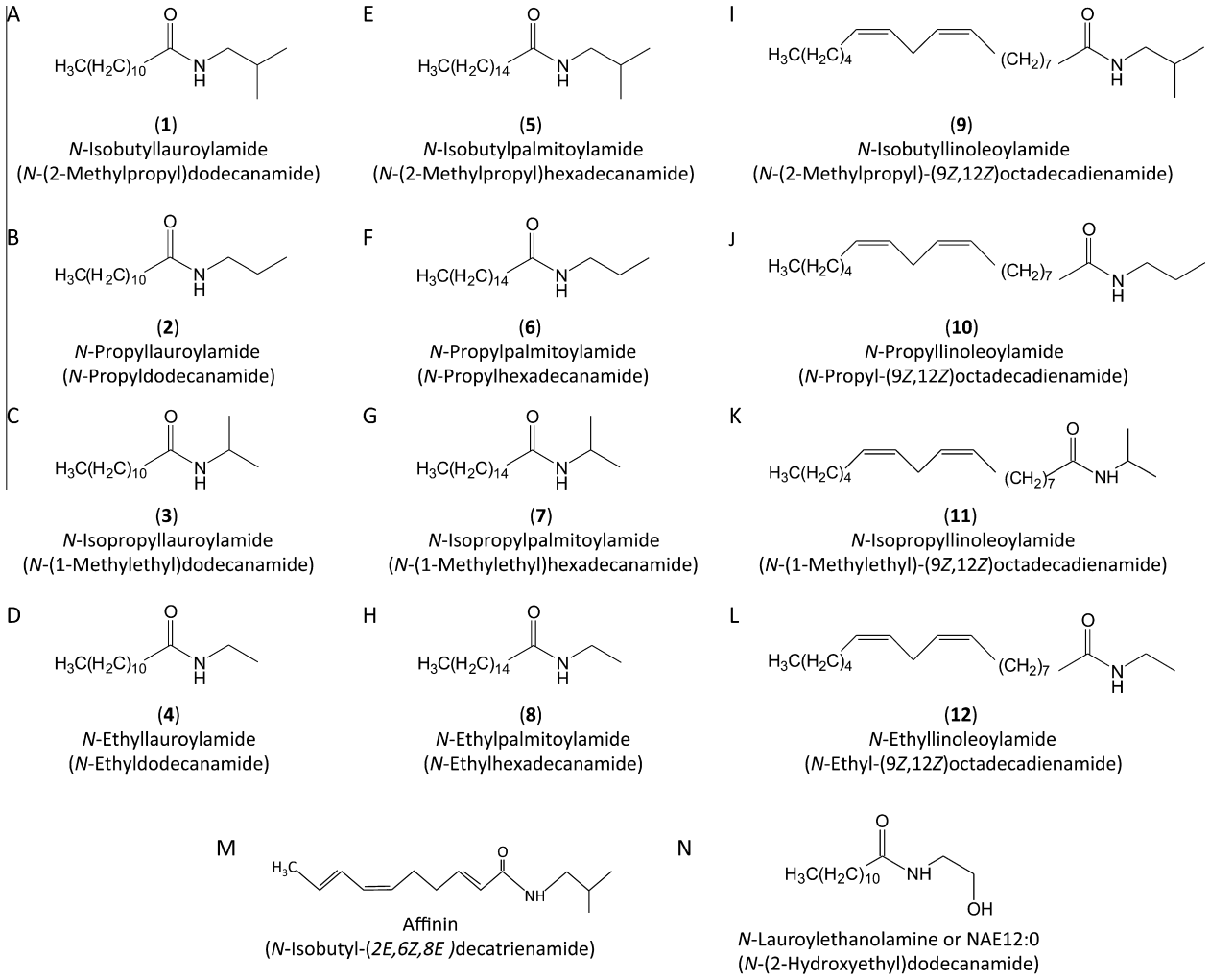

Fig. 1. Chemical Structure of "hybrid" alkamides. Three different categories of alkamide like compounds: (A–D) acyl chain with 12:0 (number of carbon atom:number of double bonds); (E–H) 16:0; (I–L) 18:2; (M) 10:3; (N) 10:0.

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compared with different alkamides demonstrated similarities in the biological activities between NAEs and alkamides (López-Bucio et al., 2007). Since both types of lipids share a similar acylamide structures, and in some instances exhibit similar negative effects on seedling growth, it has been suggested that alkamides and NAEs may act through common signaling mechanisms (López-Bucio et al., 2007), but this remains to be determined.

Enzymes capable of metabolizing the different alkamides and potentially terminate their different functions have not been reported *in planta*. On the other hand, different enzymes are known to metabolize NAEs including lipoxygenases (Keereetaweep et al., 2013; Kilaru et al., 2011; Shrestha et al., 2002), *N*-acylethanolamine-hydrolyzing acid amidase (Ueda et al., 2010) and fatty acid amide hydrolases (FAAHs) (Cravatt et al., 1996; Shrestha et al., 2003; Wei et al., 2006). FAAHs have been described as key enzymes terminating the effects of NAEs by hydrolyzing these lipids to their corresponding free fatty acids (FFA) and ethanolamine.

In animals, different structural analogs of the endocannabinoid, NAE 20:4 or the sleep-inducing primary amide, oleamide, with alkyl "head-groups" similar to plant alkamides were tested for their

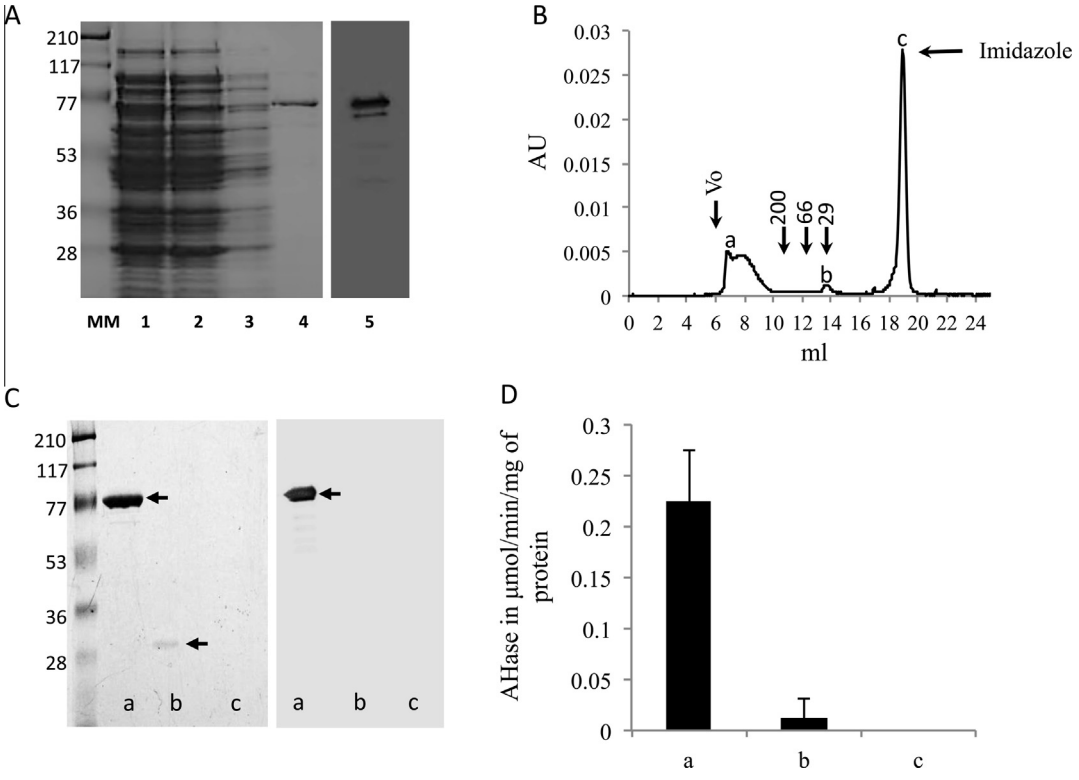

Fig. 2. Characterization of purified FAAH protein. (A) SDS–PAGE and Western blot of enriched FAAH from *E. coli* on a 12.5% polyacrylamide gel or PVDF membrane (respectively). (B) Migration of purified FAAH protein on a Superdex-200 gel filtration. Molecular mass standards are indicated in kilodaltons. (C) SDS–PAGE (left panel) and Western-blot (right panel) of fractions a, b and c. (D) Enzymatic activity in µmol min^{-1} mg of $protein^{-1}$ of the different fractions (a, b and c), reaction carried out in 50 mM Bis-Tris propane–HCl (pH 9.0) at 30 °C, 30 min, in a final volume of 0.15 ml. Data points represent means ± S.D. of triplicate assays. Vo, void volume. Proteins were separated by SDS–PAGE (10% resolving gels) and visualized in gels by Coomassie-blue staining. Western blot was probed with mouse monoclonal antibodies. The recombinant proteins expressing the HIS tag at the C-terminus were detected by chemiluminescence using a 1-to-2000 dilution of mouse monoclonal anti-HIS antibodies (ABGENT San Diego, CA) and a solution of 1-to-4000 dilution of goat anti-mouse IgG conjugated to a peroxidase (Bio-Rad).

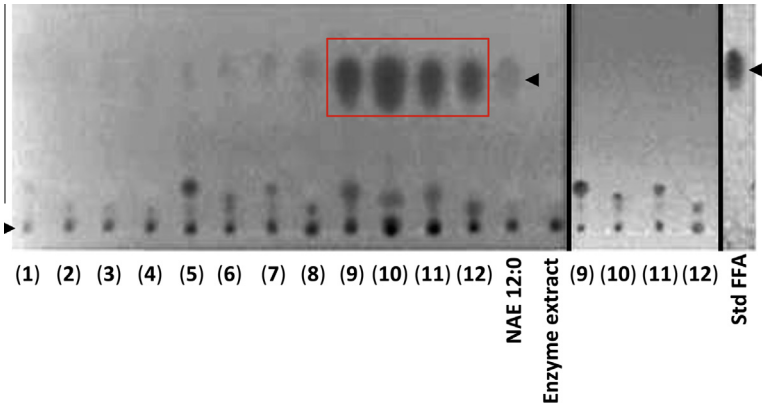

Fig. 3. TLC analysis of the lipid composition of the amidohydrolase assay with FAAH protein and different alkamide substrates (**1**–**12**). Reactions were initiated by the addition of purified FAAH protein (0.9 µg) with 50 mM Bis-Tris propane–HCl, (pH 9.0), at 30 °C for 30 min, 120 rpm, and 100 µM of different alkamides or NAE 12:0 in a final volume of 0.45 ml. Lipids were extracted and then separated by TLC (hexane/Et_2O/AcOH, 80:20:2, v/v/v). Lipids were visualized by spraying the plate with a solution of primuline and exposure under a UV lamp (Testet et al., 2005). Position of the origin, free lauric acid formed by hydrolysis of NAE 12:0 and free fatty acid (FFA) 12:0 standard are indicated (arrows).

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competitive effects on the mammalian FAAH activities (Boger et al., 2000; Lang et al., 1999; Vandevoorde et al., 2003). None of these alkamide-like compounds were described as good substrates for rat FAAH protein. Here this concept was expanded by synthesizing a broad range of NAE-alkamide "hybrid" compounds (**1**–**12**), and their impact tested on FAAH activity and plant growth. Specifically, a group of alkamides with different acyl chains (12:0, 16:0 and 18:2) and different head groups (isobutylamine, propylamine, isopropylamine and ethylamine) was synthesized. The synthetic alkamides (Fig. 1) could be hydrolyzed by FAAH *in vitro*, although with lower efficiency compared to NAEs. The *N*-dodecyl series (**1**–**4**) actually enhanced the Arabidopsis FAAH activity *in vitro*. None of the synthetic "hybrid" compounds were as potent as the "native" NAE (12:0) or alkamide (affinin) at inhibiting seedling root elongation. However, for the *N*-dodecyl series (**1**–**4**), there were reductions in cotyledon size that appeared to follow patterns of either natural NAE in a manner that was consistent through the action of endogenous FAAH activity (*e.g.*, *N*-ethyldodecanamide), or with natural alkamide (*e.g.*, affinin). Taken together, these results provide insights about the potential interaction of FAAH with alkamide metabolism, and provide a novel set of compounds that may be useful for manipulating endogenous NAE levels *in vivo* through their inhibition or enhancement of FAAH activity.

2. Results

2.1. Synthetic "hybrid" alkamides

A series of synthetic alkamides were designed with common acyl chains found in plants (NAEs-12:0 (lauric), 16:0 (palmitic), and 18:2 (linoleic)) and each of four different amino alkyl head-groups, found in naturally-occurring alkamides (*i.e.*, ethyl-, propyl-, isopropyl- and isobutyl-amine) (Fig. 1). Details of the synthesis and characterization of the compounds are described in the experimental procedures and in the supplemental documents. Stock solutions (10 mM in DMSO) were prepared and diluted to the desired concentration for enzyme or growth assays. For

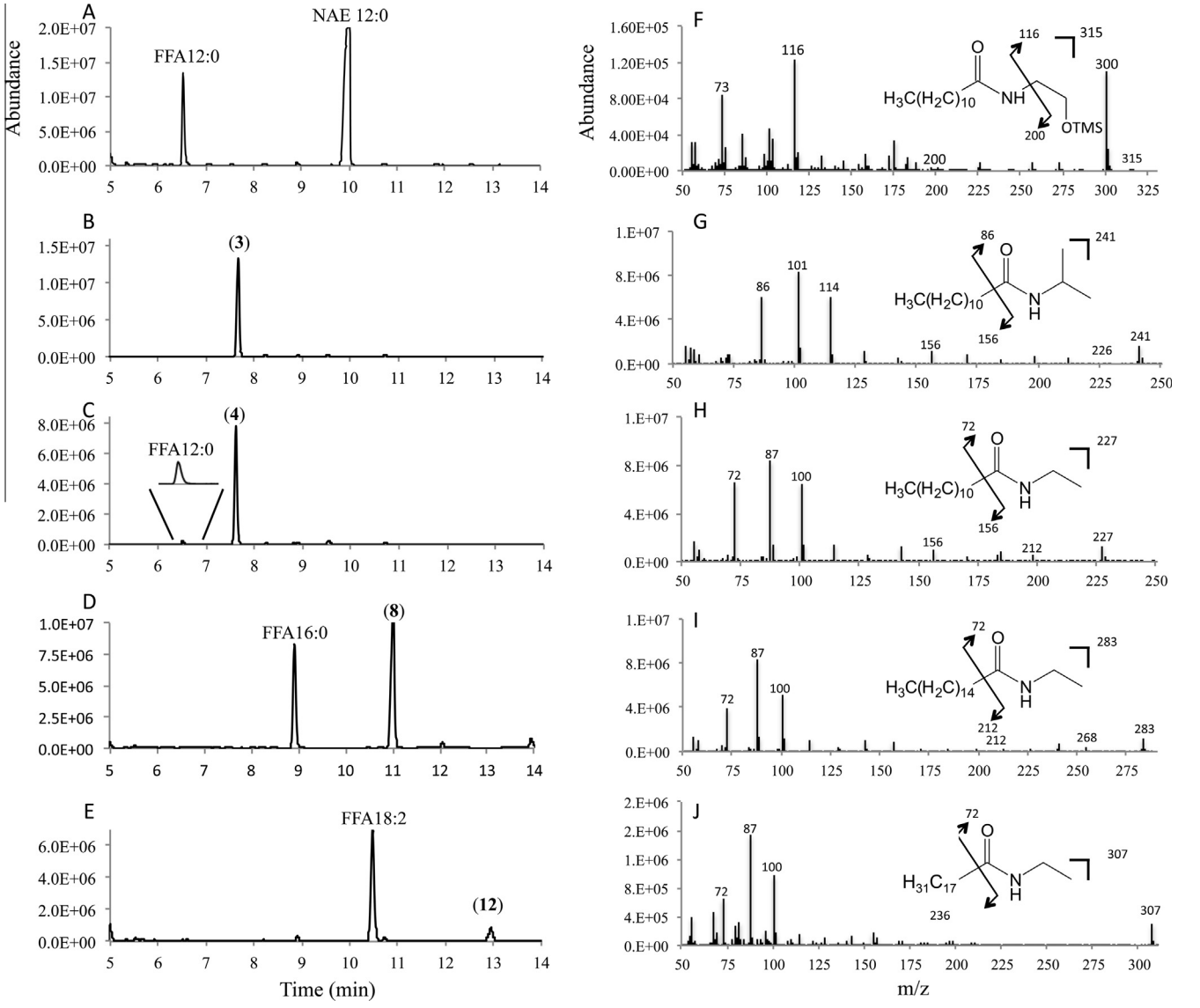

Fig. 4. Representative gas chromatography–MS analysis of the lipid composition of the amidohydrolase assay with FAAH protein and different *N*-acyl-amide substrates or NAE 12:0. Reactions were initiated by the addition of FAAH protein (0.3 μg) with 50 mM of Bis-Tris propane–HCl, (pH9.0), at 30 °C for 30 min, 120 rpm and 100 μM of different alkamide substrates in a final volume of 150 μl. Lipids were extracted and analyzed by GC–MS. (A–E) Total ion chromatograms of extracted lipids from the reactions with, NAE 12:0, (**3**), (**4**), (**8**), and (**12**). (F–J) mass spectra of NAE 12:0, (**3**), (**4**), (**8**), and (**12**), respectively.

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simplicity, the different alkamides are referred to by a number from 1 to 12, in addition to their suggested names in SciFinder® and IUPAC designations also are provided in Fig. 1 for reference.

2.2. Protein purification and amidohydrolase assays

Enzymatic assays were performed with recombinant FAAH protein (UniProt # Q7XJJ7, At5g64440p) purified to near-homogeneity by affinity chromatography using a Ni-column (Qiagen®) and by gel filtration with a Superdex 200 column. Protein purity after elution from the Ni–NTA column was determined by SDS–PAGE and Western blotting using an anti-HIS tag monoclonal antibody (Fig. 2A). Bands observed in both SDS–PAGE and Western blotting were consistent with the molecular mass calculated for FAAH protein (*i.e.*, 70 kDa). Results of the purified FAAH fraction by gel filtration analysis (Fig. 2B) using a Superdex 200 column (GE health care life sciences) displayed the presence of three major regions of absorbance (280 nm). Different fractions from the gel filtration (Fig. 2B) (denoted a, b and c) were analyzed by SDS–PAGE and Western-blotting (Fig. 2C). The amidohydrolase activity (AHase) (μmol min^{-1} mg of $protein^{-1}$) of each fraction was measured by following the hydrolysis of [1-^{14}C]NAE 12:0 to its respective [1-^{14}C]FFA (Fig. 2D). Fraction "c" was assigned to the imidazole used to elute the protein from the Ni–NTA column because it contained no protein and exhibited no AHase activity, (Fig. 2C and D). Fraction "a" contained a high molecular mass protein complex (around 700 kDa, Fig. 2B), assigned to FAAH protein by SDS–PAGE and Western blotting analysis (Fig. 2C). FAAH enzymatic assays of this fraction confirmed robust AHase activity (Fig. 2D). Together, these results indicate that Arabidopsis FAAH formed an oligomeric complex similar to purified rat FAAH protein (Patricelli et al., 1998). The low molecular mass found in fraction "b" (30 kDa) may have been a truncated form of FAAH, as it retained some low AHase activity (Fig. 2D); however, it did not bind the monoclonal antibody to the C-terminal His-tag in Western-blotting (Fig. 2C).

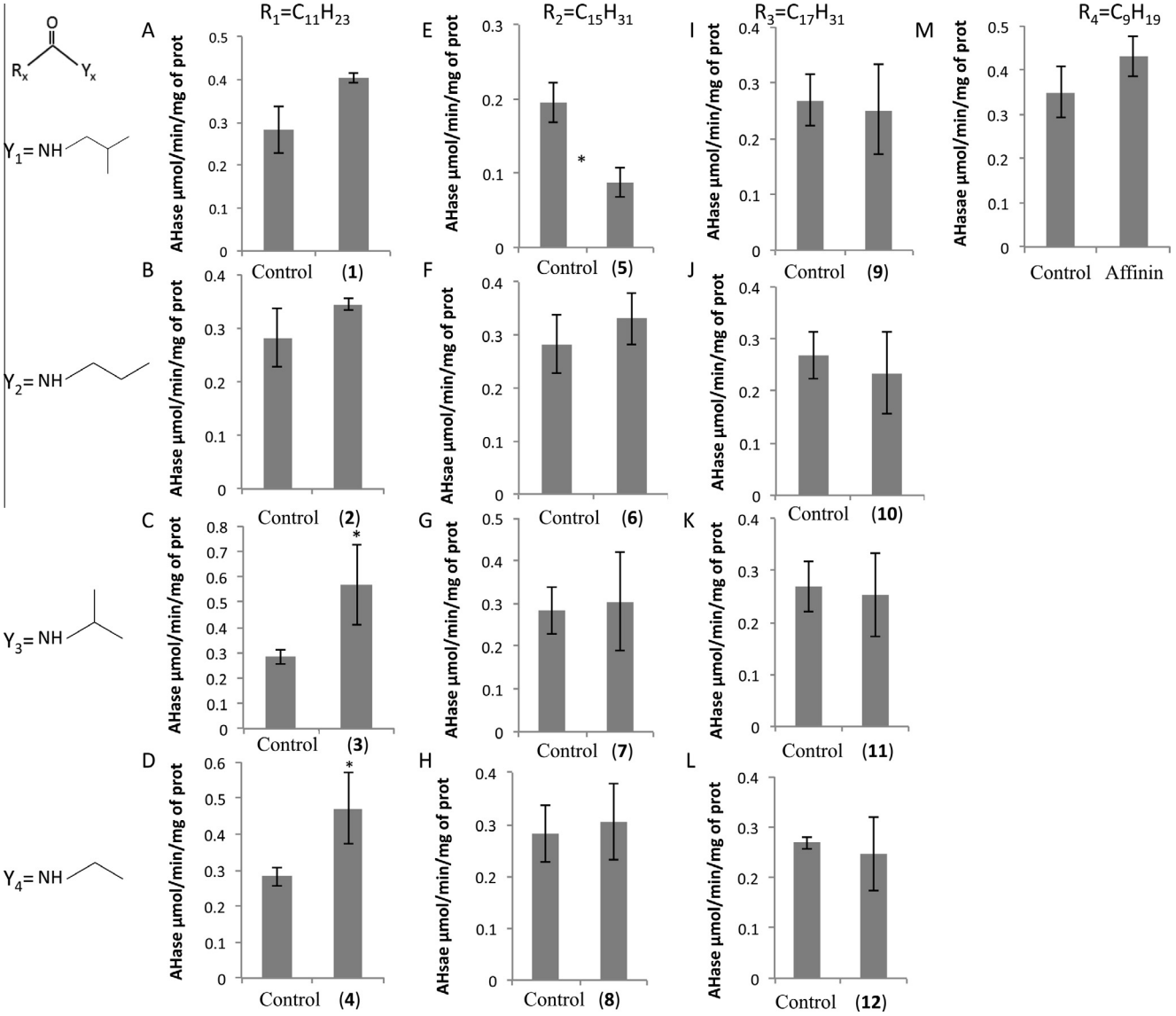

Fig. 5. Amidohydrolase activity (μmol min^{-1} mg^{-1} of protein) of purified FAAH with different [1-^{14}C]NAEs in presence (or absence) of different alkamides compounds (**1**–**12**). Reactions were initiated by the addition of purified FAAH protein (0.3 μg) with 50 mM Bis-Tris propane–HCl, (pH 9.0), and 100 μM of different radioactive NAEs. Reaction proceeded at 30 °C, 30 min, 120 rpm shaking in a final volume of 0.15 ml. Data points represent means ± S.D. of triplicate assays. (A–D) 100 μM of [1-^{14}C]NAE 12:0 was used as substrate; (E–H) 100 μM of [1-^{14}C]NAE 16:0; (I–L) 100 μM of [1-^{14}C]NAE 18:2; (M) 100 μM of [1-^{14}C]NAE 16:0. *p*-Value of <0.05 is indicated by * as determined by Student's *t* test.

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2.3. Effect of synthetic alkamides on FAAH

One goal of this work was to determine if any of the newly-synthesized alkamides (**1**–**12**) would be hydrolyzed by the recombinant FAAH proteins. With prolonged reaction times and high protein concentrations, all the alkamides were hydrolyzed to some degree. However, using conditions previously reported for the characterization of NAE hydrolytic activity in plants (Faure et al., 2014; Shrestha et al., 2003), it appeared that the newly-synthesized alkamides were relatively poor substrates for recombinant FAAH protein relative to NAEs (Fig. 3). Primuline staining of acyl lipids on the TLC plates (White et al., 1998) provided a visual representation of free fatty acid (FFA) formation from the various alkamides, but was not intended to be quantitative in these assays. Most of the alkamides appeared to be hydrolyzed by purified FAAH although formation of FFA from the *N*-12:0 series (**1**–**4**) was difficult to detect by TLC (Fig. 3).

To confirm the hydrolysis of the alkamides to FFA, GC–MS analysis was also performed of the lipids in the reaction mixtures after 30 min incubation with purified FAAH protein confirming the formation of FFA (*e.g.*, lauric acid) from the alkamides (Fig. 4 and Supplemental Fig. S1). When analyzed by GC–MS, more FFA was released from NAE than from any of the alkamides (*i.e.*, FFA 12:0 from NAE 12:0 at relative ion counts of $1.5E^{+07}$ was higher by an order of magnitude than FFA recovered from the alkamides, Fig. 4, Supplemental Fig. S1), although this should be interpreted with some caution due to differences in chromatography and ionization properties among the different alkamides and NAEs. Total ion chromatograms suggested that amidohydrolase activity was higher toward alkamides with longer acyl chain lengths (18:2 > 16:0 > 12:0, Fig. 4). Alkamides with alpha or beta methylation in the amino alkyl head group (isobutylamine, isopropylamine) (**1, 5, 9, 3, 7, 11**) were generally poorer substrates than linear analogs (ethylamine, propylamine) (**2, 6, 10, 4, 8, 12**) (Fig. 4; Supplemental Fig. S1). Similarly, affinin with its branched isobutyl group showed little hydrolysis by FAAH under these experimental conditions (Supplemental Fig. S1).

To more directly compare the FAAH-mediated hydrolysis of the synthetic alkamides and affinin with NAE, equimolar concentrations of alkamides and radiolabeled NAEs were tested with purified enzyme (Fig. 5). For each assay, the acyl chain of the [1-^{14}C]NAE substrate was matched with corresponding alkyl group of the alkamide being tested (*e.g.*, [1-^{14}C]NAE 12:0 was used with the *N*-12:0-alkamide series; **1**–**4**). Of twelve compounds tested, only one showed a significant inhibition of FAAH activity, *N*-isobutylpalmitoylamide (**5**) (Fig. 5E), suggesting that these

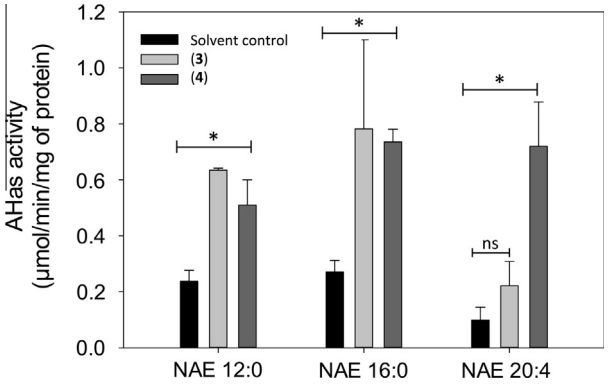

Fig. 7. Amidohydrolase activity (μmol min^{-1} mg of protein^{-1}) of purified FAAH protein with different [1-^{14}C]*N*-acylethanolamines (NAEs) in presence of 100 μM of *N*-isopropyllauroylamide (**3**) or *N*-ethyllauroylamide (**4**). Reactions were initiated by the addition of purified FAAH (0.3 μg) in 50 mM Bis-Tris propane–HCl (pH 9.0) and different radiolabeled NAEs (12:0, 16:0 and 20:4) in a final volume of 0.15 ml. Reactions proceeded at 30 °C, 30 min, with shaking (120 rpm). Data points represent means ± S.D. of triplicate assays. Plots were generated with SigmaPlot software version 12.0. *p*-Value of <0.05 is indicated by * as determined by Student's *t* test. ns, not significant.

synthetic alkamide compounds competed poorly with their corresponding NAEs. Overall and somewhat surprisingly, alkamides with shorter acyl chains actually induced an increase in AHase activity with significant increases measured for the *N*-isopropyllauroylamide (**3**) and *N*-ethyllauroylamide (**4**) (Fig. 5C and D, respectively), while those with longer acyl chains (*e.g.*, 18:2) tended to decrease in Arabidopsis FAAH activity (Fig. 5I–L). The naturally occurring affinin exhibited no significant effects on NAE hydrolysis by FAAH (Fig. 5M). These results confirmed that although FAAH was able to hydrolyze the synthetic alkamides, they were considerably less suitable substrates when compared to NAEs. Of particular interest was the apparent enhancement of NAE hydrolysis by FAAH that was conferred by some of the shorter chain alkamides (Figs. 5–8).

Enhancement of FAAH activity by (**3**), by way of example, was observed in radiometric scans following the conversion of radiolabeled NAE 12:0–12:0 free fatty acid (Fig. 6), where contributions from non-enzymatic processes were ruled out using heat-denatured controls (Fig. 6B). Similar enhancements in FAAH activity were observed for other NAE substrates including [1-^{14}C]*N*-palmitoylethanolamine (NAE 16:0), or [1-^{14}C]anandamide (NAE 20:4) when the hydrolysis was conducted in the presence of 100 μM of (**3**) or (**4**) (Fig. 7). An approximately threefold enhancement was

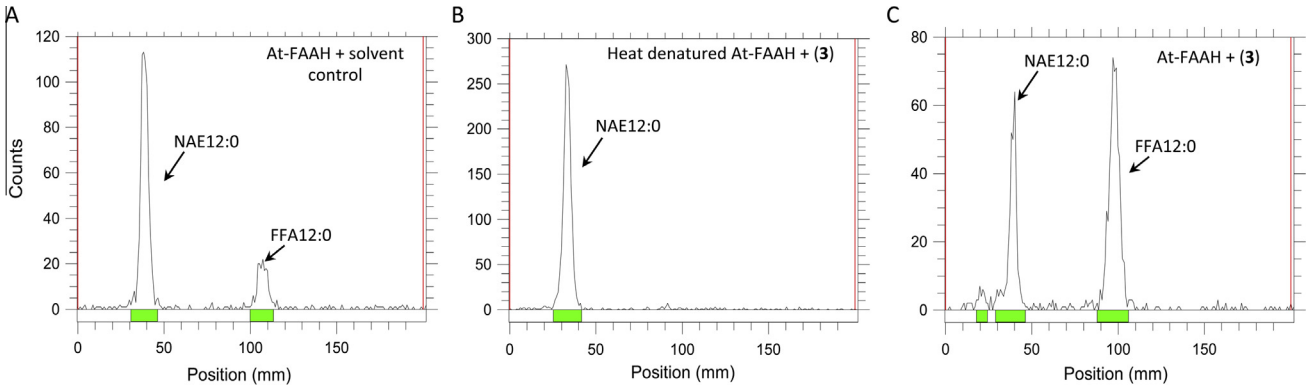

Fig. 6. Radiochromatograms of the total [1-^{14}C]lipid components of following FAAH reactions with 100 μM of [1-^{14}C]NAE 12:0 in presence (or absence) of 100 μM of *N*-isopropyllauroylamide (**3**). Reactions were initiated by the addition of purified FAAH protein (0.3 μg) with 50 mM of Bis-Tris propane–HCl (pH 9.0), and 100 μM of [1-^{14}C]NAE 12:0 in a final volume of 0.15 ml. Reaction proceeded at 30 °C, for 30 min with shaking (120 rpm). (A) Assay with 0.01% of DSMO (solvent control); (B) assay with heat denatured FAAH protein (5 min at 100 °C) and 100 μM of (**3**); (C) assays with 100 μM of (**3**). Total lipid content has been extracted has described by Shrestha et al. (2006). Chromatograms were obtained by radiometric scanning of the TLC plate with a BioScan instrument.

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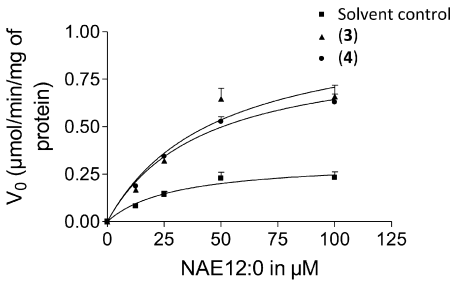

B

	K_m^{app} µM	V_{max}^{app}	K_{cat} sec^{-1}	K_{cat}/K_m^{app} $M^{-1}.sec^{-1}$
Solvent control	29 ± 3.2	0.31 ± 0.04	1.21 ± 0.03	$4.1X10^4$
(**3**)	47 ± 5.9	1.04 ± 0.19	4.1 ± 0.08	$8.7X10^4$
(**4**)	41 ± 2.4	0.912 ± 0.07	3.61 ± 0.04	$8.8X10^4$

Fig. 8. Kinetic characterization of purified recombinant AtFAAH protein in presence of 100 µM of *N*-isopropyllauroylamide (**3**) or *N*-ethyllauroylamide (**4**). (A) Initial velocities were measured at increasing concentrations of [1-^{14}C]NAE 12:0. Reactions were initiated by the addition of purified protein (0.3 µg) with 50 mM Bis-Tris propane–HCl (pH 9.0), at 30 °C in a final volume of 0.15 ml. (B) Represent the apparent kinetic parameters of the enzymes estimated by transformation of these original data (*i.e.*, double-reciprocal plots). Data points represent means ± S.D. of triplicate assays. Plots were generated with Prism software version 3.0 (GraphPad Software, San Diego) and data were fitted to a nonlinear regression (curve fit) using one site binding (hyperbola) equation with an R^2 between 0.96 and 0.97.

observed in assays containing compounds (**3**) or (**4**) for each of the radiolabelled NAE tested except for the [1-^{14}C]anandamide for which only (**4**) was able to significantly increase FAAH activity (Fig. 7). Anandamide (NAE 20:4) is not an endogenous NAE in higher plants, so perhaps the minimal modulation of Arabidopsis FAAH activity toward anandamide is not altogether surprising.

2.4. N-Isopropyllauroylamide (*3*) and N-ethyllauroylamide (*4*) and FAAH activity

To more accurately characterize the enhancement effect of the alkamide compounds on FAAH activity, the kinetic parameters of the purified, recombinant Arabidopsis FAAH enzyme were measured in the presence of 100 µM of (**3**) or (**4**) at increasing concentrations of [1-^{14}C]NAE 12:0 (Fig. 8). Arabidopsis FAAH exhibited typical Michaelis–Menten kinetics when initial velocity measurements were made at increasing concentrations of NAE 12:0. Apparent kinetic parameters of the FAAH-catalyzed enzymatic reaction were calculated and are summarized in Fig. 8B. Values for kinetic parameters of the recombinant FAAH protein (K_m^{app} and V_{max}^{app}) obtained without alkamides were similar to V_{max}^{app} and K_m^{app} determined elsewhere (0.23 µmol min^{-1} mg^{-1} and 17.6 µM) (Shrestha et al., 2003). There was a significant increase in the V_{max}^{app} with either (**3**) or (**4**) (Fig. 8B; *t*-test, confidence level 95%). Only modest differences in the K_m^{app} were observed in the presence of either alkamide compound (Fig. 8). The catalytic efficiency (k_{cat}/K_m) of the FAAH protein increased by a factor of ~2 in presence of either (**3**) or (**4**) indicating that the increase of the enzyme activity in presence of these two alkamides appeared to occur through an increase in turnover rate of the recombinant FAAH enzyme. Similar enzymatic assays were performed with free amino alkyl head groups alone (ethylamine, propylamine, isopropylamine and

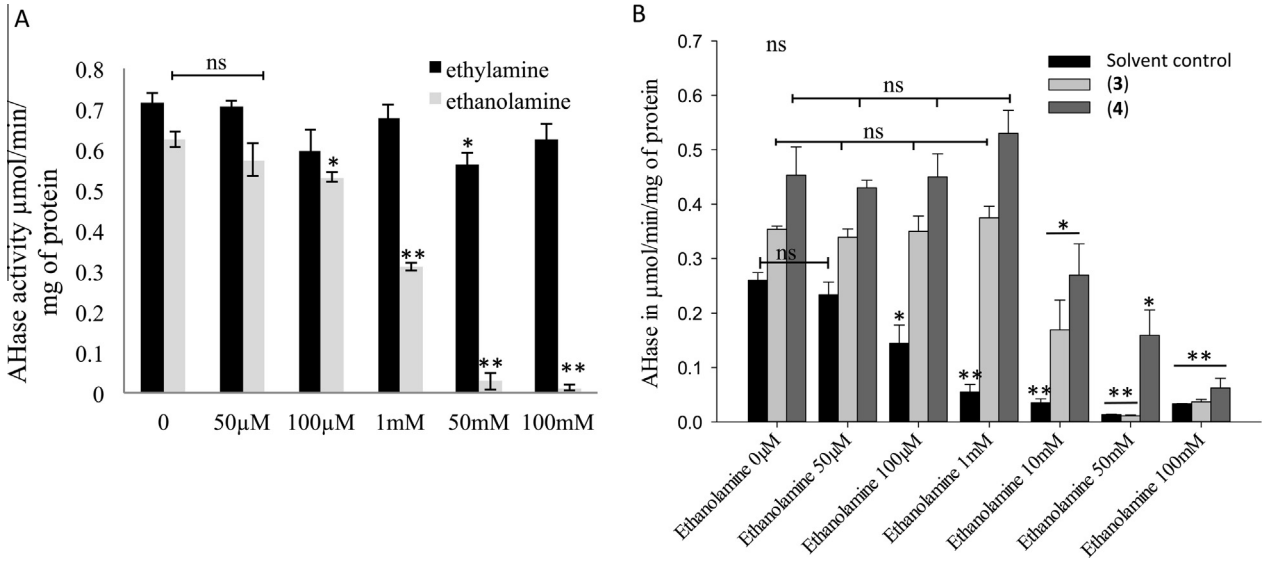

Fig. 9. Effect of ethanolamine or ethylamine on FAAH activity and protection from inhibition by 100 µM of *N*-isopropyllauroylamide (**3**) or *N*-ethyllauroylamide (**4**). Reactions were initiated by the addition of purified FAAH protein (0.3 µg) in 50 mM Bis-Tris propane–HCl (pH 9.0), at 30 °C for 30 min, with shaking (120 rpm), and 100 µM of [1-^{14}C]NAE 12:0 in a final volume of 0.15 ml. (A) With different concentrations of ethylamine or ethanolamine. (B) Plus 100 µM of (**3**) or (**4**). Solvent control corresponds to 0.01% (v/v) of DMSO. Data points represent means ± S.D. of triplicate assays. Plots were generated with SigmaPlot software version 12.0. *p*-Value of <0.05 and <0.03 are indicated by * and **, respectively, as determined by Student's *t* test. ns, not significant.

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isobutylamine), with no effect on FAAH activity being observed. Further, gel filtration separations conducted in the presence of the synthetic alkamides showed no influence of the alkamides on the oligomerization state of FAAH (Supplemental Fig. S2).

Recently, two *N*-phenoxyacylethanolamines were described that exhibited similar enhancement effects toward both rat and Arabidopsis FAAH, which appeared to be through a relief from product inhibition by free ethanolamine (Faure et al., 2014). Here these alkamide compounds were tested to establish if they might have a similar function. There was a dose-dependent inhibition in the AHase activity of FAAH at increasing ethanolamine concentrations (Fig. 9A). By sharp contrast, there was no reduction in hydrolase activity in presence of ethylamine (Fig. 9A), a molecule structurally similar to ethanolamine but lacking the hydroxyl group. Both (**3**) and (**4**) relieved ethanolamine inhibition up to a concentration of about 1 mM ethanolamine and increased FAAH activity up to 10 and 50 mM ethanolamine for (**3**) and (**4**), respectively (Fig. 9B). No changes in solution pH were observed (pH 9) up to 1 mM ethylamine or ethanolamine. While the pH of the reactions did rise to 10 at 100 mM ethanolamine, this was not appreciably higher than the pH optimum of 9 for the Arabidopsis FAAH enzyme (Shrestha et al., 2003). Because inhibition of FAAH was substantial at 1 mM ethanolamine (at pH 9), and because both (**3**) and (**4**) relieved this inhibition, it was concluded that enhancement of FAAH activity by these alkamides is mediated by a protection from product inhibition, and not through a pH effect on the enzyme.

2.5. Comparison of effects of the synthetic alkamides, NAE 12:0 and affinin on plant growth

Quantitative measurements of seedling growth (cotyledon expansion and primary root length) were recorded in the presence of these different alkamides (Figs. 10–12). Overall, cotyledon area was reduced in seedlings grown in two different concentrations of the synthetic alkamides (20 and 50 μM, Fig. 10). Within the *N*-12:0 series (**1**–**4**), three compounds, (**2**), (**3**), and (**4**), showed greater growth-inhibition in *faah1*-knockouts and less inhibition in *FAAH1*-overexpressing seedlings compared to wild-type (WT, Col(0); Fig. 10), suggesting that these alkamide compounds may be partially metabolized by the endogenous FAAH protein *in vivo*. WT seedlings treated with two different concentrations of affinin (20 and 50 μM) also showed significant reduction in cotyledon

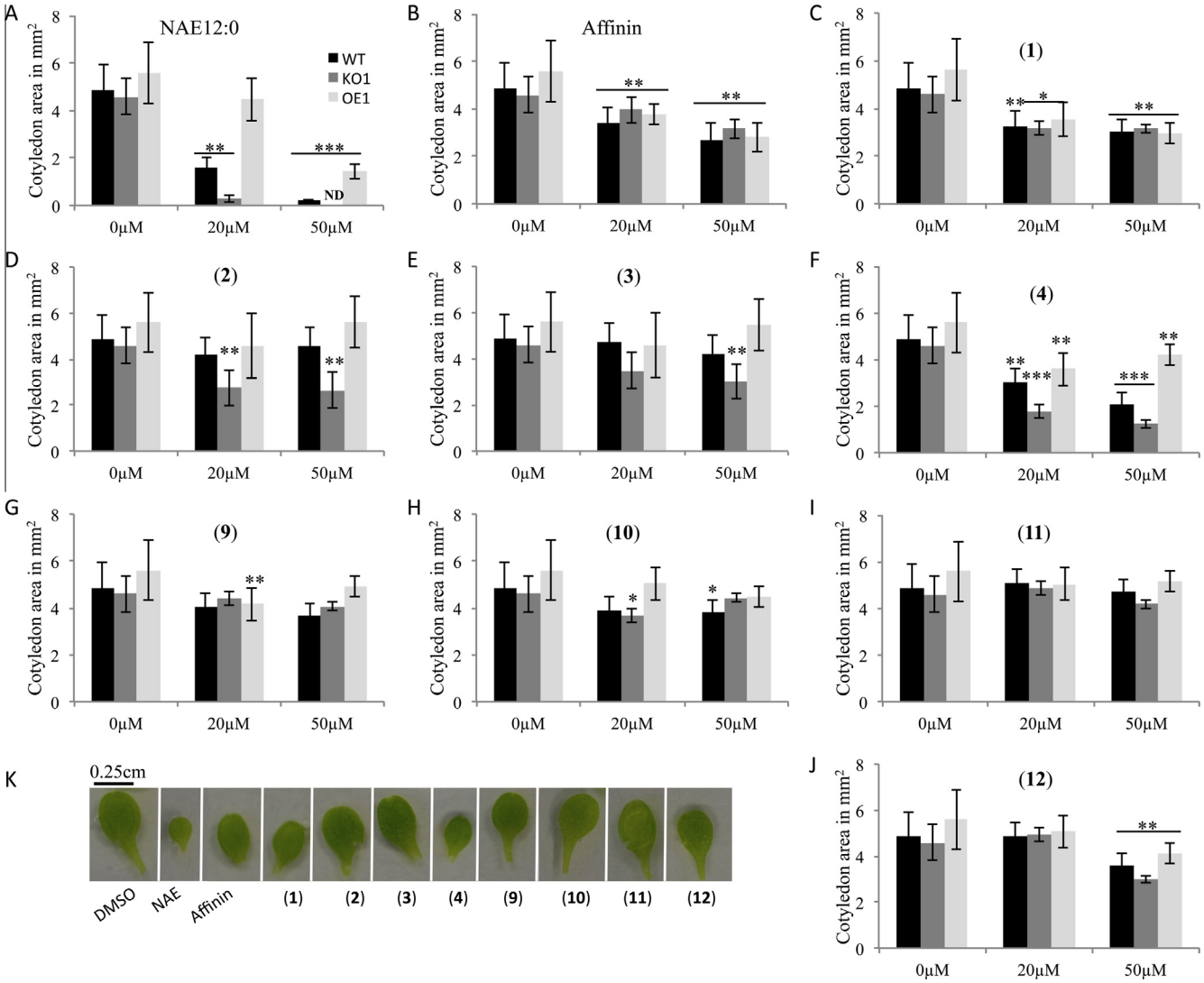

Fig. 10. NAE sensitivity of Arabidopsis seedlings in presence of different concentrations of new synthetic alkamides (**1**–**12**), NAE 12:0 or affinin. (A–J) Cotyledon size of 10 day old Arabidopsis seedlings treated with increasing concentrations of NAE 12:0 or different alkamides (WT, KO1, OE1, black, dark gray, light gray bars, respectively). (K) Representative images of 10 day old WT cotyledon seedlings treated with 50 μM of different alkamides, NAE 12:0 or affinin. The area of each cotyledon was measured with the software ImageJ. *p*-Value of <0.05,<0.03,<0.001 are indicated by *, **, ***, respectively, as determined by Student's *t* test. ND, not determined. WT, wild type (Col-0); KO1, *faah1* KO; or OE1, *FAAH1* over-expressor.

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development compared to untreated seedlings, but FAAH appeared to have no influence on growth inhibition by this naturally occurring alkamide (Fig. 10), consistent with activity assays *in vitro*. These results suggest that neither affinin nor *N*-isobutyllauroylamide (**1**) are likely to be hydrolyzed by Arabidopsis FAAH *in vivo*.

Compared to the *N*-12:0 series (**1**–**4**), most of the *N*-18:2-alkamide compounds (**9**–**12**) were not as effective at inhibiting seedling growth (Fig. 10G–J). Only the *N*-ethyllinoleoylamide (**12**) at a concentration of 50 μM showed some statistically significant inhibition of cotyledon expansion particularly in the *faah1* knockouts (Fig. 10). Independent of plant genotype, growth inhibition (root or cotyledon) was not observed for the *N*-16:0-series of alkamide compounds (**5**–**8**) (data not shown). There were only modest (*t*-test, confidence level 95%), or in some cases no, inhibitory effects of these synthetic alkamides on seedling root length for the *N*-12 series (**1**–**4**) (Fig. 11) or the *N*-18:2 series (**9**–**12**) (Fig. 12) in comparison to the notable growth inhibition by NAE 12:0 (Blancaflor et al., 2003; Wang et al., 2006) or affinin (Ramírez-Chávez et al., 2004). However, addition of NAE 12:0 (30 μM) with any of the *N*-12:0 or *N*-18:2-alkamides compounds (**1**–**4**; **9**–**12**) (30 μM) increased the severity of the growth inhibition (cotyledons and primary root) indicating a cumulative inhibition effect of these alkamide compounds in plant development (data not shown).

3. Discussion

The new synthetic alkamides used here are hybrid molecules, sharing some structural similarities with NAEs (Blancaflor et al., 2014) and also with the naturally occurring alkamides (Ramírez-Chávez et al., 2004) (Fig. 1). Because of the broad substrate selectivity of FAAH, it was not surprising that the different synthetic alkamides functioned as substrates for Arabidopsis FAAH (Fig. 3). Hydrolysis of the alkamides visualized by primuline on TLC plates was not intended to be a quantitative assay, but demonstrated that these alkamides could be converted to free fatty acids, and this was further confirmed by GC–MS (Fig. 4 and Supplemental Fig. S1). It appeared that synthetic alkamides with an α- or β-methylation (isopropyl and isobutyl) were not hydrolyzed as well as the compounds with linear alkyl "head" groups (ethyl and propyl) (Supplemental Fig. S1). Interestingly, these same α- or β-methylated compounds also were resistant to hydrolysis by rat FAAH (data not shown) consistent with previous reports with branched analogs of *N*-palmitoylethanolamine or oleamide (Boger et al., 2000; Vandevoorde et al., 2003).

Perhaps most intriguing was the increase of FAAH activity toward NAEs that was observed in the presence of (**3**) or (**4**) (Figs. 5–8). These synthetic alkamides were rather poor substrates for FAAH themselves, but it was unexpected that they would

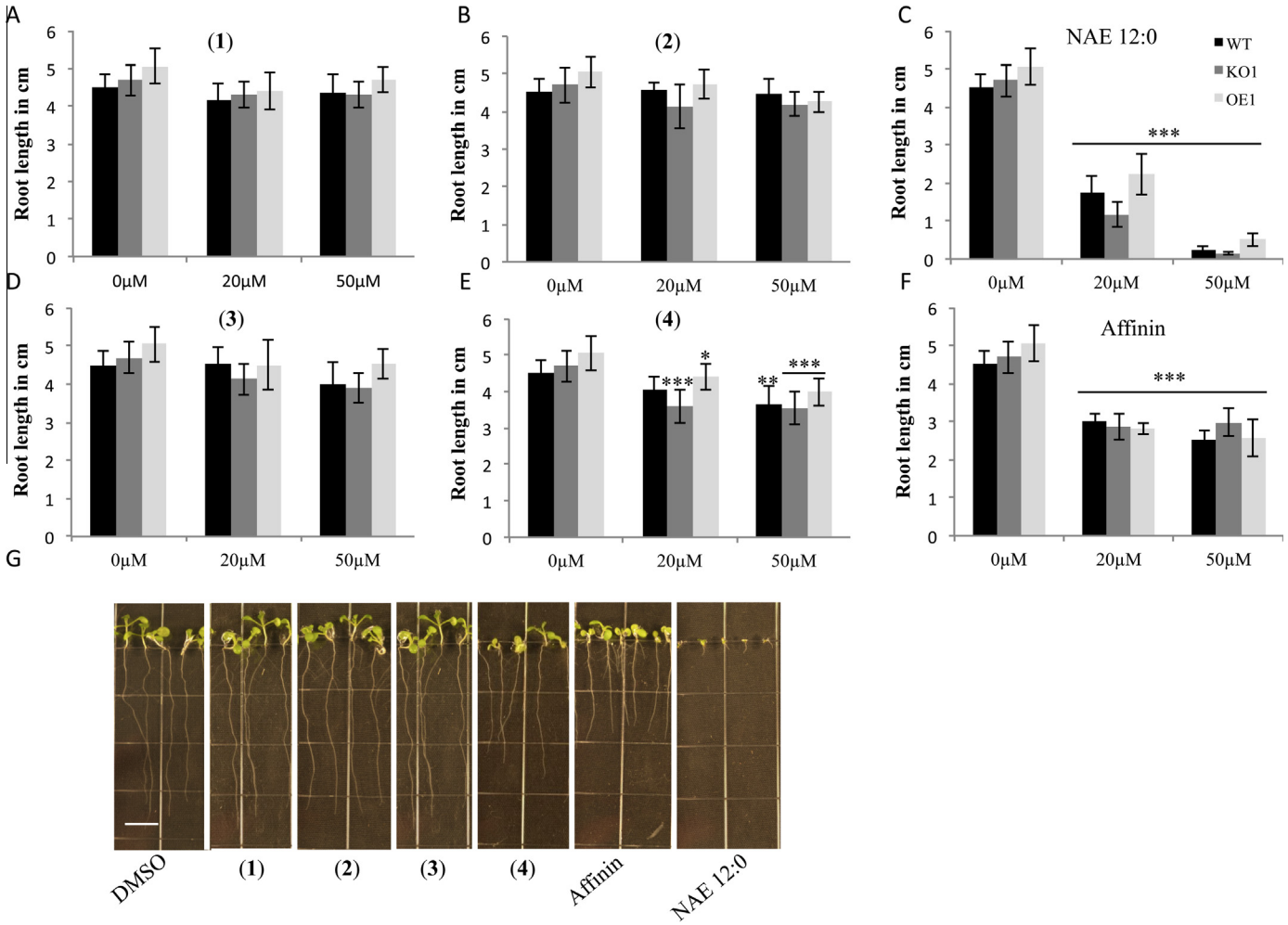

Fig. 11. Effect of different concentrations of the *N*-12:0-amide series (**1**–**4**), NAE 12:0 or affinin on the primary root length. (A–F) Root lengths of seedlings treated with increasing concentration of NAE 12:0 or different alkamides (WT, KO1, OE1, black, dark gray, light gray bars, respectively). (G) Representative images of 10 days WT seedlings treated with 50 μM of different alkamides or NAE 12:0. The length of each primary root was measured with the software ImageJ. *p*-Value of <0.05, <0.005, <0.001 are indicated by *, **, ***, respectively, as determined by Student's *t* test. WT, wild type (Col-0); KO1, *faah1* KO; or OE1, *FAAH1* over-expressor. Scale bar 1 cm.

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enhance significantly the FAAH activity toward NAEs (Figs. 5–7). Both compounds appeared to increase the V_{max} and turnover rate of the enzyme (Fig. 8). The enhanced NAE hydrolase activity was explained by the observation that the alkamides provided protection from product inhibition by free ethanolamine (Fig. 9), as opposed to an influence by these compounds on the newly-revealed oligomeric state of Arabidopsis FAAH (Fig. 2, Supplemental Fig. S2). The product inhibition by ethanolamine was recently identified in studies where *N*-phenoxyacylethanolamines showed a similar enhancement effect on both Arabidopsis and rat FAAH (Faure et al., 2014). The structures of the alkamides synthesized here were quite different from the *N*-phenoxyacylethanolamines (*i.e.*, NAE containing a phenol group in acyl chain (Faure et al., 2014); so the similar effect on FAAH was still somewhat surprising. One difference was that neither (**3**) nor (**4**) increased the activity of purified rat FAAH (data not shown), suggesting a specific feature of Arabidopsis FAAH protein toward these new hybrid alkamides. The precise mechanism by which (**3**) and (**4**) enhance FAAH activity will require additional structural insights. These alkamides may bind to a specific site on the FAAH protein and block binding of ethanolamine directly. Or, they may act to alter the overall conformation of FAAH so as to prevent this ethanolamine feedback inhibition. It is also possible that these compounds could alter substrate availability as a general detergent effect. Nonetheless, the protection from feedback regulation of FAAH by these compounds has revealed a previously unappreciated property of FAAH with respect to its inhibition by ethanolamine. The specificity of this inhibition becomes evident by the comparison with ethylamine, lacking the OH group, which had no effect on FAAH activity (Fig. 9). This negative feedback regulation suggests that free ethanolamine in tissues may influence the NAE signaling pathway in plant systems. It is worth noting that unlike animal systems, ethanolamine is formed from serine decarboxylation in plant tissues, and free ethanolamine levels in Arabidopsis leaf tissues are reported to be about 250 nmol/g fresh weight (Rontein et al., 2003). Still, it remains to be determined whether free ethanolamine is an important regulatory feature of NAE metabolism *in planta*.

The naturally occurring affinin was not hydrolyzed appreciably by FAAH under conditions used here (Fig. 3 and Supplemental Fig. S1). Moreover, inhibition of NAE 12:0 hydrolysis could not be observed when 100 μM of affinin was added in the presence of purified FAAH protein (Fig. 5M). This suggests that affinin may be metabolized by enzymes other than FAAH *in planta*, or that it may be difficult to identify conditions for hydrolysis of affinin by FAAH *in vitro*. *N*-decanoyl-homoserine lactone (C10-HL), a quorum-sensing molecule produced by bacteria, was shown to be a substrate for FAAH, and acts similarly to affinin in plant tissues (Ortíz-Castro et al., 2008). Growth assays in the presence of 50 μM of exogenous affinin to the plant media showed an overall decrease in cotyledon size and root elongation (Ramírez-Chávez et al., 2004). However, unlike C10-HL (Ortíz-Castro et al., 2008), no tolerance or sensitivity toward affinin was observed with *FAAH* overexpressing- or *faah1* knockout-seedlings, respectively (Figs. 10 and 11). It may be that additional metabolic pathways other than FAAH are involved in the metabolism of alkamides in plants, indicating the need for further studies in this area. Notably, affinin showed a trend towards increasing FAAH's capacity to metabolize NAE 12:0 when added at equimolar concentrations, although this increase was not significant at $p < 0.05$ (Fig. 5). However, affinin's structural similarity to (**1**), suggests that affinin might influence FAAH activity directly in plants, but this will also require further investigation.

Because of the structural similarities to both NAEs and naturally-occurring alkamides, it was anticipated that the new synthetic compounds reported here would negatively influence seedling growth in a manner similar to the parent compounds. Further, Arabidopsis plants were used with either a T-DNA disruption in FAAH (*faah* knockout) or overexpressing *FAAH* to test whether sensitivities to the synthetic alkamides would suggest their

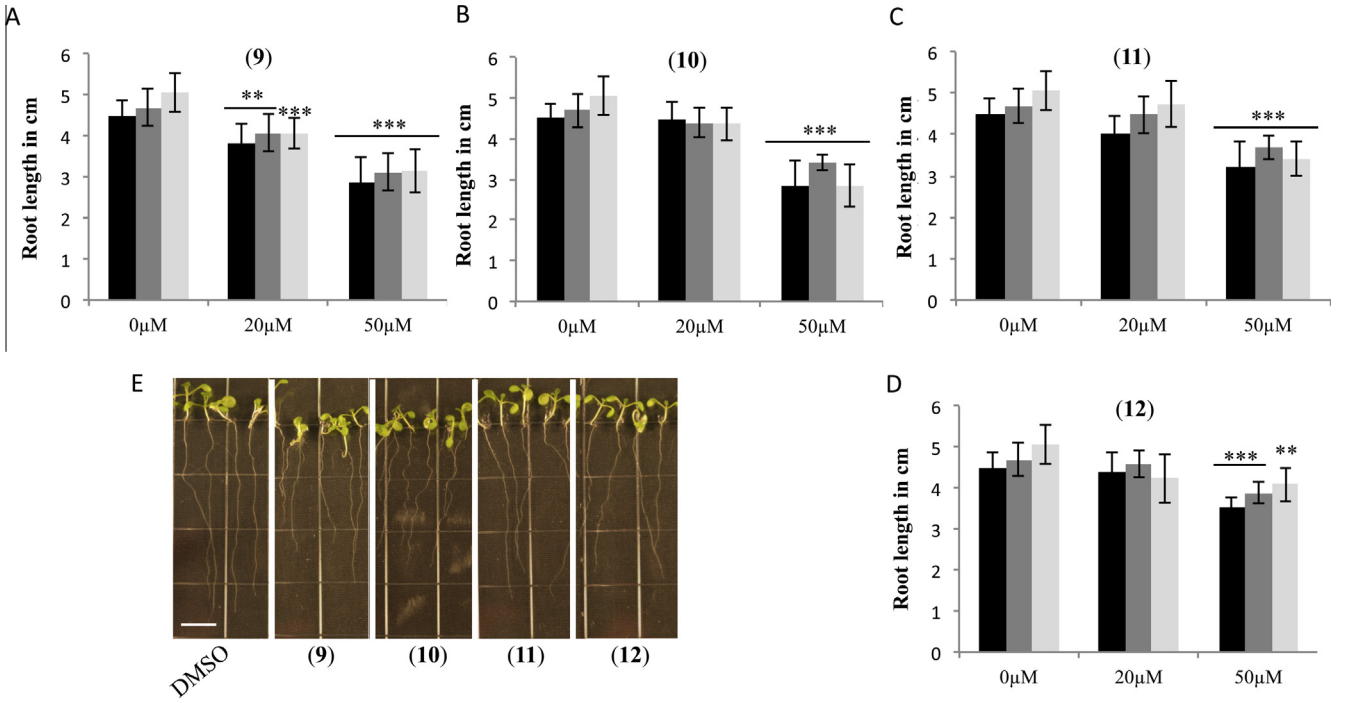

Fig. 12. Effect of different concentrations of the *N*-18:2-alkamide series (**9**–**12**) on the primary root length. (A–D) Root lengths of seedlings treated with increasing concentration of the different *N*-18:2-amide compounds (WT, KO1, OE1, black, dark gray, light gray bars, respectively). (E) Representative images of 10 days WT seedlings treated with 50 μM of different *N*-18:2-amide series compounds. The length of each primary root was measured with the software ImageJ. *p*-Value of <0.05,<0.005,<0.001 are indicated by *, **, ***, respectively, as determined by Student's *t* test. WT, wild type (Col-0); KO1, *faah1* KO; or OE1, *FAAH1* over-expressor. Scale bar 1 cm.

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hydrolysis by FAAH *in planta* in these different genotypes, similar to NAEs (Wang et al., 2006). Indeed, the synthetic alkamide compounds inhibited plant growth development in a manner similar to NAE 12:0 treatment (Blancaflor et al., 2003), although the effects of the alkamides were not as pronounced as NAE 12:0 (Figs. 10–12). It is presumed that endogenous FAAH could mediate the tolerance of seedlings to several of these alkamides. For example, *faah1*-KO seedlings treated with micromolar amounts (20–50 μM) of the *N*-12:0 alkamides displayed a more severe inhibition of growth with respect to both root length and cotyledon area when compared to WT, an effect that was not seen for (**1**); whereas, the *FAAH*-OE seedlings showed a better tolerance for this series of compounds (Figs. 10–12). Consistent with their structural similarities, (**1**) showed growth inhibition effects very similar to affinin on seedling development (Ramírez-Chávez et al., 2004), and these inhibitory effects were independent of the FAAH transcript or enzyme activity levels (Fig. 11). It will be interesting to test this new synthetic alkamide with mutants like *drr*1 or *dhm*1 that are tolerant or hypersensitive to affinin and decanamide (Morquecho-Contreras et al., 2010; Pelagio-Flores et al., 2013) or to determine if (**1**) will influence cytokinin or jasmonate signaling, pathways that interact with natural alkamides (López-Bucio et al., 2007; Méndez-Bravo et al., 2011) but not with NAEs.

3.1. Concluding remarks

Recently, additional, naturally-occurring alkamides have been identified in extracts of *Lepidium* and *Heliopsis* species with effects on the cellular uptake and/or the hydrolysis of anandamide by FAAH, continuing to point to cross-over between plant alkamides and endocannabinoid signaling in mammals (Hajdu et al., 2014). It will be of interest to test the effects of these new potent alkamides like *N*-benzyl-(9*Z*,12*Z*)octadecadienamide on plant development, perhaps with the different FAAH mutants. Taken together the synthetic alkamides described here have several unusual properties in plant systems: some, like (**1**) act similarly to natural alkamides, others, like (**4**) or (**12**) act in a manner more similar to NAEs and appear to be hydrolyzed by FAAH *in planta*, others, like the *N*-16:0 series (**5**–**8**) have very little activity *in vitro* or *in vivo*, and still others, like (**3**) enhance FAAH activity *in vitro* by preventing feedback inhibition of ethanolamine. As such, these synthetic alkamides represent a range of potentially useful pharmacological tools for the further dissection of NAE and alkamide functions in plants, or in the biochemical characterization of the FAAH protein and represent potential novel compounds for regulating cellular physiology, growth and lipid signaling. Similarly, it may be of interest to evaluate the effects on endocannabinoid signaling in mammals of these synthetic "hybrid" alkamides.

4. Experimental

4.1. Materials

[1-^{14}C] Lauric acid was from Amersham Biosciences, [1-^{14}C] palmitic acid was purchased from NEN (New England Nuclear, Boston, MA), and [1-^{14}C] arachidonic acid was purchased from PerkinElmer Life Sciences. Ethanolamine, anandamide, isopropyl-β-D-thiogalactopyranoside (IPTG), Triton-X100, ethylamine, propylamine, isobutylamine, isopropylamine and acyl chlorides, primuline dye (yellow 59) were from Sigma–Aldrich Chemical Co. (St. Louis). *N*-dodecyl-β-D-maltoside (DDM) was from Calbiochem (LA Jolla, CA). Silica Gel G (60 Å)-coated glass plates for thin-layer chromatography (TLC, 10 × 20 cm or 20 × 20 cm, 0.25 mm thickness) were from Whatman (Clifton, NJ). Different *N*-[1-^{14}C] acylethanolamines (and non-radiolabeled NAEs) were synthesized from ethanolamine and corresponding [1-^{14}C] fatty acids (and non-radiolabeled FFAs) by first producing the fatty acid chloride (Hillard et al., 1995) and purifying by TLC as described elsewhere (Shrestha et al., 2002).

4.2. Synthesis of alkamides

Synthesis of compounds was performed using published methods (Vandevoorde et al., 2003). All solvents were purified and dried under standard methods. Organic extracts were dried using anhydrous $MgSO_4$. NMR spectra were recorded on a Varian 400 MHz (^{1}H) and 500 MHz (^{13}C) NMR spectrometers. Chemical shifts were recorded as δ values against tetramethylsilane as an internal standard.

Typical synthetic procedure: to a 2-neck dry round-bottom flask, dry CH_2Cl_2 (50 mL was added, followed by addition of corresponding acyl chloride (1 M equivalent)). The reaction vessel was cooled to 0 °C followed by drop-wise addition of 10 M equivalents of the corresponding amine. The reaction mixture was allowed stir at room temperature for 1 h. Extraction of synthetic alkamides was performed using two successive aqueous extractions (50 mL) of 5% Na_2CO_3, followed by 1 M HCl and brine. The organic phase was dried over $MgSO_4$. Evaporation of solvent provided a white solid product or a viscous yellow liquid depending on the fatty acyl series. High resolution mass spectra (HRMS) were obtained for each compound on a Thermo Scientific Hybrid MALDI-LTQ Orbitrap XL mass spectrometer in positive ion mode with 2,5-dihydroxybenzoic acid (DHB) as a MALDI matrix. Ions corresponding to $[M+H]^+$ and $[M+Na]^+$ were identified for each compound and observed masses differed from expected masses between 0.0001 and 0.0006. HRMS data for observed and calculated $[M+H]^+$ are provided below for each compound after NMR information.

4.2.1. *N*-(2-Methylpropyl)dodecanamide or *N*-isobutyllauroylamide (**1**)

White solid. 60.4% yield. ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 5.43 (bs, 1H, NH), 3.10 (*t*, 1H, –CH_2), 2.17 (*t*, 2H, –CH_2), 1.76 (mp, 2H, –CH_2), 1.63 (mp, 2H, –CH_2), 1.29–1.25 (overlapping signals, 16H, –CH_2), 0.92–0.90 (overlapping signals, 9H, –CH_3). HRMS: $[M+H]^+$ of *m*/*z* 256.2638; calculated mass: 256.2640 for $C_{16}H_{33}NO$.

4.2.2. *N*-Propyldodecanamide or *N*-propyllauroylamide (**2**)

White solid. 81.7% yield. ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 5.42 (bs, 1H, NH), 3.22 (*q*, 1H, –CH_2), 2.15 (*t*, 2H, –CH_2), 1.62 (mp, 2H, –CH_2), 1.52 (mp, 2H, –CH_2), 1.29–1.25 (overlapping signals, 16H, –CH_2), 0.94–0.86 (overlapping signals, 6H, –CH_3). HRMS: $[M+H]^+$ of *m*/*z* 242.2482; calculated mass: 242.2484 for $C_{15}H_{31}NO$.

4.2.3. *N*-(1-Methylethyl)dodecanamide or *N*-isopropyllauroylamide (**3**)

White solid. 64.1% yield. ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 5.227 (bs, 1H, NH), 4.091 (mp, 1H, –CH), 2.114 (*t*, 2H, –CH_2), 1.608 (mp, 2H, –CH_2), 1.277–1.252 (overlapping signals, 16H, –CH_2), 1.46 (*d*, 6H, –CH_3), 0.877 (mp, 3H, –CH_3). ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm): 172.6, 41.3, 37.2, 29.8–29.5 (6 overlapping signals), 26.1, 23.0, 22.9, 14.3. HRMS:$[M+H]^+$ of *m*/*z* 242.2481; calculated mass: 242.2484 for $C_{15}H_{31}NO$.

4.2.4. *N*-Ethyldodecanamide or *N*-ethlyllauroylamide (**4**)

White solid. 77.6% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.403 (bs, 1H, NH), 3.230 (mp, 2H, –CH_2), 2.137 (*t*, 2H, –CH_2), 1.613 (mp, 2H, –CH_2), 1.286–1.246 (overlapping signals, 16H, –CH_2), 1.30 (*d*, 3H, –CH_3), 0.873 (mp, 3H, –CH_3). ^{13}C NMR (in $CDCl_3$, 500 MHz) δ (ppm): 173.5, 37.1, 34.5, 32.1, 29.9–29.6 (6 overlapping signals), 26.1, 23.0, 15.1, 14.3. HRMS:$[M+H]^+$ of *m*/*z* 228.2324; calculated mass: 228.2327 for $C_{14}H_{29}NO$.

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*4.2.5. N-(2-Methylpropyl)hexadecanamide or N-isobutylpalmitoylamide **(5)***

White solid. 91.4% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.453 (bs, 1H, NH), 3.076 (mp, 2H, $-CH_2$), 2.162 (*t*, 2H, $-CH_2$), 1.753 (mp, 1H, –CH), 1.637 (mp, 2H, $-CH_2$), 1.246 (overlapping signals, 24H, $-CH_2$), 0.951–0.857 (overlapping signals, 9H, $-CH_3$). ^{13}C NMR (in $CDCl_3$, 500 MHz) δ (ppm): 173.5, 47.1, 37.2, 32.2, 29.9–29.6 (9 overlapping signals), 28.8, 26.2, 23.0, 20.4, 14.4. HRMS: $[M+H]^+$ of *m/z* 312.3263; calculated mass: 312.3266 for $C_{20}H_{41}NO$.

*4.2.6. N-Propylhexadecanamide or N-propylpalmitoylamide **(6)***

White solid. 82.2% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.42 (bs, 1H, NH), 3.20 (mp, 2H, $-CH_2$), 2.15 (*t*, 2H, $-CH_2$), 1.62 (mp, 2H, $-CH_2$), 1.50 (mp, 2H, $-CH_2$), 1.28–1.25 (overlapping signals, 24H, $-CH_2$), 0.94–0.86 (overlapping signals, 9H, $-CH_3$). HRMS: $[M+H]^+$ of *m/z* 298.3107; calculated mass: 298.3110 for $C_{19}H_{39}NO$.

*4.2.7. N-(1-Methylethyl)hexadecanamide or N-isopropylpalmitoylamide **(7)***

White solid. 62.2% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.20 (bs, 1H, NH), 4.09 (mp, 1H, –CH), 2.11 (*t*, 2H, $-CH_2$), 1.61 (mp, 2H, $-CH_2$), 1.28–1.25 (overlapping signals, 24H, $-CH_2$), 1.15 (*d*, 6H, $-CH_3$), 0.88 (mp, 3H, $-CH_3$). HRMS: $[M+H]^+$ of *m/z* 298.3106; calculated mass: 298.3110 for $C_{19}H_{39}NO$.

*4.2.8. N-Ethylhexadecanamide or N-ethylpalmitoylamide **(8)***

White solid. 43.5% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.39 (bs, 1H, NH), 3.29 (mp, 2H, $-CH_2$), 2.14 (*t*, 2H, $-CH_2$), 1.61 (mp, 2H, $-CH_2$), 1.28–1.25 (overlapping signals, 24H, $-CH_2$), 1.13 (*t*, 3H, $-CH_3$), 0.88 (*t*, 3H, $-CH_3$). HRMS: $[M+H]^+$ of *m/z* 284.2949; calculated mass: 284.2953 for $C_{18}H_{37}NO$.

*4.2.9. N-(2-Methylproyl)-(9Z,12Z)octadecadienamide or N-isobutyllinoleamide **(9)***

Yellow oil. 78.1% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.40–5.28 (overlapping signals, 5H, NH and C=C—H), 3.08 (*t*, 2H, $-CH_2$), 2.77 (*t*, 2H, $-CH_2$), 2.17 (*t*, 2H, $-CH_2$), 2.06–2.04 (overlapping signals, 4H, $-CH_2$), 1.76 (mp, 1H, –CH), 1.63 (mp, 2H, $-CH_2$), 1.35–1.31 (overlapping signals, 14H, $-CH_2$), 0.92–0.90 (overlapping signals, 9H, $-CH_3$). HRMS: $[M+H]^+$ of *m/z* 336.3262; calculated mass:336.3266 for $C_{22}H_{41}NO$.

*4.2.10. N-Propyl-(9Z,12Z)octadecadienamide or N-propyllinoleamide **(10)***

Yellow oil. 73.9% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.52 (bs, 1H, NH), 5.40–5.28 (overlapping signals, 4H, C=C—H) 3.19 (mp, 2H, $-CH_2$), 2.75 (*t*, 2H, $-CH_2$), 2.14 (*t*, 2H, $-CH_2$), 2.02 (overlapping signals, 4H, $-CH_2$), 1.61 (mp, 2H, $-CH_2$), 1.50 (mp, 2H, $-CH_2$), 1.35–1.29 (overlapping signals, 14) $[M+H]^+$ of *m/z* 322.3107; calculated mass: 322.3110 for $C_{21}H_{39}NO$.

*4.2.11. N-(1-Methylethyl)-(9Z,12Z)octadecadienamide or N-isopropyllinoleamide **(11)***

Yellow oil. 80.1% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.40–5.28 (overlapping signals, 5H, NH and C=C—H), 4.06 (mp, 1H, –CH), 2.75 (*t*, 2H, $-CH_2$), 2.11 (*t*, 2H, $-CH_2$), 2.04 (overlapping signals, 4H, $-CH_2$), 1.60 (mp, 2H, $-CH_2$), 1.35–1.29 (overlapping signals, 14H, $-CH_2$), 1.14–1.12 (*d*, 6H, $-CH_3$), 0.87 (*t*, 3H, $-CH_3$). HRMS: $[M+H]^+$ of *m/z* 322.3104; calculated mass: 322.3110 for $C_{21}H_{39}NO$.

*4.2.12. N-Ethyl-(9Z,12Z)octadecadienamide or N-ethyllinoleamide **(12)***

Yellow oil. 59.5% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): Yellow oil. 73.9% yield. ^{1}H NMR (in $CDCl_3$, 400 MHz), δ (ppm): 5.53 (bs, 1H, NH), 5.40–5.28 (overlapping signals, 4H, C=C—H) 3.27 (mp, 2H, $-CH_2$), 2.75 (*t*, 2H, $-CH_2$), 2.13 (*t*, 2H, $-CH_2$), 2.02 (overlapping signals, 4H, $-CH_2$), 1.61 (mp, 2H, $-CH_2$), 1.35–1.29 (overlapping signals, 14H, $-CH_2$), 1.12 (*t*, 3H, $-CH_3$), 0.87 (*t*, 3H, $-CH_3$). HRMS: $[M+H]^+$ of *m/z* 308.2949; calculated mass: 308.2953 for $C_{20}H_{37}NO$.

4.3. Plant materials and growth

Ten mg of different *Arabidopsis thaliana* seeds (*faah1*-KO, *FAAH*-OE, Col(0); Wang et al., 2006) were surface sterilized and then stratified in the dark for 2 days at 4 °C prior to sowing in solid MS (Murashige and Skoog) medium containing different concentrations of NAE 12:0 or alkamides (Teaster et al., 2007). Growth of seedlings was in 16 h-light/8 h-dark cycle (60 μmol m^{-2} s^{-1}) for 11 days at 20 °C. Images of primary roots and cotyledons were captured using a Nikon DX camera. Root lengths and cotyledon areas were measured using ImageJ software (1.4.3.67 version). Statistical tests (Student's *t*-test) were conducted by using the Microsoft Excel 2010 software.

4.4. Protein expression and purification

The recombinant plasmid FAAH1-pTrcHis2 (At5g64440, Uniprot # Q7XJJ7) was constructed as described in Shrestha et al. (2003). Arabidopsis-FAAH proteins (FAAH) were expressed and purified in a 50 mM Bis-Tris propane–HCl (pH 9), Triton X-100 (1% v/v) using an a QiQexpress® NI–NTA Fast Start (Qiagen®) column as described elsewhere (Faure et al., 2014; Kim et al., 2013).

An FPLC® system (Amersham Pharmacia Biotech) was used for all gel filtration chromatography separations. The purified protein from the Ni-column was concentrated by filtration–centrifugation using a Centricon YM-30 (Millipore, Bedford, MA) device to a final volume of 200 μl and then loaded onto a Superdex 200 gel filtration column (GE health care life sciences). The column was equilibrated with 50 mM Bis-Tris propane–HCl, (pH 9), 0.1 M NaCl, 0.2 mM DDM and the eluted proteins were monitored by UV absorbance at 280 nm. Fractions were collected and assayed for enzyme activity and analyzed by SDS–PAGE and Western blotting to confirm the location of the eluted FAAH protein (Faure et al., 2014). Molecular mass calibration of the column was done using blue dextran (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa).

4.5. FAAH assays

The NAE amidohydrolase assays were conducted as previously described (Kim et al., 2013) with few modifications. The reactions were conducted for 30 min at 30 °C, in BTP buffer (150 μl, 50 mM Bis-Tris propane–HCl, pH 9.0), with different concentrations of cold or radiolabelled NAEs, and different concentrations of alkamides at varying concentrations of purified protein (see legend of Figs. for details in reaction compositions). The reactions were terminated by adding hot *i*PrOH (2 ml, 70 °C). The lipids were extracted and the distribution of lipids was evaluated by radiometric scanning of TLC plates as described (Shrestha et al., 2006). For the non-radioactive assays, lipids were located by UV of the TLC plate after spraying a solution of primuline as described (Testet et al., 2005) or they were analyzed by GC–MS. Extracted lipid after enzymatic reactions were evaporated under N_2, and derivatized with BSTFA (50 μl, Fisher Scientific, Houston, TX, USA) for 30 min at 55 °C. TMS-ether derivatives were dried under N_2 and suspended in hexane 50 μl) for injection. Lipid analyses were performed using a GC–MS model Agilent GC 7890A/MSD 5975C system and a capillary HP-5 MS column (30 m × 0.250 mm, 0.25-mm coating thickness; Agilent Technologies) in full mass scan mode as previously described (Keereetaweep et al., 2013). Identification of the FFAs or alkamides was based on identification of characteristic

molecular and fragment ions in comparison with known standards. To test ethanolamine inhibition, assays containing purified FAAH protein (0.3 μg) were first incubated with 100 μM of the different alkamides and then with different concentrations of ethanolamine or ethylamine (0–100 mM) as described (Faure et al., 2014). Assays were conducted in the presence of detergent to facilitate solubility of lipophilic compounds for enzyme assays (Shrestha et al., 2003).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.11.011.

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