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N-acylethanolamines in seeds of selected legumes

Barney J. Venables *, Cheryl A. Waggoner, Kent D. Chapman

Department of Biological Sciences, University of North Texas, P.O. Box 310559, Denton, TX 76203, USA

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Abstract

Seven molecular species of *N*-acylethanolamines were quantified in seeds from selected members of the legume family. Total concentrations for the 14 taxa studied ranged over approximately three orders of magnitude with no consistent overall relationship to phylogeny. Elevated concentrations observed in some species make them good candidates for natural sources of these compounds which are of increasing therapeutic interest in the modulation of the mammalian endocannabinoid system.

Keywords: Fabaceae; Legumaceae; Legume; *N*-acylethanolamine; Endocannabinoid

1. Introduction

N-acylethanolamines (NAEs) have been recognized as minor components of plant lipids with anti-inflammatory activity in mammals for almost 50 years. The various biological roles of this family of compounds began to be explored in more detail with the discovery that several NAEs and related lipids accumulated in damaged tissues (Schmid et al., 1990) and that anandamide (*N*-arachidonylethanolamine; AEA) is an endogenous ligand for the cannabinoid (CB) receptor in mammalian neurons (Di Marzo, 1998). The resulting research has demonstrated that NAEs have fundamental roles in defense signaling in plants (Chapman, 2004), immune responses in both invertebrates and vertebrates (Berdyshev, 2000; Salzet et al., 2000; Klein et al., 2003; Walter and Stella, 2004) and mammalian neuromodulation (Di Marzo, 1998; Freund et al., 2003). The potent biological activity of NAEs has led to exploration of therapeutic applications including anti-inflammatory and anti-tumor activities (De Petrocellis et al., 2000; Croxford, 2003).

As interest in the biological activity and possible therapeutic applications of NAEs has grown, our laboratory has begun to search for enriched natural sources of these compounds. Earlier we found that several seed types had NAE concentrations much higher than usually found in vegetative plant tissues or normal animal tissues (Chapman et al., 1999). Seeds examined in this study had total NAE concentrations in the μg/g range. Subsequently we identified a cottonseed refining fraction with enriched NAE content and demonstrated that principal NAE species found in these plant sources [ethanolamides of palmitic (16:0) and linoleic (18:2) acids] exhibited neuromodulatory activity at micromolar concentrations in murine neuronal networks that were cultured and evaluated on microelectrode arrays (Chapman et al., 2003). Here we expand on this earlier work by examining seeds of selected species of the legume family for total NAE content and relative proportions of individual NAE types. Legumes are the third largest flowering plant family with more than 700 genera and 20,000 species. They exhibit tremendous variety in morphological and ecological characteristics and the

* Corresponding author. Tel.: +1 940 369 7708; fax: +1 940 565 4297.
E-mail address: venables@unt.edu (B.J. Venables).

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phylogenetic relationships among the various lineages have been updated based on recent molecular analyses (Doyle and Luckow, 2003). We selected 14 taxa representing 12 species and 10 genera distributed among 4 major sub-families (Table 1).

2. Results and discussion

Total NAE concentrations in the seeds selected for this study were extremely variable ranging over approximately 3 orders of magnitude on a fresh weight basis and 2 orders of magnitude on an extracted lipid weight basis (Table 2). Extracted lipid weight ranged widely from less than 1% (w/w) for *P. sativum* to more than 40% for *A. hypogaea*. The *Pisum, Phasaelus* and *Vigna* genera accounted for most of the low lipid content taxa represented and these genera also had modest total NAE content on a fresh weight basis. The most primitive taxa from the Cercidae, Caesalpinia and Mimosoideae sub-families had consistently low total NAE content on both a fresh and extracted lipid weight basis, even though their extracted lipid mass was in the middle of the observed range (about 4–10%). The highest NAE content taxa were all among the Papilionoideae sub-family. Within this group, unusually high NAE content was seen in *A. hypogaea*, which also had the highest total lipid content and *G. max*, with the second highest total lipid content. The genus *Medicago* was represented by 2 species, *sativa* (cv. 7107) and *truncatula* (cv. A17 and cv. Jemalong). The three *Medicago* taxa had similar total extracted lipid content (10–12% w/w), but the *M. truncatula* cv. Jemalong had the highest NAE content observed in this study (or in any of our previous work). Jemalong had total NAE concentrations (fresh weight as well as lipid weight) about an order of magnitude higher than the con-specific

Table 1
Legumes selected for seed survey for NAEs

Sub-family	Clade/sub-grouping	Genus and species	Common Name	Cultivar
Cercideae		*Bauhinia congesta* (Britton & Rose) Lundell	Anacacho Orchid tree	
Caesalpinieae		*Caesalpinia gilliesii* (Hooker) Bentham	Bird of Paradise tree	
Mimosoideae		*Mimosa borealis* Gray	Fragrant Mimosa	
Papilionoideae	Genistoids	*Lupinus succulentus* Linnaeus	Arroyo Lupine	
		Lupinus texensis Hooker	Texas Bluebonnet	
	Aeschynomenoid/dalbergioids	*Arachis hypogaea* Linnaeus	Peanut	
	IRLC "inverted repeat-loss clade" (Wojciechowski et al., 2000)	*Medicago sativa* Linnaeus	Alfalfa	7101
		Medicago truncatula Gaertner	Barrelclover	A17
				Jemalong
		Pisum sativum Linnaeus	Garden Pea	Early Alaska
				Taos
		Phaseolus vulgaris Linnaeus	Kidney bean	Amarillo del Norte
		Vigna unguiculata Linnaeus	Black-eyed Pea	Tohono O'odham
		Glycine max Merrill	Soybean	Dare

Groupings taken from Doyle and Luckow (2003).

Table 2
Total lipid and NAE content observed for the selected taxa

Taxa	Replicate analyses (*N*)	Extracted lipid content (% w/w)	Total NAE content μg/g; mean (s.d.)	
			Fresh wt. basis	Extracted lipid wt. basis
Bauhinia congesta	5	10	0.306 (0.034)	3.09 (0.52)
Caesalpinia gilliesii	4	9	0.342 (0.084)	3.70 (0.99)
Mimosa borealis	4	4	0.845 (0.156)	20.5 (4.12)
Lupinus succulentus	5	5	1.52 (0.094)	28.5 (1.85)
Lupinus texensis	5	3	1.52 (0.177)	73.6 (26.4)
Arachis hypogaea	5	45	17.1 (0.568)	39.5 (1.33)
Medicago sativa cv. 7101	5	11	7.43 (0.280)	68.0 (5.10)
Medicago truncatula cv. A17	5	11	4.29 (0.35)	40.0 (4.20)
Medicago truncatula cv. Jemalong	5	12	44.6 (2.81)	350 (26.8)
Pisum sativum cv. Early Alaska	5	2.4	3.44 (0.212)	144 (3.37)
Pisum sativum cv. Taos	5	0.5	0.580 (0.020)	124 (9.74)
Phaseolus vulgaris cv. Amarillo del Norte	5	2.3	0.173 (0.028)	6.84 (0.96)
Vigna unguiculata cv. Tohono O'odham	5	3.5	0.462 (0.031)	13.4 (1.39)
Glycine max cv. Dare	3	18.8	31.8 (2.29)	169 (10.8)

N = number of seed preparations (0.1–0.5 g fresh weight each) analyzed.

M. truncatula cv. A17. Interestingly, *P. sativum* cv. Early Alaska and cv. Taos, which were among the taxa with the lowest total extracted lipid and total NAE content on the basis of fresh weight, were among the taxa with the highest NAE content on a lipid weight basis, second only to *G. max* and *M. truncatula* cv. Jemalong.

Most seeds in our study exhibited NAE profiles (Tables 3 and 4) similar to patterns observed previously in non-leguminous seeds (Chapman et al., 1999). The typical pattern is dominated by NAEs 18:2 and 16:0 followed by 18:1 and 18:3 with minor contributions from all remaining NAEs. However, some unusual patterns were also observed. The *B. congesta, L. texensis, A. hypogaea and P. sativum* cv. Taos NAE profiles were dominated by NAE 18:1. All the representatives of the *Medicago* genus had elevated NAE 18:3 concentrations and this was the predominant NAE in *M. sativa* cv. 7101 and *M. truncatula* cv. A17 as well as *P. acutifolius*. The short/medium chain NAEs were minor components for all profiles. Like total NAE content, the taxa examined exhibited a wide range in the relative distribution of NAE types. This distribution appears to reflect the general abundance of the corresponding fatty acids among the various taxa represented. For example, the dominance of NAE 18:1 followed by NAE 18:2 in *A. hypogaea* parallels the fatty acid distribution in storage triacylglycerols in this species (40.0 and 36.3%, respectively) while *G. max* domination by NAE 18:2 followed by NAE 18:1 parallels its fatty acid distribution (18:2 and 18:1 = 51.4% and 26.7%, respectively). *M. sativa* is unusually rich in 18:3 (29.4%) [fatty acid data from BAGKF Institute for Chemistry and Physics of Lipids http://www.bagkf.de/sofa/]. This may indicate that the NAE profiles are a reflection of the general lipid biosynthetic capacity of these seeds (for storage oil and biomembranes).

Table 3
Detailed NAE profiles in desiccated seeds: ng/g fresh seed weight (s.d.)

Taxa	NAE molecular species (acyl chain)							
	N	12:0	14:0	16:0	18:0	18:1	18:2	18:3
Bauhinia congesta	5	25.6(18.2)	10.6(7.1)	37.4(1.3)	7.51(0.9)	135(9.1)	66.2(7.1)	22.1(2.2)
Caesalpinia gilliesii	4	5.70(0.5)	3.6(0.3)	54.0(11.1)	26.4(3.0)	48.2(6.1)	138(25.0)	63.1(46.3)
Mimosa borealis	4	8.6(2.6)	7.1(2.7)	224(46.7)	53.3(4.7)	131(26.5)	358(55.9)	56.9(25.9)
Lupinus succulentus	5	11.9(2.2)	17.7(0.9)	387(23.1)	209(10.8)	245(26.6)	460(47.0)	96.9(10.2)
Lupinus texensis	8	12.5(2.6)	12.2(5.9)	370(139)	55.8(37.6)	737(231)	814(378)	96.3(70.5)
Arachis hypogaea	5	<10	18.4(1.1)	3730(135)	686(68)	7960(378)	4540(501)	122(23)
Medicago sativa cv. 7101	5	<10	<10	1150(123)	469(32)	1030(68)	1990(54)	2760(66)
Medicago truncatula cv. A17	5	<10	<10	181(105)	65(11)	321(74)	981(102)	2740(83)
Medicago truncatula cv. Jemalong	5	<10	374(23.8)	12,700(683)	2030(146)	9560(811)	12,154(876)	7630(419)
Pisum sativum cv. Early Alaska	5	<2	16.3(1.1)	665(31)	186(16.2)	1060(52.1)	1160(133)	331(70)
Pisum sativum cv. Taos	5	2.0(0.6)	3.7(1.0)	103(9.3)	37.9(1.5)	216(9.4)	174(15.8)	43.0(3.0)
Phaseolus vulgaris cv. Amarillo del Norte	4	3.5(4.3)	5.3(5.2)	53.5(11.1)	18.9(7.43)	24.4(3.1)	28.8(5.7)	37.6(12.5)
Vigna unguiculata cv. Tohono O'odham	5	<2	3.8(0.4)	138(13.1)	51(3.3)	71.0(7.3)	125(7.0)	69(10.8)
Glycine max cv. Dare	3	463(113)	510(79)	6720(534)	1620(148)	4900(376)	12,740(995)	3500(250)

N = number of seed preparations (0.1–0.5 g fresh weight each) analyzed.

Table 4
Detailed NAE profiles in desiccated seeds: μg/g lipid weight (s.d.)

Taxa	NAE molecular species (acyl chain)							
	N	12:0	14:0	16:0	18:0	18:1	18:2	18:3
Bauhinia congesta	5	0.26(0.20)	0.11(0.08)	0.38(0.03)	0.08(0.01)	1.36(0.16)	0.67(0.11)	0.22(0.01)
Caesalpinia gilliesii	4	0.06(0.01)	0.04(0.01)	0.58(0.13)	0.28(0.02)	0.52(0.07)	1.49(0.26)	0.69(0.53)
Mimosa borealis	4	0.21(0.05)	0.17(0.07)	5.45(1.27)	1.29(0.17)	3.19(0.75)	8.66(1.42)	1.38(0.66)
Lupinus succulentus	5	0.22(0.041)	0.33(0.02)	7.29(0.62)	3.93(0.20)	4.60(0.51)	8.64(0.81)	1.82(0.17)
Lupinus texensis	8	0.43(0.08)	0.43(0.20)	13.03(4.52)	1.95(1.27)	26.0(7.40)	28.7(12.54)	3.36(2.38)
Arachis hypogaea	5	<0.02	0.04(0.01)	8.62(0.41)	1.58(0.16)	18.4(0.73)	10.5(1.18)	0.28(0.05)
Medicago sativa cv. 7101	5	<0.02	<0.02	10.4(1.18)	4.28(0.53)	9.4(0.69)	18.1(1.51)	25.1(1.74)
Medicago truncatula cv. A17	5	<0.02	<0.02	1.70(1.01)	0.60(0.10)	2.90(0.68)	9.10(1.18)	25.3(1.72)
Medicago truncatula cv. Jemalong	5	<0.02	2.94(0.26)	99.7(6.38)	15.9(1.03)	75.1(6.09)	95.5(8.29)	60.0(5.66)
Pisum sativum cv. Early Alaska	5	<0.02	0.68(0.03)	27.8(0.99)	7.75(0.52)	44.4(0.28)	48.4(4.84)	13.8(2.51)
Pisum sativum cv. Taos	5	0.40(0.13)	0.78(0.26)	21.9(2.68)	8.02(0.51)	45.54(2.41)	37.0(4.78)	9.10(1.04)
Phaseolus vulgaris cv. Amarillo del Norte	5	0.15(0.19)	0.20(0.18)	2.11(0.37)	0.76(0.36)	0.96(0.09)	1.13(0.14)	1.46(0.36)
Vigna unguiculata cv. Tohono O'odham	5	<0.02	0.11(0.03)	3.97(0.48)	1.46(0.16)	2.04(0.31)	3.61(0.48)	1.99(0.23)
Glycine max cv. Dare	3	2.48(0.69)	2.72(0.54)	35.7(3.13)	8.60(0.43)	26.02(1.65)	67.6(2.97)	18.6(1.01)

N = number of seed preparations (0.1–0.5 g fresh weight each) analyzed.

Although the more primitive members of the group tended to be among those with the lowest NAE content, there was little consistent relationship among the phylogenetic position of the taxa studied and the observed NAE concentration. Even con-specific cultivars had NAE concentrations that varied as greatly as that seen across major phylogenetic groupings. Similarly, the observed NAE profiles were quite variable, even among closely related taxa. These surprising differences could not be attributed to known sources of variation in storage, stress, experimental or analytical conditions. Great care was taken to ensure that all seed preparations were representative, well homogenized and free of plant debris or other sources of external contamination. Internal standards were deuterated species confirmed to be free of native NAEs by GC/MS. All analyses were conducted on desiccated seeds and it is unlikely that any metabolism during the determinations could have accounted for such differences, but rather these differences in quiescent seeds were likely due to differences in NAE accumulation during seed development, prior to desiccation. We noted significant cultivar differences (approximately 4-fold) in cottonseeds previously (Chapman et al., 1999).

The biological significance of the distinct differences in the quantitative patterns of these closely related lipids in the seeds of this diverse group of plants is unknown. The principal functions attributed to NAEs in plants have focused on membrane stabilization/protection and signaling (Chapman, 2004). NAEs accumulate in damaged tissues (Schmid et al., 1990) and may contribute to cytoprotection by removing membrane destabilizing precursors in their formation (free fatty acids and ethanolamine) and may play a role in seed germination and seedling growth (Chapman et al., 1999) as well as in the activation of plant defense genes (Tripathy et al., 1999). The elevated NAE content of seeds suggests they play a special role in dormancy which may be quite distinct from their role in adult vegetative tissues. Some NAE types may be of particular importance for membrane protection during water loss stress experienced by desiccated seeds, while other molecular types may dominate in signaling functions in other tissues. As in animals where important signaling is attributed to the relatively minor NAE constituent, AEA, plant signaling may be dominated by minor short/medium chain NAEs (Chapman, 2004).

Most research on the biological function of NAEs has been conducted on mammals and has resulted in interest in the therapeutic applications for NAE manipulation. Although most of the research in this area has focused on AEA (which we have not found in plants) and the closely related 2-arachidonoylglycerol (2-AG), a wide variety of physiological effects of potential therapeutic interest have been described for the NAE types that are abundant in seeds. For most seeds we have examined, the NAE profiles are dominated by NAE 16:0 and NAE 18:2. There is a long history of interest in NAE 16:0 as an anti-inflammatory agent (Coburn et al., 1954; Long and Martin, 1956; Kuehl et al., 1957; Berdyshev, 2000), and more recently as an analgesic (Calignano et al., 1998; Jaggar et al., 1998). NAE 16:0, although perhaps not a ligand for the cannabinoid (CB) receptors described to date, appears to modulate endocannabinoid activity through other means including down-regulation of the fatty acid amide hydrolase (FAAH) enzyme (Di Marzo et al., 2001) responsible for the breakdown of both AEA and 2-AG potentiating the action of these two endogenous cannabinoids. NAE 16:0 has also been shown to accumulate in induced cerebral ischemia (Franklin et al., 2003) and to be neuroprotective (Skaper et al., 1996). Recently, peroxisome proliferator- activated receptor-α (PPAR-α) was identified as the molecular target responsible for the anti-inflammatory properties of NAE 16:0 (Verme et al., 2005). NAE 18:2 has been shown to similarly potentiate endocannibinoid activity by competing with endogenous ligands for FAAH (Cravatt and Lichtman, 2003). Thus, dominant NAEs in seeds, NAEs 16:0 and 18:2, appear to hold promise as modulators of endocannibinoid activity by providing two distinct forms of potentiation: inhibition of expression of FAAH in the first place coupled with competitive inhibition of FAAH once expressed. Preliminary data on the suppression of tumor necrosis factor α by lipopolysaccharide-stimulated monocytes (THP-1 cell line; Venables, unpublished data) indicate that these two NAEs have potent interactive cannabimimetic activity(ies). Although other NAE types are less well studied, there is evidence that the family of NAE compounds may act collectively to produce an ''entourage'' effect to modulate endocannabinoid-induced physiological functions (Mechoulam et al., 1998).

3. Experimental

3.1. Seed sources

B. congesta, C. gilliesii and *M. borealis* were obtained from Sweet Briar Nursery and Gardens, Belton, TX. *L. succulentus* and *L. texensis* were obtained from Wildseed Farms Unlimited, Fredricksburg, TX. *A. hypogaea* and *P. sativum* cv. Early Alaska were obtained from Gurney's Seed and Nursery Company, Yankton, SD. *P. sativum* cv. Taos, *P. acutifolius, P. vulgaris* and *V. unguiculata* were obtained from Native Seeds SEARCH, Tucson, AZ. *M. sativa, truncatula* cv. A-17 and cv. Jemalong were kindly provided by Dr. Rebecca Dickstein, University of North Texas. *G. max*cv. Dare was a gift from Dr. Richard Wilson, University of North Carolina.

3.2. NAE quantification

NAE extraction, enrichment and identification/quantification by GC/MS are described in our previous studies (Chapman et al., 1999, 2003; Tripathy et al., 1999), except that deuterated NAEs were used here as internal standards. Briefly, deuterated NAEs used as internal standards for isotope dilution quantification were prepared by reacting d4 ethanolamine (New England Nuclear, Boston, MA, USA) with appropriate acylchlorides (Nu-Check Prep, Elysian, MN): 12:0, 14:0, 16:0, 18:0, 18:1$^{cis\Delta 9}$, 18:2$^{cis\Delta 9,12}$ and 18:3$^{cis\Delta 9,12,15}$ (Giuffrida and Piomelli, 1999). Purity of standards was verified by GC/MS.

Desiccated seed samples were ground to a fine powder in a high-speed coffee grinder. Sub-samples of 0.1–0.5 g along with 50 ng of each internal standard were placed in hot 2-PrOH to inactivate endogenous phospholipases (Chapman et al., 1998), extracted into $CHCl_3$, filtered and evaporated under N_2 for gravimetric determination of extracted lipid content. Extracts were then subjected to normal-phase HPLC (Gilson model 712, Middleton, WI) using an Alltech (Deerfield, IL) semi-preparative silica column (10 × 250 mm, 10 m) and UV detection (214 nm) as follows. Total lipid residues were solubilized in 200 mL $CHCl_3$ and injected on a linear gradient of 2-PrOH in hexanes (up to 16% 2-PrOH over 18 min at 4.5 mL/min) resulting in NAE elution between 11 and 15 min. The enriched NAE fraction was collected, evaporated to dryness under N_2 and derivatized in 50 mL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 65 °C for 15 min. The reaction vessel was cooled, BSTFA evaporated and replaced with 20 μL of hexanes for final analysis by GC/MS (Hewlett–Packard 5890 II GC with 5970 mass selective detector operated in selected ion mode, 70 eV). Injector temperature was 260 °C and the column (WCOT DB-5.625, J&W Scientific, Folsom, CA; 30-m × 0.25 mm i.d., 0.25 μm film thickness) was programmed from 40 to 300 °C at 10 °C/min. Carrier gas was helium at a constant inlet pressure of 8.0 psi. The standard curve was prepared by injection of 5.0 ng of each internal standard with target NAEs ranging from 0.01 to 10 ng. Masses used for quantification and secondary masses used for confirmation of identification are listed for internal standards and native NAEs in Table 5. Final NAE concentrations were calculated on the basis of fresh seed weight and total extracted lipid weight.

Table 5
Quantification and confirming ions (m/z) used for TMSi derivatives of internal standards and native NAEs

NAE species		Quantification ions $[M - CH_3]^+$	Confirming masses (% abundance relative to quantification mass)
Internal standards	Native NAEs		
NAE 12:0 d4		304	228(14), 273(8)
	NAE 12:0	300	225(10), 272(8)
NAE 14:0 d4		332	256(19), 301(8)
	NAE 14:0	328	253(14), 300(26)
NAE 16:0 d4		360	284(20), 329(8)
	NAE 16:0	356	281(22), 328(11)
NAE 18:0 d4		388	312(37), 357(11)
	NAE 18:0	384	309(80), 356(10)
	NAE 18:1	382	307(18), 397(48)
	NAE 18:2	380	305(34), 395(54)
	NAE 18:3	378	303(20), 393(31)

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