Benefits of Using L-AEE in Various Applications

Abstract

This technical whitepaper highlights L-Arginine ethyl ester dihydrochloride's applications as a nitric oxide (NO) signaling precursor and protein aggregation suppressor, including situations where L-AEE has been more useful than L-arginine (L-Arg).

Background

L-Arginine ethyl ester dihydrochloride (L-AEE; CAS# 36589-29-4) is a water-soluble[1,2,3] powder with a melting point of ~115–118 °C[4]. The structure of this amino-acid (AA) derivative with HCl groups can be seen in Figure 1, which illustrates guanidino (pK_a ~12.23)[i] and amino moieties along with an ethyl-ester-modified carboxylic end. The latter lipophilic C-terminal protection of perhaps the most[5,6] hydrophilic of the 20 canonical AAs enables[7,8] this L-arginine (L-Arg) prodrug to more easily[9,10] cross cell membranes for passive cellular entry, which may also be facilitated by L-AEE's being "less charged" at the carboxylic end[11,12] and possible[13,14] charge-pairing with anionic phospholipid-bilayer headgroups[15]. Following intracellular esterase-catalyzed hydrolysis[16], NOS-catalyzed oxidation of the resulting L-Arg can generate nitric oxide (NO)[17]. This EDRF may subsequently be involved in vasodilation (widening of blood vessels), platelet break-up, and a host of other *in vivo* signaling-derived applications[18,19,20]. Like L-Arg, a common refolding[21,22] additive, L-AEE also has the ability to suppress aggregation of proteins and other peptide-containing molecules through guanidinium-induced cation-π and hydrogen-bonding interactions with their AA surface residues[23,24]. However, the latter additive's greater hydrophobicity and AA-body net charge may comparatively enhance the aggregation inhibition[25,26]. Meanwhile, L-AEE's supply as a dihydrochloride protects[27] the ethyl ester from hydrolytic degradation, extends shelf life, and stabilizes guanidinium-stacking clusters while providing an acidic pH adjustment that may be crucial for stabilizing macromolecules[24,28]. It may also enhance absorption by the digestive system when administered orally[29]. As a reactant, L-AEE is easily convertible[ii] into (HCl-less) ligand or structural moieties, with the fatty acid ester 'LAE' being a prominent example[30]. However, several promising antimicrobial alternatives (e.g., oleic arginate) for gram-positive bacteria were synthesized from raw-material L-AEE in a recent (ca. 2022) USDA patent[31,32]. Meanwhile, the guanidinium +-charge of CDCArg, a potential agent for treating NAFLD/NASH, most likely meant that this L-AEE-generated bile-acid conjugate, taken up by hepatocytes, could not be efficiently absorbed back into the liver but would instead drag absorbed fat into the colon[33]. Covered in greater detail below is L-AEE's considerable, untapped potential in NO/EDRF-derived signaling applications and peptide-containing molecule aggregation inhibition.

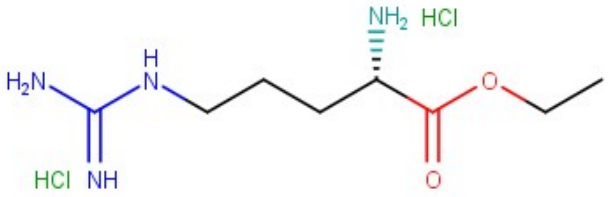

Figure 1. Structure of L-AEE.

Representative Cases of L-AEE as a Value-added Differentiator

Usage 1: Biological Applications Related to Nitric Oxide (NO) Signaling

Online-forum discussions attest to L-AEE's[34,35] considerable effectiveness in topical sexual health application, which may[36] very well continue to be the case even in the *absence* of clinical conditions such as erectile dysfunction, as a vasodilator and smooth-muscle relaxant via NO/EDRF-induced signaling[11,17].

[i] As calculated in MarvinSketch.

[ii] Compared to a very high localized pH at point-of-contact causing ester hydrolysis when 20% NaOH solution was added to aq. L-AEE, a uniform solution[30] of moderate alkalinity (e.g., triethylamine) considerably reduced any L-Arg impurity formation (non-hydrolytic media can yet further improve the free-ester yield).

With its chloride ions perhaps binding, in a paste of minimum water, with the protonated prodrug via Coulomb forces[37,38], L-AEE's electronic neutrality[39] should facilitate its efficient permeation through the skin's stratum corneum, which is decidedly more[40] hydrophobic than the basal epidermal layer that the ethyl ester could passively diffuse through while acting as a pseudo-carrier for other agents[34,41,42]. Not only may L-AEE enhance the transdermal delivery of other therapeutic agents (e.g., salicylic acid) negatively charged at physiological pH, but their tissue absorption and/or distribution might even, as believed, be improved by L-AEE-derived NO production increasing skin permeability, which could be a topic for future research in, e.g., acne-prone individuals[41]. Either way, topical dosages might be better optimized after taking hydrolysis assays (e.g., K_m, V_{max}) inside the stratum basale's keratinocytes which hold the bulk of the skin's esterase activity, considering the potential of the ethyl ester, based on its chemical structure, to be a nonspecific carboxylesterase, or else a CES1, substrate[43]. Meanwhile, L-AEE's use in production (kilogram-scale)[44] of capsules, tablets, and granules for men's sexual health could be related to L-Arg's extensive catabolism by arginase in the gut-liver pathway (and elimination by bacteria in the gut)[45,46] limiting its oral bioavailability[47] as a NOS substrate[48]. In contrast, arginase cannot[49] degrade the ethyl ester, whose arginine may become far[50] more bioavailable partially[51,52,53,54] as a result of the ethyl ester passing through the small intestine largely[55] intact—a possible indicator of only slight esterase-catalyzed hydrolysis of the ethyl ester occurring prior to this prodrug's crossing of the gut-vascular barrier[56,34,57,58]. In fact, significantly lower amounts of oral L-AEE compared to L-Arg could be ingested for an equivalent NO/EDRF-derived therapeutic effect[34,36]. As such, when studying NO-signaling through eNOS (NOS3) for L-AEE's potential application in workout supplements (e.g., muscle pump)[59,55,60], rodent specimens that are plasma-esterase-deficient would better mimic, possible intestinal/hepatic enzyme activity differences notwithstanding, potential human *in vivo* responses to oral L-AEE[61].

Meanwhile, when applied to the subarachnoid space outside the brain, L-arginine ethyl ester better sustained the vasodilation of pial arterioles in piglets, and was ~10-fold more potent, than L-Arg[18,10]. L-AEE could therefore enable extended EDRF-derived therapeutic responses[8,55]. Through an NO-dependent mechanism, *in vitro* studies show[62] L-AEE being just as good as (if not better than) the L-Arg at enhancing, besides preadipocyte proliferation and differentiation, human-adipose-tissue-derived endothelial cell proliferation and thus having at least equivalent angiogenic power[63]. One bonus feature of using L-AEE-releasing[62] polymeric scaffolds to support endothelialization and wound healing by EPC culturing—i.e., via cell adhesion within the open pores previously occupied by L-AEE as a porogen—would likely be the ethyl ester's anti-thrombogenic properties both *in vitro*, such as in isolated human blood for thromboelastography, and potentially *in vivo*[64]. Such scaffolding could also be used for culturing other cells (e.g., dermal fibroblasts), and is pertinent with regard to the ethyl ester and/or L-AEE's higher liposolubility making it incorporable, in contrast to L-Arg, into commonly used biopolymers (e.g., PDLLA)[62,64]. Finally, L-AEE-modified[65] Arg-ZnPc was designed as a non-NOS-catalyzed, ROS-responsive NO donor for photodynamic therapy (Arg-ZnPc was also a photosensitizer) in cancer treatment[66].

Usage 2: Inhibition of Peptide-containing Molecule Aggregation

Discovered[67,68] to be a more potent aggregation inhibiting alternative to L-Arg via Δ-treated lysozyme (0.2–1.0 mg/mL), L-AEE was also found[69] to better prevent this model protein's thermal (98 °C, pH 6.5–7.1) inactivation[25,70]. Compared to 100–200 mM L-AME, 100–200 mM L-AEE recovered higher residual activity and yielded greater oxidative refolding (25 °C), respectively (Na-phosphate buffer)[68,25]. In the latter case, 200 mM L-AEE was also more effective than 200 mM L-Arg for retrieving native lysozyme[25]. Considering L-Arg's suppression[71] of Δ-treated lysozyme aggregation at 4.4 mg/mL, L-AEE's aggregation-suppressing effect could likewise be tested on higher concentrations of this model protein[72]. Though it could be a helpful heuristic[73], a +-charge for L-AEE's α-amino group (pK_a ~7.4)[67] is not required for successful inhibition of Δ-induced aggregation given the success of the ethyl ester (100 mM) in aggregation suppression (pH 10) near lysozyme's isoelectric point (pI ~11.0)[74]. L-AEE's reported success[75] in inhibiting aggregation of crosslinker-polymerized lysozyme during concentration-by-ultrafiltration of a

> 30-kDa fraction, beside its prevention of aggregation when added to a prior supernatant mixture that was subsequently size-fractionated (25 °C) to the 30-kDa cut-off, suggests possible efficacy for suppressing aggregation in column chromatography[76].

Meanwhile, while L-Arg and L-AME were not effective enough, L-AEE efficiently induced PrP^{Sc} amplification *in vitro* in bodily fluids like CSF and urine by accelerating $PrP^{C} \rightarrow PrP^{Sc}$ structural conversion[77]. This was thought to be by preventing excessive aggregation of the infectious-prion molecules, whose larger/non-soluble aggregates are minimally toxic and cannot convert PrP^{C} molecules to their misfolded cousins in contrast to smaller/soluble oligomers[77,78]. Compared to L-Arg, the ethyl ester also better suppressed DTT-induced aggregation (45 °C, pH 7.0) of bovine serum albumin where the rate-limiting step of the general aggregation process was protein unfolding[79,80,81]. Likewise, L-AEE enhanced the suppression of high-[IgG] solution opalescence—whose increase otherwise might have indicated larger antibody associates[26]. The ethyl ester's higher yield over the base-arginine for oxidative refolding of recombinant mink growth hormone may be especially noteworthy for the closeness of the buffer's pH 8.0 to rmGH's *p*I (6.83)[82]. L-AEE has also been used in low-temperature LURE/MEPF peptide refolding[83,84,85,86]. Found to be a model therapeutic agent against Httex1-peptide aggregation *in vitro* besides cell models (e.g., yeast, neuro-2a cells) of Huntington's disease, L-AEE moreover prevented NT_{17} oligomerization and poly(Gln) molecule interactions more effectively than L-Arg through secondary-structure modulation of Httex1's NT_{17} domain, and more effectively disrupted preformed aggregates through possibly hydrogen-bonding alteration[23]. In fact, as demonstrated with low-solubility caffeic acid, L-AEE may be a better hydrotrope than L-Arg at the same concentration and thus a "potent" solubility enhancer[23].

Varsal Advantage

Varsal is a leading producer of extremely-high-purity L-AEE. We are differentiated from the competition as Varsal's proprietary manufacturing logistics processes allows us to produce consistent, stable, extremely-high-purity material—leading to maximal yield and product quality for Varsal's customers.

Extremely high purity in L-AEE is important, given this prodrug's untapped potential as a therapeutic agent, in order to, e.g., ensure safety, avoid unexpected interactions with L-AEE or biological systems, and demonstrate the highest therapeutic efficacies. Considering the publication of a novel hard-capsule dosage form for a small, select group of hygroscopic active materials including L-AEE, impurities could alter L-AEE's release profile and/or kinetics besides compromise the encapsulated L-AEE dispersion's stability[87]. In protein purification meanwhile, L-AEE impurities could throw off the intricate interplay of effects—e.g., thermodynamics, surface residue-additive interactions, crowding, etc.[88,24,22]—that may otherwise lead to successful protein stabilization but remains incompletely understood for even L-Arg[89]. Otherwise, L-AEE's unusual status as both a prodrug *and* an aggregation inhibitor necessitates an extremely-high-purity product due to this chemical's many potential uses in a range of high product quality operating environments (e.g., *in vivo*, large-scale protein manufacture and other bio/pharmaceutical applications).

Varsal is able to serve a wide variety of end-markets and applications, as our intimate knowledge of the manufacturing process allows us to custom-manufacture various grades of L-AEE tailored to our customers' requirements. Please contact us at info@varsal.com to learn more about how Varsal can help you solve your complex chemical and specialty intermediates challenges!

[1] "Preparation method of lauroyl arginine ethyl ester hydrochloride." 2017. Feng, et al.
[2] "Process for the synthesis of hydrochloride salt." 2010. Ghare
[3] "Is there any possibility of making a salt in the amino group from an amino acid ester?" 2018. ResearchGate.
[4] "L-Arginine ethyl ester dihydrochloride." 2023. Chemical Book.
[5] "Coating composition and medical device." 2018. Yamashita and Nozawa.
[6] "A Look at Arginine in Membranes." 2010. Hristova and Wimley.
[7] "Enzyme and receptor modulation." 2021. Davidson, et al.
[8] "Stimulation of 125I-3-iodo-α-methyl-l-tyrosine uptake." 2010. Shikano, et al.
[9] "Acidic Microenvironments Found in Cutaneous Leishmania Lesions." 2022. Frick, et al.
[10] "Time-dependent action of carbon monoxide." 2010. Knecht, et al.
[11] "Compositions and methods for treating sexual dysfunction." 2001. Sacks and Van Dyke.
[12] "Elucidating Ubiquitin Chain Formation." 2018. Carpenter.
[13] "In situ examination of a charged amino acid." 2018. Zhang, et al.
[14] "Interaction of aliphatic amino acids." 2020. Kanwa, et al.
[15] "Intracellular boron accumulation." 2014. Sato, et al.
[16] "Methylesters of L-Arginine and N-Nitro-L-arginine." 1998. Choi, et al.
[17] "Role of Nitric Oxide in the Cardiovascular and Renal Systems." 2018. Ahmad, et al.
[18] "Mono-L-arginine-containing compounds." 1990. Busija, et al.
[19] "Human Alveolar Macrophage Mediated Vasodilation." 1994. Thomas, et al.
[20] "Nitric Oxide: A Guardian for Vascular Grafts?" 2011. De Mel, et al.
[21] "Protein refolding using chemical refolding additives." 2012. Yamaguchi, et al.
[22] "Role of Arginine in the Stabilization of Proteins against Aggregation." 2005. Baynes, et al.
[23] "Discovery of Arginine Ethyl Ester." 2019. Singh, et al.
[24] "Interactions Between L-Arginine/L-Arginine Derivatives and Lysozyme." 2013. Gao, et al.
[25] "Indispensable structure of solution additives." 2009. Matsuoka, et al.
[26] "Arginine and its Derivatives Suppress the Opalescence of an Antibody Solution." 2022. Oki, et al.
[27] "Why are alkyl esters of amino acids?" 2023. Micke.
[28] "Role of arginine salts in preventing freezing-induced increase in subvisible particles in protein formulations." 2022. Munjal, et al.
[29] "What Is L-Arginine HCL?" 2023. King.
[30] "Process for the synthesis of highly pure cationic surfactant products." 2012. Mehta and Shastri.
[31] "Biobased-fatty acid arginates." 2021. Lew, et al.
[32] "Synthesis of Sustainable Iso- and n-Fatty Acid." 2022. Zhang, et al.
[33] "L-arginine conjugates." 2014. Voloshin, et al.
[34] "Emu oil and l-arginine ethyl ester." 2011.
[35] "L-arginine ethyl ester + Castor Oil." 2010.
[36] "Review of Bulk Arginine Ethyl Ester." 2006.
[37] "Salts of Therapeutic Agents." 2018. Gupta, et al.
[38] "Dipole-dipole Forces." 2023. Eames.
[39] "Topical delivery of arginine to overcome pain." 1997. Fossel.
[40] "Welcome to Histology." 2023. King.
[41] "Topical compositions for the treatment of dermatological disorders." 2021. Weinberger, et al.
[42] "Pharmaceutical Salts." 2002. Wermuth and Stahl.
[43] "Specificity of Procaine and Ester Hydrolysis." 2007. Jewell, et al.
[44] "Health food suitable for male and female." 2017. Qing, et al.
[45] "Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine." 2008. Schwedhelm, et al.
[46] "Enzymes of arginine metabolism." 2004. Morris.
[47] "Manufacturer study adds evidence supporting more bioavailable form of arginine." 2018. Menayang.
[48] "Oral supplementation with a combination of L-citrulline." 2014. Morita, et al.
[49] "The specificity of the enzyme arginase." 1934. Calvery and Block.
[50] "Bulk 100 g Arginine Ethyl Ester." 2023.
[51] "Arginine and Citrulline and the Immune Response in Sepsis." 2015. Wijnands, et al.
[52] "Source and fate of circulating citrulline." 1981. Windmueller and Spaeth.
[53] "Adverse Gastrointestinal Effects of Arginine and Related Amino Acids." 2007. Grimble.
[54] "Controlled release nitric oxide producing agents." 1999. Kuhrts.
[55] "Clayton's Health Facts: Arginine Ethyl Ester." 2018. South.
[56] "The blood–brain and gut–vascular barriers." 2021. Scalise, et al.
[57] "What is Arginine Ethyl Ester?" 2023. Legazpi.
[58] "BRONX WILD BULL ARGININE ETHYL ESTER HCL." 2023.
[59] "THE SCIENCE BEHIND THE PUMP." 2023. Gauvreau.
[60] "NutraBio L-Arginine Ethyl Ester Powder." 2023.
[61] "Carboxylesterases: General detoxifying enzymes." 2016. Hatfield, et al.
[62] "L-arginine and arginine ethyl ester." 2015. Paul, et al.
[63] "Polymer-Based Nitric Oxide Therapies: Recent Insights for Biomedical Applications." 2014. Jen, et al.
[64] "A Material Conferring Hemocompatibility." 2016. Everett, et al.
[65] "Arginine-Substituted Phthalocyanine." 2016. Wang, et al.
[66] "Multiple Functions Integrated inside a Single Molecule." 2020. Shi, et al.
[67] "Arginine ethylester." 2004. Shiraki, et al.
[68] "Amino Acid Esters." 2005. Shiraki, et al.
[69] "Amidated amino acids." 2007. Shiraki, et al.
[70] "Small Amine Molecules." 2016. Shiraki, et al.
[71] "Effects of arginine on heat-induced aggregation." 2011. Shah, et al.
[72] "Challenges in the development of high protein concentration formulations." 2004. Shire, et al.
[73] "Charge state of arginine as an additive on heat-induced protein aggregation." 2016. Miyatake, et al.
[74] "Protein stabilization method." 2002. Kentaro, et al.
[75] "Mechanistic investigation of capability of enzymatically synthesized polycysteine to cross-link proteins." 2016. Narai, et al.
[76] "Protein aggregation under high concentration/density state." 2017. Arakawa, et al.
[77] "l-Arginine ethylester enhances in vitro amplification." 2016. Murayama, et al.
[78] "What Is Our Current Understanding of PrPSc-Associated Neurotoxicity and Its Molecular Underpinnings?" 2017. Hughes and Halliday.
[79] "Quantification of Anti-Aggregation Activity of Chaperones." 2013. Borzova, et al.
[80] "Relationship between the initial rate of protein aggregation and the lag period for amorphous aggregation." 2014. Borzova, et al.
[81] "Kinetic regime of dithiothreitol-induced aggregation of bovine serum albumin." 2015. Borzova, et al.
[82] "Studies of the Refolding Processes of Recombinant Growth Hormones." 2010.
[83] "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." 2009. Okuda, et al.
[84] "Direct interaction of ligand–receptor pairs specifying stomatal patterning." 2012. Lee, et al.
[85] "A receptor heteromer mediates the male perception of female attractants in plants." 2016. Wang, et al.
[86] "Method for improving drought resistance of apples." 2020. Ma, et al.
[87] "Stable dosage form articles for oral administration." 2017. Casi, et al.
[88] "Protein-Associated Cation Clusters." 2013. Vagenende, et al.
[89] "The Effect of Arginine on the Phase Stability of Aqueous Hen Egg-White Lysozyme Solutions." 2023. Brudar and Hribar.