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.

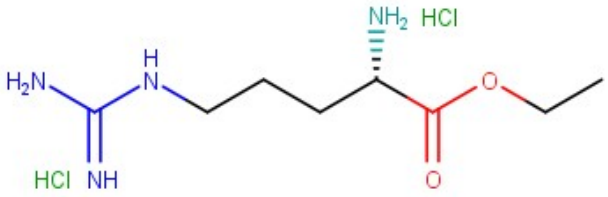

Figure 1. Structure of L-AEE.

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 provider of extremely-high-purity L-AEE. We are differentiated from the competition as Varsal's proprietary manufacturing logistics processes allows us to provide 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 provide 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!

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