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Multiple-step, one-pot synthesis of 2-substituted-3-phosphono-1-thia-4-aza-2-cyclohexene-5-carboxylates and their corresponding ethyl esters

Dunxin Shen [a], Kenneth Hensley [b], Travis T. Denton [a,*]

[a] Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Spokane, WA 99201, United States
[b] Arkansas College of Osteopathic Medicine, Fort Smith, AR 72916, United States

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ABSTRACT

The multiple-step, one-pot procedure for a series of 2-substituted-3-phosphono-1-thia-4-aza-2-cyclohexene-5-carboxylates, analogues of the natural, sulfur amino acid metabolite lanthionine ketimine (LK), its 5-ethyl ester (LKE) and 2-substituted LKEs is described. Initiating the synthesis with the Michaelis-Arbuzov preparation of α-ketophosphonates allows for a wide range of functional variation at the 2-position of the products. Nine new compounds were synthesized with overall yields range from 40 to 62%. In addition, the newly prepared 2-isopropyl-LK-P, 2-*n*-hexyl-LKE-P and 2-ethyl-LKE were shown to stimulate autophagy in cultured cells better than that of the parent compound, LKE.

Lanthionine ketimine (LK, (5*R*)-3, 5-dicarboxy-1-thia-4-aza-2-cyclohexene) is a natural compound first described as an alternative product of the mammalian transsulfuration pathway arising from the metabolism of homocysteine, in which cystathionine beta synthase (CβS) and glutamine transaminase K (GTK) are integral components.[1,2] Although the specific target of LK remains elusive, LK, and its more cell membrane permeable analogue, lanthionine ketimine ethyl ester (LKE), have been shown to possess multiple neuroprotective, anti-neuroinflammatory and neurotrophic activities.[2–11] Chief amongst the biological actions of LKE is its ability to promote autophagy in cells and *in vivo*.[12,13] LKE penetrates the blood-brain barrier and is more potent than LK in cell culture assays, possibly due to increased lipophilicity, inherent to the esterified 5-carboxylate group.[7,12,13] LKE increases lifespan and slows decline of motor function in the $SOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis (ALS),[14] decreases production of native Aβ(1-40) in SH-SY5Y cells,[5] decreases amyloid burden inside neurons and in plaques,[5] decreases protein phosphorylated-tau accumulation,[5] decreases microglial activation[9] and slows cognitive decline in the 3×Tg-AD mouse model of Alzheimer's disease (AD).[5,7] Besides neurodegenerative diseases, preclinical rodent studies indicate stroke and glioma as additional disease targets of LKE and its analogues.[6,15] Although the exact molecular target of LK(E) has not yet been identified, past studies have identified collapsin response mediator protein-2 (CRMP2), an adaptor protein involved in neurite outgrowth via interaction with tubulin dimers, cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK-3β), synaptic plasticity, vesicular trafficking and autophagy regulation.[16,17] The effects of lanthionine ketimine may result from the compound's ability to engage CRMP2 pathways to alter localization of the protein mTOR (mammalian target of rapamycin) and, thus, promote beneficial autophagy.[2,12]

Due to the necessity for new compounds in the fight against neurological disorders such as ALS, AD and Parkinson's disease, we set out to prepare new analogues of LK and LKE. To do so, modifications were made to the preexisting synthetic strategy to incorporate substituents on the 2-position of LK(E)s (Scheme 1). The synthesis of 2-substituted-LKEs begins by the bromination of α-ketocarboxylic acids, **1**, by treatment with bromine for one hour in refluxing dichloromethane. After removal of the solvent and excess bromine, the brominated acid is reacted with an aqueous solution of cysteine ethyl ester hydrochloride (**3**) to afford the 2-substituted LKEs (**4**). An important feature of LKE as a drug

* Corresponding author.
E-mail address: travis.denton@wsu.edu (T.T. Denton).

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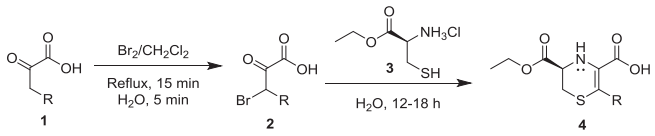


Scheme 1. Synthetic sequence for the preparation of 2-substituted-LKEs.

candidate is its relatively small size and possession of functional groups that can be further modified, while remaining within the accepted parameters of the rule of five for druglikeness.[18] We reasoned that amongst the potential modes of target engagement, electrostatic interaction of the 3-carboxylate group has a high probability of importance, which could possibly be further enhanced by increasing the charge density or the hydrogen bonding opportunities near this corner of the molecule. Thus, we designed a synthetic sequence to prepare 3-phosphonate analogues of LK(E), namely lanthionine ketimine (ester) phosphonates (LK(E)-Ps, Scheme 2). The synthesis of 2-substituted-LK(E)-Ps begins with the preparation of dimethyl α-ketophosphonates (DMAKPs, **6**), utilizing standard Michaelis-Arbuzov (MA) reaction conditions. α-Ketophosphonate diesters are extremely reactive towards nucleophiles, therefore, to avoid any unnecessary side reactions, the remaining transformations were all performed in the same pot. Accordingly, following the MA reaction, the methyl chloride by-product and excess trimethyl phosphite were removed by simple rotary evaporation. The crude dimethyl α-ketophosphonates (**6**) were dissolved in excess trimethylsilyl bromide (TMS-Br) and heated to 100 °C, using microwave irradiation, for ten minutes to afford the intermediate bis-TMS esters (**7**). After removal of the excess TMS-Br and methyl bromide, intermediates **7** were brominated by treatment of the crude reaction mixture with bromine in refluxing dichloromethane for one hour. After removal of the solvent and excess bromine, the crude intermediates were treated with water for three minutes to hydrolyze the bis-TMS esters to the diacid form before reacting with cysteine ethyl ester hydrochloride (**3**) or cysteine hydrochloride (**9**) to afford the LKE-Ps or LK-Ps, respectively. Most products precipitated from the final, aqueous reaction system as white to off-white solids.

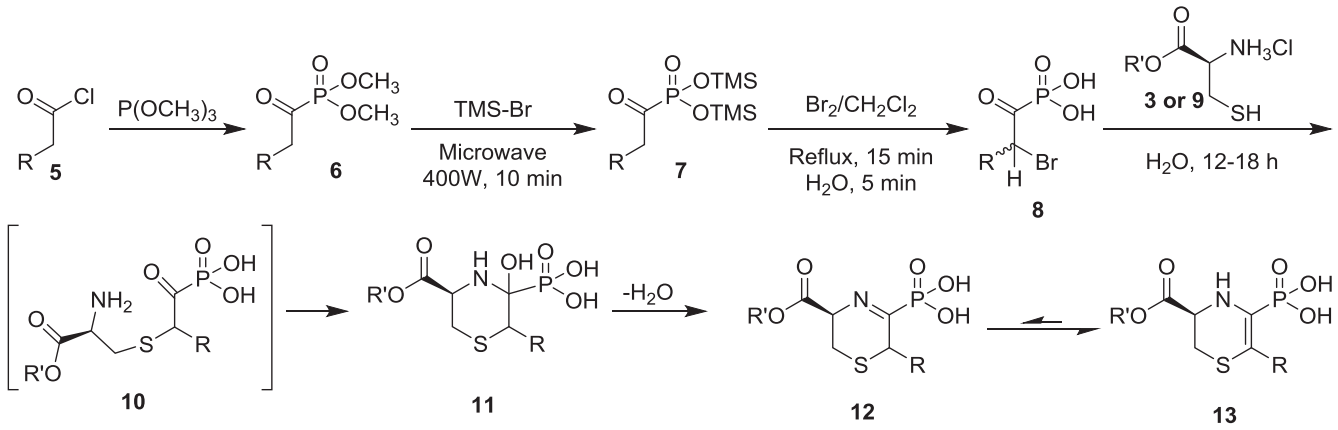
During conditions screening, the reaction mixtures would often turn very dark after the bromination step of the sequence. It was suspected that the sulfur containing intermediates were being oxidized, and ultimately polymerized, by the bromine that was not completely removed from the previous reaction. In an effort to eliminate this problem, in the final step, where the brominated intermediate is reacted with the amino acid, a 5% aqueous sodium bisulfite solution was used as the reaction solvent in place of pure water to serve as a reductant to neutralize any remaining bromine from the previous reaction. The addition of the reducing agent both protected the reaction from becoming dark in color and ultimately increased the overall yield of the precipitated products. The final, solid materials were collected by centrifugation, triturated with ethyl ether and isolated by vacuum filtration.

Using these procedures, two 2-substituted LKEs and seven 2-substututed LK(E)-Ps were prepared (Table 1) and their structures were confirmed by ^{1}H, ^{13}C and ^{31}P NMR and liquid chromatography tandem UV spectrophotometry high-resolution mass spectrometry (LC/UV/HRMS).

Considering the existence of imine-enamine tautomerism, the final product could be in either imine or enamine form. The enamine form was confirmed by one- and two-dimensional NMR analysis. The ^{1}H and ^{13}C NMR spectra for 2-*n*-hexyl-LKE-P are shown in Fig. 1.

Proton assignments were confirmed by the COSY cross peaks between methine proton 1 and the diastereotopic methylene protons 3 and 4 (Supplementary Fig. 1). The smaller, vicinal coupling constant for peak 3 (3.3 Hz) indicates the cis (staggered) orientation of protons 1 and 3 whereas the larger, vicinal coupling constant (6.3 Hz) indicates the trans (antiperiplanar) orientation of protons 1 and 4. The ^{13}C NMR peak assignments for 2-*n*-hexyl-LKE-P are shown in Fig. 1, B. Carbons 1, 2 and 3 were confirmed to be proton-free by DEPT analysis (Supplementary Fig. 2. The chemical shift of carbon 1 is 170.5 ppm, indicative of a carbonyl carbon. The remaining two, proton-free, alkene (enamine) carbons give rise to the doublet at 127.0 ppm with a coupling constant (J = 192.5 Hz) significantly larger than the doublet at 110.3 ppm (J = 17.5 Hz) resulting in the assignments made. Carbons 4–11 were also confirmed by DEPT and HSQC analysis (Supplementary information). According to the peak assignment of 2-*n*-hexyl-LKE-P, all other synthesized LK(E)-Ps were characterized accordingly.

Upon analysis of 2-benzyl-LK-P and 2-ethyl-LKE, some intriguing features were revealed in the ^{1}H NMR spectra. The two methylene (benzyl) protons in 2-benzyl-LK-P are split into a quartet. This peak should be, theoretically, a simple singlet integrating to two protons. This quartet was confirmed to represent the benzyl protons by HSQC analysis (Supplementary Fig. 4). Correspondingly, the methylene protons in 2-ethyl-LKE, which should be split into a quartet, are split into two multiplets, each integrating to a single proton. The two multiplets were confirmed to be the methylene protons of the 2-ethyl group by HSQC analysis (Supplementary Fig. 5). We hypothesize that the benzyl and ethyl groups have restricted rotation about the CH_2-alkene (enamine) bond, thus generating two distinguishable rotamers at room temperature, although this does not explain the multiplicity of the benzyl pro-



Scheme 2. Synthetic sequence for the preparation of 2-substituted-LK(E)-Ps.

Table 1
2-substituted LKEs and LK(E)-Ps prepared and yield.

Name	Yield (%)
2-methyl-LKE	40%
2-ethyl-LKE	47%
2-isopropyl-LK-P	61%
2-*n*-butyl-LK-P	55%
2-*n*-butyl-LKE-P	58%
2-*n*-hexyl-LK-P	58%
2-*n*-hexyl-LKE-P	62%
2-phenyl-LK-P	51%
2-benzyl-LK-P	57%

tons of 2-benzyl-LK-P. In the case of 2-benzyl-LK-P rotamers, the region of the spectrum corresponding to the benzyl protons should contain two singlets that, together, would integrated to two protons Yet, these protons are, as indicated, split into a quartet. In regards to 2-ethyl-LKE, this phenomenon is also observed. In theory, these protons should be split into a single quartet, yet, the splitting pattern is a complicated doublet of multiplets. These multiplets, together, integrate to the proper value of two protons and, if rotamers are the purpose for this complicated spitting pattern, the multiplets should coalesce at higher temperature as the rotational barrier is overcome. As indicated by ^{1}H NMR analysis (Supplementary Fig. 6), when increasing the temperature of the sample from room temperature to 35 °C, the signals began to coalesce, endorsing the likelihood of this assessment. Although this is only a partial explanation for this phenomenon, all corresponding NMR analyses support the structures assigned. In future rounds of synthesis, as the library of compounds is increased, this phenomenon will be examined in further detail.

With the synthesis of 2-alkyl-LK(E)s and 2-alkyl-LK(E)-Ps established, an initial biological evaluation was performed. 2-ethyl-LKE, 2-isopropyl-LK-P and 2-*n*-hexyl-LKE-P were selected to test their effect on autophagy enhancement *in vitro*, marked by an increase in LC3-II (the phosphatidylethanolamine-appended microtubule associated protein 1, light chain 3). Using this assay, the increased LC3-II signal may be due to the late-stage blockade of autophagy clearance. To account for this, bafilomycin-A1 (baf), a vacuolar ATPase inhibitor, was included to fully block autophagy clearance and, therefore, any increase in signal, above this induced baseline, indicates stimulation of autophagy, not blockage of autophagosomal clearance.[19] RG2 glioma cells were treated with LK, 2-isopropyl LK-P, 2-ethyl-LKE and 2-*n*-hexyl LKE-P and 50 nM bafilomycin-A1 and analyzed for stimulation of autophagy (Figs. 2 and 3).

The results indicate a number of biological consequences including 1) the cellular permeability of the new compounds and 2) the successful stimulation of autophagy in the presence of bafilomycin. The increase in autophagy activity of 2-isopropyl-LK-P was greater than LK at 10 μM but showed less of an effect at 100 μM. This biphasic relationship may indicate that, due to the high potency of the new compounds, 100 μM may be above the optimum concentration for autophagy stimulation and in the range of being toxic to the cells. This is also indicated in the testing of 2-ethyl-LKE and 2-*n*-hexyl-LKE-P (Fig. 3). In these preliminary experiments, it is assumed that the optimum concentration for autophagy stimulation of 2-*n*-hexyl-LKE-P is between 1 and 10 μM while that of 2-ethyl-LKE is above 10 μM. With the preliminary potencies estimated, future analyses will be performed on these, and future analogues, with additional doses, in replicate experiments, to specifically determine the concentrations at which the compounds most effectively stimulate autophagy.

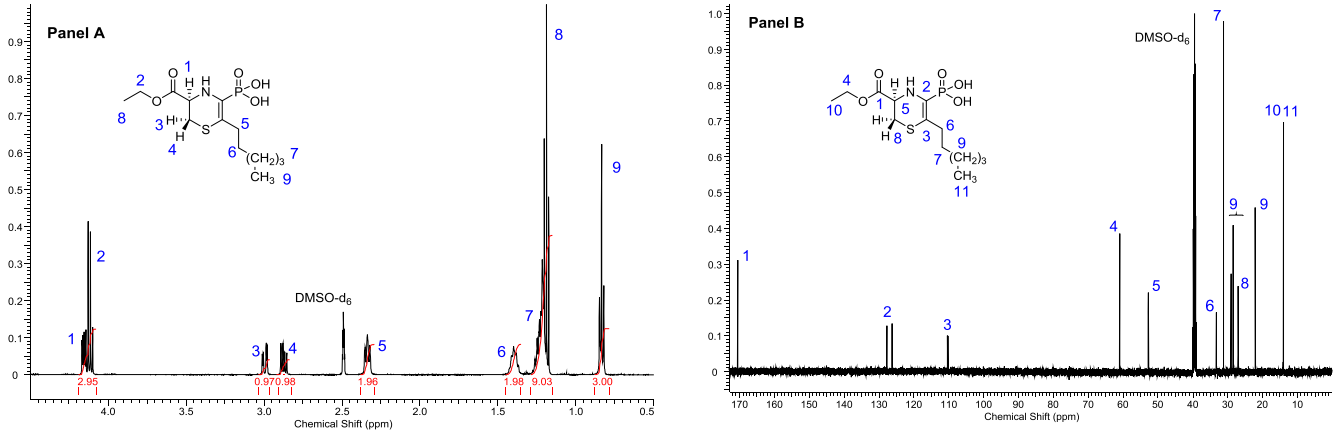


Fig. 1. ^{1}H (Panel A, Left) and ^{13}C (Panel B, Right) NMR spectra of 2-*n*-hexyl-LKE-P.

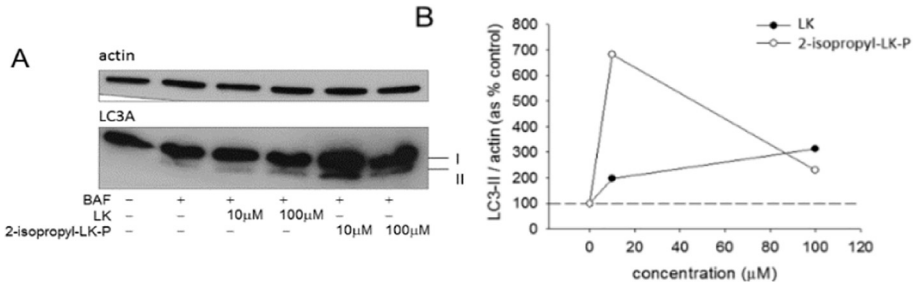


Fig. 2. Autophagy enhancement of LK and 2-isopropyl-LK-P.

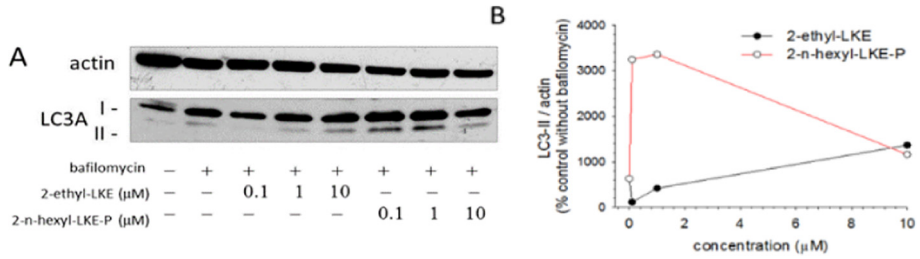


Fig. 3. Autophagy enhancement of 2-ethyl LKE and 2-*n*-hexyl-LKE-P.

The biological results obtained, in the cell culture model, verify the compounds ability to permeate into living cells to elicit their biological response. In an effort to define a comparable experimental value for cell-membrane permeating ability, LKE, 2-ethyl-LKE, 2-*n*-butyl-LK-P, 2-*n*-butyl-LKE-P, 2-*n*-hexyl-LK-P and 2-*n*-hexyl-LKE-P were screened for permeability across Caco-2 epithelial monolayers (Supplementary data). This assay revealed that, out of all compounds tested, LKE was the only compound to have quantifiable membrane permeability. This indicates that the phosphonates are even more potent ligands/substrates for their autophagy stimulating target, relative to LKE, than cell based assays indicate. In other words, if cellular membrane permeability for the phosphonates is lower than that of LKE, when comparing LKE to a phosphonate at any given extracellular concentration, the intracellular concentration of the phosphonate is lower than LKE and, since the phosphonate activates autophagy better than LKE at this lower concentration, it is more potent than LKE.

In conclusion, a novel series of 2-substituted, phosphonate analogues of LKE and novel 2-substituted-LKEs were designed and prepared. All of the compounds tested showed increased autophagic flux in cell culture systems. Future directions include the synthesis of more lipophilic forms of the LK(E)-Ps as well as the preparation of phosphonate-ester pro-drug forms in an effort to increase the cell-permeability and overall efficacy of the compounds. Target identification is also underway by genetic dissection of the autophagy pathway by engaging the use of genetically modifiable Drosophila melanogaster as an *in vivo* tool.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.01.052.

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