

## ORIGINAL ARTICLE

# Ecdysis triggering hormone peptide in the African malaria mosquito *Anopheles gambiae*: The peptide structure for receptor activation

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**Abstract** Infections by mosquito-borne diseases represent one of the leading causes of death in third world countries. The rapid progression of resistance to conventional insecticide causes a significant threat to the highly efficient preventive methods currently in place. Insect neuropeptidergic system offers potential targets to control the insect vectors. The essential roles of the neuropeptide ecdysis triggering hormone (ETH) in insect development and reproduction led us to attempt understanding of the fundamentals of the biochemical interaction between ETH and its receptor in the African malaria mosquito *Anopheles gambiae*. One of two ETH peptides of the African malaria mosquito (AgETH1), a small peptide hormone with 17 amino acid residues (SESPGFFIKLSKSVPRI-NH<sub>2</sub>), was studied to elucidate its molecular structure. N-termini deletions and mutations of conserved amino acids in the ligand revealed the critical residues for the receptor activation. The solution structure of AgETH1 using 2D <sup>1</sup>H-<sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy and nuclear overhauser effect (NOE) derived constraints revealed a short alpha helix between residues 3S and 11S. The NMR solution structure of AgETH1 will be of significant assistance for designing a new class of insecticidal compounds that acts on the AgETH receptor aiming for *in silico* docking studies.

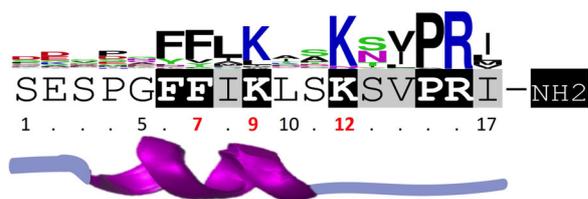
**Key words** *Anopheles gambiae*; ecdysis triggering hormone; G protein-coupled receptor; insect; malaria; neuropeptide; NMR spectroscopy

## Introduction

Numerous species of hematophagous insects including mosquitoes are vectors of many human pathogens causing the diseases such as malaria, Dengue, yellow fever, Chikungunya, and Zika fever. Up to 700 million cases of

infections by mosquito-borne diseases, which is ~10% of the world population, are reported each year (Campbell-Lendrum *et al.*, 2015). Mosquito control has been heavily relying upon the use of chemical insecticides, which has been highly efficient for the disruption of the pathogen transmission cycle by suppressing the vector population. However, the evolution of insecticide resistance has hampered the efficacy of currently available classes of insecticides, and the development of new classes of insecticides has been delayed, in part, by regulations requiring safe and environmentally benign chemicals (Ranson & Lissenden, 2016).

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**Fig. 1** Sequence alignment of ecdysis triggering hormone (ETH) peptides. The sequence logo was made with 81 ETH peptides listed in the DiNeR (Yeoh *et al.*, 2017) and aligned on the top of the *Anopheles gambiae* ETH1 (AgETH1) sequence that is decorated by inverted letters for those highly conserved with other ETHs and gray background for similar amino acid residues. The numbering is based on the AgETH1. The structure on the top is the nuclear magnetic resonance (NMR) structure determined in this study containing an alpha-helical structure in 3S to 11S.

Ecdysis is a unique biology of Ecdysozoa including insects, shedding old cuticles for size growth during development. The ecdysis behavior involves complex neuropeptidergic signals (Kim *et al.*, 2006). The top command peptide is the ecdysis triggering hormone (ETH), essential for the initiation of series of sequential behavioral events leading to the shedding of their external cuticle during molting (Zitnan *et al.*, 1996; Park *et al.*, 1999; Park *et al.*, 2002a; Dai & Adams, 2009; Lenaerts *et al.*, 2017; Shen *et al.*, 2021). Additional functions of the ETH have recently been uncovered for the regulation of another crucial endocrine factor, juvenile hormone (JH) in Dipteran insects (Areiza *et al.*, 2014; Nouzova *et al.*, 2018) as well as for the control of reproduction (Areiza *et al.*, 2014; Nouzova *et al.*, 2018; Shi *et al.*, 2019). Most of all, disruption of this signaling system has been demonstrated to be lethal to many species of insects (Park *et al.*, 2002a; Dai *et al.*, 2008; Lenaerts *et al.*, 2017; Shi *et al.*, 2017; Shen *et al.*, 2021), offering a potential target system for development of an insecticidal strategy.

Insect species have an *eth* gene encoding two similar ETH peptides ETH1 and ETH2 commonly characterized by the C-terminal motif PRXamide where X is a hydrophobic amino acid (Fig. 1) (Park *et al.*, 1999; Zitnan *et al.*, 2003; Dai & Adams, 2009). The conserved sequences are further extended to a number of amino acids extended to the N-terminal region of the PRXamide (Fig. 1). In this study, we modeled the ETH1 of African malaria mosquito *Anopheles gambiae* (AgETH1, 17mer SESPGFFIKLSKSVPRI-NH<sub>2</sub>) (Park *et al.*, 2002b; Park *et al.*, 2003; Jindal *et al.*, 2021). We investigated the structural components of the ETH for activation of the AgETHR-A. We hereby report the nuclear magnetic resonance (NMR) spectroscopy solution structure and lig-

and activities of the deletion series and mutants of the AgETH1 on the receptor AgETHR-A.

## Materials and methods

### Peptide synthesis

The 17-residue AgETH1 peptide was prepared by stepwise solid-phase synthesis using the standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry which was acquired from PepMic Ltd, Jiangsu, China. The peptide was purified to >95% purity using reverse-phase high-performance liquid chromatography on a C18 column. The mass and purity of each peptide were confirmed by matrix-assisted-laser desorption time-of-flight (MALDI-TOF) mass spectroscopy analysis. The mass of the AgETH1 peptide determined by mass spectroscopy (1891.60 Da) was consistent with the mass calculated from the sequence (1891.24 Da). The mutated ligands and N-terminally deleted variants were also prepared and purified following similar protocols.

### Circular dichroism (CD)

All the CD experiments employing titration with trifluoroethanol (TFE) were performed to determine the secondary structure of AgETH1 using a Jasco-8155 spectropolarimeter with a 0.1-cm path length cell over the 190–260 nm wavelength range as previously described (Soulages *et al.*, 2001). The spectra were acquired at 25 °C using the continuous mode with 1 nm bandwidth, a 2-s averaging time per point and a scan speed of 100 nm/min. Five scans were accumulated and averaged for each spectrum. The peptide samples (2 μmol/L) were prepared in water with increasing TFE percentage (0%, 10%, 20%, 30%, 40%, and 50%). The mean residue ellipticity was expressed as percent ellipticity. The CD spectra for peptide variants were measured in 30% TFE.

### NMR spectroscopy

The high-resolution 1D and 2D <sup>1</sup>H-<sup>1</sup>H NMR experiments were performed at 25 °C with a 11.75 Tesla (499.84 MHz for <sup>1</sup>H) Varian VNMRS spectrometer (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a 5 mm cryogenic triple resonance inverse detection pulse field gradient probe. All of the NMR spectral data were acquired at the Biomolecular NMR Core Facility in the Department of Biochemistry and Molecular Biophysics at Kansas State University. Purified AgETH1

was dissolved in 650  $\mu\text{L}$  of 30% deuterated TFE (TFE- $d_3$ ) and 70%  $\text{H}_2\text{O}$  reaching a concentration of 2 mmol/L. A total of 16 increments of 2K data points were recorded for the 2D  $^1\text{H}$ - $^1\text{H}$  double quantum filtered correlation spectroscopy (DQF-COSY) (Rance *et al.*, 1983) experiments whereas 2D  $^1\text{H}$ - $^1\text{H}$  total correlation spectroscopy (TOCSY) (Bax & Davis, 1985) and nuclear Overhauser effect spectroscopy (NOESY) (Kumar *et al.*, 1980) experiments were performed using 2048 t2 and 256 t1 data points with spectral width of 12 ppm in each dimension and 16 transients per increment. Spin-lock time of 80 msec at B1 field strength of 7 kHz was used for 2D  $^1\text{H}$ - $^1\text{H}$  TOCSY experiments using MLEV-17 pulse sequence (Bax & Davis, 1985), and mixing times of 100, 300, and 500 msec were used for 2D  $^1\text{H}$ - $^1\text{H}$  NOESY experiments. All data sets were collected in hypercomplex phase-sensitive mode. When necessary, spectral resolution was enhanced by Lorentzian–Gaussian apodization. The water peak was suppressed by presat method and the residual water peak was assigned a value of 4.72 ppm and thus used as an internal reference for chemical shifts assignments. NMR data processing was done using VnmrJ3.2a (Agilent Technologies, Palo Alto, CA, USA) and analyzed using Sparky software (Goddard & Kneller, 2004). Before processing, the t2 dimension of DQF-COSY data sets was zero-filled to 4k and t1 dimension to 2K. Side-chain proton resonances were assigned as previously discussed (Schrage *et al.*, 2017) by overlaying cross peaks in the TOCSY spectra with those in the NOESY spectra acquired under similar conditions. NOE cross peaks were classified as strong, medium, or weak intensity based on the number of contour lines observed.

#### Chemical shifts assignments and structure calculations

The proton chemical shift assignments were made, using standard two-dimensional NMR methods, on the basis of through bond and through space connectivities (Wuthrich, 1986). We used 2D  $^1\text{H}$ - $^1\text{H}$  DQF-COSY and TOCSY NMR spectra for intraresidue and 2D  $^1\text{H}$ - $^1\text{H}$  NOESY spectra for interresidue spin systems connectivities. For short side-chain residues, such as serine, valine, isoleucine, and so forth, through bond connectivities were straightforward. For long side-chain residues, such as lysine, arginine, and proline, through bond connectivities were made in a progressive manner. A total of 147 distance constraints obtained from the NOESY spectrum were used for structure calculations. The distance constraints were determined from NOESY cross peak intensities observed in spectrum obtained with a mixing time of 500 msec and also appeared in spectrum with mixing

time of 100 msec. The cross peak intensities were classified as strong (1.8–2.7 Å), medium (1.8–3.4 Å), weak (1.8–4.0 Å), and very weak (1.8–5.0 Å). Upper distance limits for NOEs involving methyl protons and nonstereo specifically assigned methylene protons were corrected appropriately, by adding 1 Å to the constraints for center averaging (Wüthrich *et al.*, 1983). In all cases, the lower boundaries were restricted to the sum of Van der Waals radii. The distance constraints obtained were then used to create initial peptide structures starting from extended structures using the Crystallography and NMR System (CNS), version 1.3 (Brünger *et al.*, 1998), which uses both simulated annealing protocol and molecular dynamics to produce low-energy structures with minimum distance and geometry violations. Default parameters were used to generate 100 structures for the initial CNS run. A second round of calculations generated 20 structures, from which the 10 lowest energy structures with no restriction violations were selected and analyzed with VMD 1.9.3 (Humphrey *et al.*, 1996)

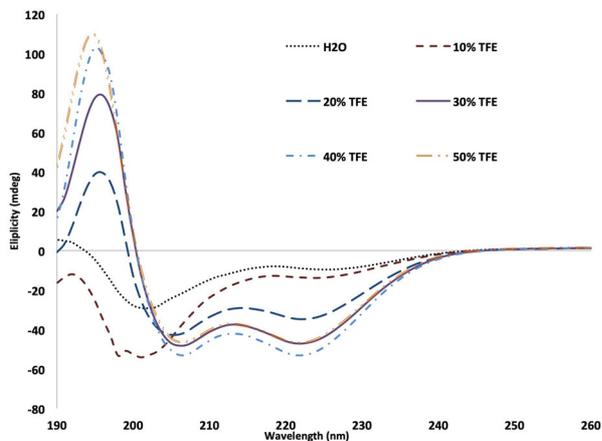
#### Cloning and expression of AgETHR-A

ETH sequence alignments were using the sequences for 81 insect ETH captured in the DIneR (Yeoh *et al.*, 2017). The procedure for gene cloning and heterologous expression of AgETHR-A1 was previously described (Jindal *et al.*, 2021). We chose AgETHR-A, not the isoform AgETHR-B, for the study because the former provided higher specificities to the AgETH-1 in the preliminary study (Jindal *et al.*, 2021). Briefly, the receptor clone was expressed in Chinese Hamster Ovarian cell (CHO-K1) that contains apoaequorin and Galpha 16. Calcium mobilization was detected with 1-s interval for 20 s after the ligand is treated. The accumulated luminescence was converted to the percent responses. The highest activity of wild-type AgETH1 (10  $\mu\text{mol/L}$ ) was used as the standard 100% activity for calculations of the comparative activities of different ligands.

## Results and Discussion

#### CD data and preliminary structural analysis

CD data were acquired for the AgETH peptides at increasing concentrations of TFE in  $\text{H}_2\text{O}$  at 25 °C. As indicated on the CD spectra shown in Fig. 2, in the absence of TFE, a negative band was observed near 200 nm due to  $\pi$  to  $\pi^*$  electronic transition, suggesting random coil peptide. However, as the TFE was added, the band shifted to 208 nm and an additional shoulder appeared around



**Fig. 2** Circular dichroism data per v/v percent trifluoroethanol (TFE) concentration. *Anopheles gambiae* ecdysis triggering hormone 1 (AgETH1) seems to adopt a largely unstructured state with low (<20%) TFE concentrations. An enhancement of the  $\alpha$ -helical properties is observed as the TFE concentration reaches 30%.

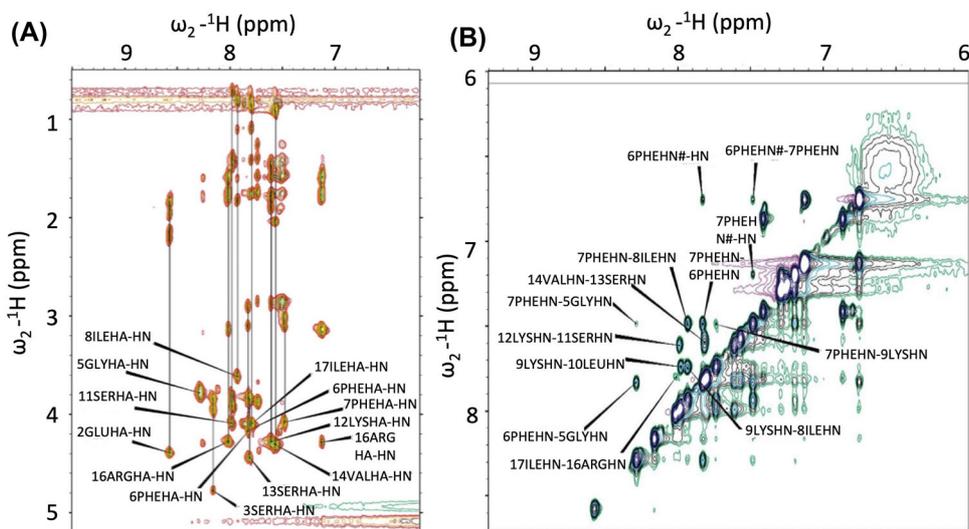
220 nm. A strong positive band appeared after the concentration of TFE exceeded 10% v/v increasing in magnitude with further increase in TFE as a percentage of the total volume. These new bands are characteristics of helical structure. Thus, our preliminary structure analysis using CD spectral data suggested helical structure for this peptide in the presence of TFE. As such, all further NMR

experiments were conducted in TFE- $d_3$ : $H_2O$  (30%:70%) solvent.

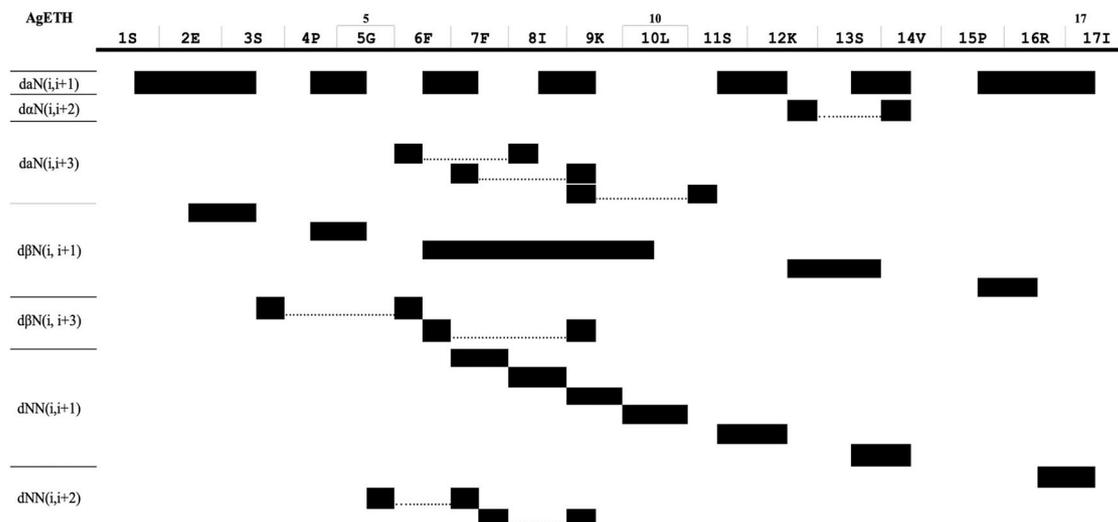
### NMR results and peptide backbone conformation

2D  $^1H$ - $^1H$  TOCSY and NOESY NMR spectra acquired under similar experimental conditions were analyzed to yield the amino acid resonances and proton chemical shift assignments. Fig. 3A shows the NH-C $\alpha$ H, NH-C $\beta$ H, NH-C $\gamma$ H, and NH-C $\delta$ H cross peak assignments in the fingerprint region of the 2D  $^1H$ - $^1H$  TOCSY spectrum used for residue assignments. Fig. 3B illustrates 2D  $^1H$ - $^1H$  NOESY HN-HN amide region critical for secondary structural component determination. In these spectra, the amide proton chemical shifts are well dispersed, indicative of a folded structure for this peptide.

Analysis of the 2D  $^1H$ - $^1H$  NOESY spectrum (Fig. 3B) allowed for the identification of  $d_{NN}(i, i+1)$  proton connectivities, which were used for sequential assignments. Structural information was provided by the short-, medium-, and long-range  $d_{\alpha N}$  and  $d_{NN}$  connectivities, which were obtained through further examination of the NOESY spectrum. Sequential  $d_{NN}$  NOEs, characteristic of alpha helices, were observed between residues 6F–7F, 7F–8I, 8I–9K, 9K–10L, 11S–12K, 13S–14V, and 16R–17I (Figs. 3B and 4). The  $^1H$  chemical shifts assignments for AgETH1 determined by the combined use of a 2D  $^1H$ - $^1H$  TOCSY, DQF-COSY, and NOESY NMR spectra are summarized in Table 1. The sequential, medium-range,



**Fig. 3** 2D  $^1H$ - $^1H$  total correlation spectroscopy (TOCSY) fingerprint region (A) and nuclear Overhauser effect spectroscopy (NOESY) HN-HN region (B). Amino acid spin systems are connected by a vertical black line and labeled with residue name and number. In B, sequential HN-HN connectivities are indicative of alpha helices.



**Fig. 4** Summary of nuclear overhauser effect (NOE) connectivity in 30% trifluoroethanol (TFE). Dotted lines represent medium-range and long-range connectivity.

**Table 1** *Anopheles gambiae* ecdysis triggering hormone 1 (AgETH1) proton chemical shift (ppm) assignments.

Column 1	HN	HA	HB	HD and others
1SER	–	3.99	3.80	–
2GLU	8.48	4.31	2.10, 1.76	–
3SER	8.07	4.69	3.86, 3.76	–
4PRO	–	4.27	2.22	$\delta H$ 3.74, 3.82 $\gamma H$ 1.94, 1.82
5GLY	8.20	3.69	–	–
6PHE	7.74	4.07	3.01, 2.82	–
7PHE	7.39	4.00	3.01, 2.93	–
8ILE	7.84	3.53	1.75	$\delta H$ 0.72 $\gamma H$ 1.53, 1.02
9LYS	7.65	3.79	1.66, 1.18	$\delta H$ 1.49 $\gamma H$ 1.31 $\epsilon H$ 2.76
10LEU	7.89	3.84	1.38	$\gamma H$ 1.39 $\delta H$ 0.63
11SER	7.90	3.99	3.70	–
12LYS	7.52	4.23	1.81, 1.69	$\delta H$ 1.49 $\gamma H$ 1.37 $\epsilon H$ 2.78
13SER	7.73	4.37	3.75	–
14VAL	7.48	4.23	1.96	$\gamma H$ 0.82
15PRO	–	4.26	2.12	$\delta H$ 3.70, 3.49 $\gamma H$ 1.84
16ARG	7.93	4.19	1.68	$\delta H$ 3.05 $\gamma H$ 1.51 $\epsilon H$ 7.07
17ILE	7.71	4.01	1.67	$\delta H$ 1.01 $\gamma H$ 1.33, 0.76

and long-range NOE connectivities are summarized in Fig. 4.

The absence of a  $d_{\alpha\alpha}(i, i+1)$  NOE connectivity for prolines 4 and 15 and their corresponding preceding residues suggest that P4 and P15 adopted the trans-conformation. The observation of sequential  $d_{NN}(i, i+1)$  NOE connectivities (Fig. 4) for residues 6–12 of AgETH1 suggest that this peptide adopts a helical conformation

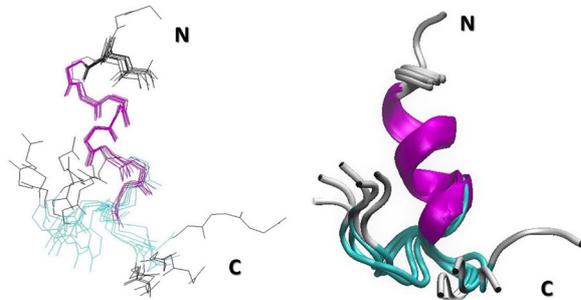
in 30:70 TFE:H<sub>2</sub>O. In addition, the observations of  $d_{\beta N}(i, i+1)$ ,  $d_{\alpha N}(i, i+2)$ , and  $d_{\alpha N}(i, i+3)$  connectivities are unique signatures of helical structure (Fig. 4). A simulated annealing molecular dynamics analysis was performed with distance constraints derived from the NOEs and found to converge to a single family of conformers regardless of the starting structure. A total of 147 NOE distance constraints including 84 intraresidue, 44 sequential, 16 medium-range, and three long-range constraints were used (Table 2). From the initial 100 structures generated, the 10 lowest energy structures were retained. Superimposition of the 10 lowest energy structures showed considerable degree of rigidity with a pairwise root mean square deviation of the backbone of 1.99 Å (Table 2). Alignment along residues 3–11 of the 10 lowest energy structures showed similar rigidity with a backbone root mean square deviation of 1.86 Å (Table 2). The dihedral angles for all the finalized energy-minimized structures fall into the stereochemically allowed regions of Ramachandran plot. A stereodiagram of the backbone atoms of the 10 lowest energy structures overlaid with one another and the average structure of AgETH1 are shown in Fig. 5. The average structure comprises a short alpha helix between residues 3S and 11S while the N and C-terminal regions are largely unstructured.

#### AgETH1 and the variants on AgETHR-A

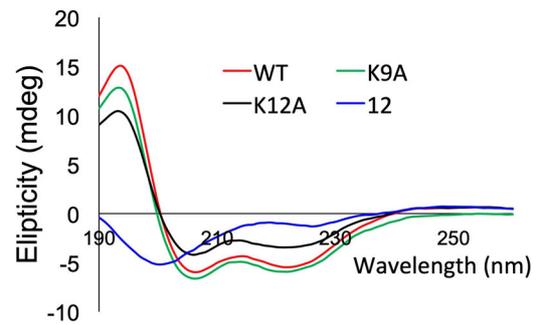
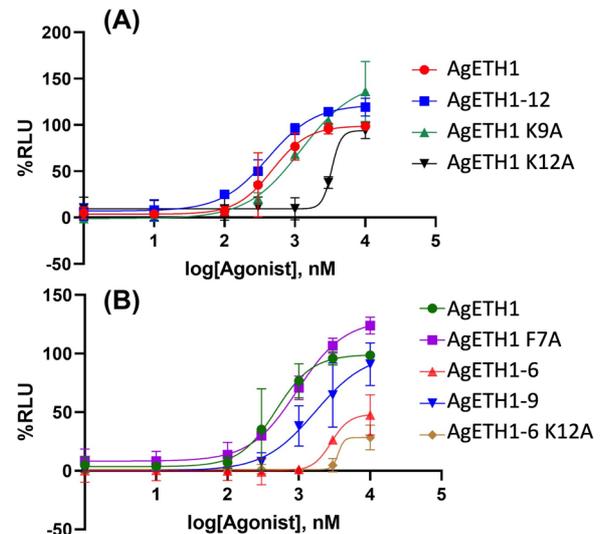
We tested AgETH1 on the receptor AgETHR-A because they are the pair showing relatively high specificity

**Table 2** Summary of total nuclear overhauser effect (NOE) constraints for the 10 lowest energy structures.

NOE constraints	Number
Total	147
Intraresidue	84
Sequential	44
Medium range	16
Long range	3
Constraints per residue	8.65
Pairwise root mean square deviation (RMSD) to mean structure (residues 1–17)	
Backbone	1.99 ± 0.55
Heavy atoms	2.48 ± 0.75
Pairwise RMSD to mean structure (residues 3–11)	
Backbone	1.86 ± 0.65
Heavy atoms	2.38 ± 0.95
Percentage of residues in $\phi$ - $\psi$ region	
Allowed	75.82
Additionally allowed	19.17
Generously allowed	4.15
Disallowed	0.83

**Fig. 5** Structural representation of *Anopheles gambiae* ecdysis triggering hormone 1 (AgETH1) in 30% trifluoroethanol (TFE). The backbones of the 10 lowest energy structures are superimposed. AgETH comprises a short  $\alpha$ -helix between residues 3S and 11S. Note that the  $\alpha$ -helix and C-terminal active motif PRXamide are connected by a flexible hinge.

to only mosquito ETHs based on our earlier study (Jindal *et al.*, 2021). The specificity of AgETHR-A was important to discriminate the essential amino acid residues involved in the receptor activation. To assess the biochemical implications of the alpha-helical structure and positively charged Lys residues of wild-type AgETH1, seven synthetic variants of AgETH1 containing mutations and truncations at various highly conserved residue positions were generated. The variant activities on the AgETHR-A were evaluated via calcium biolumi-

**Fig. 6** Circular dichroism data at 30% trifluoroethanol (TFE) concentration of *Anopheles gambiae* ecdysis triggering hormone 1 (AgETH1) wild-type and mutated variants. See Table 3 for the variant sequences.**Fig. 7** Dose-response curves of *Anopheles gambiae* ecdysis triggering hormone 1 (AgETH1) variants on the AgETHR-A. Y axis is percent relative luminescence measured in the calcium mobilization assay.

nescence reporter assays. The CD spectra in 30% TFE indicated that the AgETH1-12 (FFIKLSKSVPRI-NH<sub>2</sub>) lost the alpha-helical structure while the full lengths with the K to A mutations at the 9 and 12 position, respectively, retained the helical structures (Figs. 6 and 7 and Table 3). However, in the activities of the ligand variants on the AgETHR-A, AgETH1-12 retained almost full activity, while K9A and K12A variants showed significantly reduced activities. Shorter peptides retaining the C-terminal PRXamide motifs, AgETH1-12, -9, and -6, showed gradually reduced activities on the receptor

**Table 3** Half maximal effective concentration (EC<sub>50</sub>) values for the wild-type *Anopheles gambiae* ecdysis triggering hormone 1 (AgETH1) peptide and synthetic analogs on the AgETHR-A.

Peptide/analog	Analog sequence	nmol/L EC <sub>50</sub> (confidence interval)	R <sup>2</sup>
AgETH1 WT	SESPG <b>FFIKLSK</b> SVPRI-NH2	476 (385–595)	1.0
AgETH1 F7A	SESPG <b>F</b> AIKLSK <b>S</b> SVPRI-NH2	953 (336–520)	1.0
AgETH1 K9A	SESPG <b>FFIAL</b> SKSVPRI-NH2	1225 (840–2371)	1.0
AgETH1 K12A	SESPG <b>FFIKLSA</b> SVPRI-NH2	3324 (NA)	1.0
AgETH1-12	<b>FFI K LS K</b> SVPRI-NH2	415 (385–595)	1.0
AgETH1-9	<b>K LS K</b> SVPRI-NH2	1699 (1033–6449)	1.0
AgETH1-6	<b>K</b> SVPRI-NH2	2839 (2671–3013)	0.99
AgETH1-6 K12A	<b>A</b> SVPRI-NH2	3572 (NA)	0.98

Note: EC<sub>50</sub> values represent the average of three replicates. The conserved residues are in bold as indicated in Fig. 1. The point mutations are in bold red fonts. NA in the confidence interval is not applicable due to the large range.

(Table 3 and Fig. 7). Ala scanning for F7, K9, and K12 had reduced activities on the receptor.

The necessary ligand structure for activation of AgETHR-A was found to be the amidated C-terminal region of 12 amino acids shown by the near full activity of the AgETH1-12. In addition, F7, K9, and K12, which are all conserved amino acids in insect ETH sequences (Fig. 1), are also found to be important in the receptor activations. Exceptions in the amino acid residues of 10 and 11 (L and S), the C-terminal end of the alpha helix, appears to be a flexible hinge linking two different active regions for receptor activation. It is also worth noting that the alpha-helical structure between 3S and 11S in AgETH1 is contrasted to the case of another –PRXamide peptide, pheromonotropic neuropeptide PBAN, which predominantly adopts a beta-sheet (Nachman *et al.*, 1993; Bhattacharya *et al.*, 2015). The structural difference may contribute the specificity of the ligand–receptor interactions as ETH ligand and the receptors are generally well discriminated from other –PRXamide systems (Jiang *et al.*, 2014). The ligand accessibility to the G protein-coupled receptor binding pocket could be more complex like the case of *Tribolium* sulfakinin receptor, which proposed the importance of the outer opening of the binding cavity in the receptor (Yu *et al.*, 2015). *In silico* docking model in the ETH system will provide further insights for the ligand-binding pocket to develop the ETH-specific insecticidal strategy. This study illustrating the AgETH1 structure and the important amino acid residues provides improved knowledge for the ligand–receptor docking model of the important insect neuropeptide ETH.

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## Disclosure

The authors declare they have no conflicts of interest.

## References

- Areiza, M., Nouzova, M., Rivera-Perez, C. and Noriega, F.G. (2014) Ecdysis triggering hormone ensures proper timing of juvenile hormone biosynthesis in pharate adult mosquitoes. *Insect Biochemistry and Molecular Biology*, 54, 98–105.
- Bax, A. and Davis, D.G. (1985) MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *Journal of Magnetic Resonance*, 65, 355–360.
- Bhattacharya, D., Mishra, N., Coutinho, E.C., Srivastava, S., Pissurlenkar, R.R.S. and Shaikh, M. (2015) Conformational study on pheromonotropin neuropeptide using NMR and molecular dynamics. *Pharmaceutica Analytica Acta*, 6, 359.
- Brünger, A.T., Adams, P.D., Clore, G.M., Delano, W.L., Gros, P., Grosse-Kunstleve, R.W. *et al.* (1998) Crystallography & NMR system: a new software suite for macromolecular

- structure determination. *Acta Crystallographica Section D Structural Biology*, 54, 905–921.
- Campbell-Lendrum, D., Manga, L., Bagayoko, M. and Sommerfeld, J. (2015) Climate change and vector-borne diseases: what are the implications for public health research and policy? *Philosophical Transactions of the Royal Society B Biological Science*, 370, 20130552.
- Dai, H., Ma, L., Wang, J., Jiang, R., Wang, Z. and Fei, J. (2008) Knockdown of ecdysis-triggering hormone gene with a binary UAS/GAL4 RNA interference system leads to lethal ecdysis deficiency in silkworm. *Acta Biochimica Biophysica Sinica*, 40, 790–795.
- Dai, L. and Adams, M.E. (2009) Ecdysis triggering hormone signaling in the yellow fever mosquito *Aedes aegypti*. *General Comparative Endocrinology*, 162, 43–51.
- Goddard, T.D. and Kneller, D.G. (2004) *Sparky 3*. University of California, San Francisco.
- Humphrey, W., Dalke, A. and Schulten, K. (1996) VMD: visual molecular dynamics. *Journal of Molecular Graphics*, 14, 33–38.
- Jiang, H., Wei, Z., Nachman, R.J., Adams, M.E. and Park, Y. (2014) Functional phylogenetics reveals contributions of pleiotropic peptide action to ligand-receptor coevolution. *Scientific Reports*, 4, 6800.
- Jindal, V., Park, Y. and Kim, D. (2021) Functional characterization of ecdysis triggering hormone receptors (AgETHR-A and AgETHR-B) in the African malaria mosquito, *Anopheles gambiae*. *Frontiers in Physiology*, 12, 702979.
- Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H. and Adams, M.E. (2006) A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. *Current Biology*, 16, 1395–1407.
- Kumar, A., Ernst, R.R. and Wüthrich, K. (1980) A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. *Biochemical and Biophysical Research Communications*, 95, 1–6.
- Lenaerts, C., Cools, D., Verdonck, R., Verbakel, L., Vanden Broeck, J. and Marchal, E. (2017) The ecdysis triggering hormone system is essential for successful moulting of a major hemimetabolous pest insect, *Schistocerca gregaria*. *Scientific Reports*, 7, 46502.
- Nachman, R.J., Kuniyoshi, H., Roberts, V.A., Holman, G.M. and Suzuki, A. (1993) Active conformation of the pyrokinin/PBAN neuropeptide family for pheromone biosynthesis in the silkworm. *Biochemical and Biophysical Research Communication*, 193, 661–666.
- Nouzova, M., Michalkova, V., Hernandez-Martinez, S., Rivera-Perez, C., Ramirez, C.E., Fernandez-Lima, F. et al. (2018) JH biosynthesis and hemolymph titers in adult male *Aedes aegypti* mosquitoes. *Insect Biochemistry and Molecular Biology*, 95, 10–16.
- Park, Y., Filippov, V., Gill, S.S. and Adams, M.E. (2002a) Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development (Cambridge, England)*, 129, 493–503.
- Park, Y., Kim, Y.J. and Adams, M.E. (2002b) Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proceedings of the National Academy of Sciences USA*, 99, 11423–11428.
- Park, Y., Kim, Y.J., Dupriez, V. and Adams, M.E. (2003) Two subtypes of ecdysis-triggering hormone receptor in *Drosophila melanogaster*. *Journal of Biological Chemistry*, 278, 17710–17715.
- Park, Y., Zitnan, D., Gill, S.S. and Adams, M.E. (1999) Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. *FEBS Letters*, 463, 133–138.
- Rance, M., Sorensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wüthrich, K. (1983) Improved spectral resolution in cosy 1H NMR spectra of proteins via double quantum filtering. *Biochemical and Biophysical Research Communication*, 117, 479–485.
- Ranson, H. and Lissenden, N. (2016) Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology*, 32, 187–196.
- Schrag, L.G., Cao, X., Herrera, A.I., Wang, Y., Jiang, H. and Prakash, O. (2017) Solution structure and expression profile of an insect cytokine: *Manduca sexta* stress response Peptide-2. *Protein & Peptide Letters*, 24, 3–11.
- Shen, C.H., Xu, Q.Y., Fu, K.Y., Guo, W.C., Jin, L. and Li, G.Q. (2021) Ecdysis triggering hormone is essential for larva–pupa–adult transformation in *Leptinotarsa decemlineata*. *Insect Molecular Biology*, 30, 241–252.
- Shi, Y., Jiang, H.B., Gui, S.H., Liu, X.Q., Pei, Y.X., Xu, L. et al. (2017) Ecdysis triggering hormone signaling (ETH/ETHR-A) is required for the larva–larva ecdysis in *Bactrocera dorsalis* (Diptera: Tephritidae). *Frontiers in Physiology*, 8, 587.
- Shi, Y., Liu, T.Y., Jiang, H.B., Liu, X.Q., Dou, W., Park, Y. et al. (2019) The ecdysis triggering hormone system, via ETH/ETHR-B, is essential for successful reproduction of a major pest insect, *Bactrocera dorsalis* (Hendel). *Frontiers in Physiology*, 10, 151.
- Soulages, J.L., Arrese, E.L., Chetty, P.S. and Rodriguez, V. (2001) Essential role of the conformational flexibility of helices 1 and 5 on the lipid binding activity of apolipoprotein III. *Journal of Biological Chemistry*, 276, 34162–34166.
- Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*. John Wiley & Sons, New York.
- Wüthrich, K., Billeter, M. and Braun, W. (1983) Pseudostructures for the 20 common amino acids for use in studies

- of protein conformations by measurements of intramolecular proton-proton distance constraints with nuclear magnetic resonance. *Journal of Molecular Biology*, 169, 949–961.
- Yeoh, J.G.C., Pandit, A.A., Zandawala, M., Nässel, D.R., Davies, S.-A. and Dow, J.T. (2017) DIneR: database for insect neuropeptide research. *Insect Biochemistry and Molecular Biology*, 86, 9–19.
- Yu, N., Zotti, M.J., Scheys, F., Braz, A.S.K., Penna, P.H.C., Nachman, R.J. *et al.* (2015) Flexibility and extracellular opening determine the interaction between ligands and insect sulfakinin receptors. *Scientific Reports*, 5, 12627.
- Zitnan, D., Kingan, T.G., Hermesman, J.L. and Adams, M.E. (1996) Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science*, 271, 88–91.
- Zitnan, D., Zitnanova, I., Spalovska, I., Takac, P., Park, Y. and Adams, M.E. (2003) Conservation of ecdysis-triggering hormone signalling in insects. *Journal of Experimental Biology*, 206, 1275–1289.

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