Biostable agonists that match or exceed activity of native insect kinins on recombinant arthropod GPCRs

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The multifunctional arthropod ‘insect kinins’ share the evolutionarily conserved C-terminal pentapeptide motif Phe-X1-X2-Trp-Gly-NH2, where X1 = His, Asn, Ser, or Tyr and X2 = Ser, Pro, or Ala. Insect kinins regulate diuresis in many species of insects. Compounds with similar biological activity could be exploited for the control of arthropod pest populations such as the mosquito Aedes aegypti (L.) and the southern cattle tick Rhipicephalus (Boophilus) microplus (Canestrini), vectors of human and animal pathogens, respectively. Insect kinins, however, are susceptible to fast enzymatic degradation by endogenous peptidases that severely limit their use as tools for pest control or for endocrinological studies. To enhance resistance to peptidases, analogs of the insect kinins incorporating bulky 3,\(\alpha\)-disubstituted amino acids in positions adjacent to both primary and secondary peptidase hydrolysis sites were synthesized. In comparison with a control insect kinin, several of these analogs are highly stable to hydrolysis by degradative enzymes ANCE, neprilysin and Leucine aminopeptidase. Six analogs were evaluated by calcium bioluminescence assay on recombinant receptors from mosquito and tick. Four of these analogs either matched or exceeded the potency of the control kinin peptide agonist. One of these was about 5-fold more potent than the control agonist on the tick receptor. This analog was 8-fold more potent than the control agonist on the mosquito receptor, and twice more potent than the endogenous Aedes kinin-II. The analog also demonstrated potent activity in an in vitro Aedes Malpighian tubule fluid secretion assay. Similar comparisons of analog potency cannot be made to tick kinins because no endogenous kinin has yet been identified. These potent, biostable analogs represent ideal new tools for endocrinologists studying arthropod kinin-regulated processes in vivo, particularly for ticks in which their role remains to be established.

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1. Introduction

G-Protein-coupled receptors (GPCRs) are seven transmembrane cell surface proteins that are activated by diverse stimuli such as biogenic amines, neuropeptides and protein hormones. A distinct intracellular response, mostly through their heterotrimeric G-protein, is initiated when GPCRs are activated by their ligands leading to biological effects (Park and Adams, 2005; Kristiansen, 2004; Pierce et al., 2002; Han et al., 2007). Insects have 50–80 neurohormone GPCRs, which, together with their ligands, control most critical metabolic processes, such as reproduction, development, homeostasis and feeding (Grimmelikhuijzen et al., 2007; Hauser et al., 2006a,b; Gäde and Goldsworthy, 2003; Gäde, 2004). Because these receptors are involved in critical physiological processes they are considered good targets to control arthropod pest populations. Blocking or overstimulating these receptors could lead to reduction of pest fitness or death (Gäde and Goldsworthy, 2003).

Insect neuropeptides found in several arthropod and invertebrate groups regulate important biological functions including water balance, egg and pheromone production, blood sugar level, metamorphosis, etc. (Bede et al., 2007; Coast, 2007; Coast et al., 2002; De Loof, 2008; Gäde, 2004; Nässel, 1996; Riehle et al., 2002; Torfs et al. 1999; Altstein, 2004). In diverse species, insect kinins stimulate hindgut contractions, diuresis, digestive enzyme release and probably inhibit larval weight gain (Holman et al., 1990; Coast et al., 1990; Nachman et al., 1991, 2002; Harshini et al., 2002, 2003; Seinsche et al., 2000). Due to their specificity and their high activity at extremely low dosages, neuropeptides have been studied as potential leads for the development of new
The multifunctional arthropod ?insect kinins? share the evolutionarily conserved C-terminal pentapeptide motif Phe-X1-X2-Trp-Gly-NH2, where X1 = His, Asn, Ser, or Tyr and X2 = Ser, Pro, or Ala. Insect kinins regulate diuresis in many species of insects. Compounds with similar biological activity could be exploited for the control of arthropod pest populations such as the mosquito Aedes aegypti (L.) and the southern cattle tick Rhipicephalus (Boophilus) microplus (Canestrini), vectors of human and animal pathogens respectively. Insect kinins, however, are susceptible to fast enzymatic degradation by endogenous peptidases that severely limit their use as tools for pest control or for endocrinological studies. To enhance resistance to peptidases, analogs of the insect kinins incorporating bulky a,a-disubstituted amino acids in positions adjacent to both primary and secondary peptidase hydrolysis sites were synthesized. In comparison with a control insect kinin, several of these analogs are highly stable to hydrolysis by degradative enzymes ANCE, neprilysin and Leucine aminopeptidase. Six analogs were evaluated by calcium bioluminescence assay on recombinant receptors from mosquito and tick. Four of these analogs either matched or exceeded the potency of the control kinin peptide agonist. One of these was about 5-fold more potent than the control agonist on the tick receptor. This analog was 8-fold more potent than the control agonist on the mosquito receptor, and twice more potent than the endogenous Aedes kinin-II. The analog also demonstrated potent activity in an in vitro Aedes Malpighian tubule fluid secretion assay. Similar comparisons of analog potency cannot be made to tick kinins because no endogenous kinin has yet been identified. These potent, biostable analogs represent ideal new tools for endocrinologists studying arthropod kinin-regulated processes in vivo, particularly for ticks in which their role remains to be established.
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environmentally friendly pest control agents. However, the natural compounds cannot be directly used, as they are susceptible to degradation by endogenous peptidases present in the insect digestive system and circulating hemolymph (blood) (Cornell et al., 1995; Gäde and Goldsworthy 2003; Lamango et al., 1996). If both chemical and conformational requirements responsible for neuropeptide biological activity were fully understood, design of analogs containing unnatural moieties could overcome these limitations (Nachman et al., 1994).

The endogenous arthropod insect kinins are 6–14 amino acids long neuropeptides characterized by the evolutionary conserved C-terminal pentapeptide PhεX1X2-Trp-Gly-NH2, where X1 is His, Asn, Ser, or Tyr and X2 = Ser, Pro, or Ala (Holman et al., 1999; Torfs et al., 1999). This C-terminal pentapeptide kinin core is the minimum sequence required for full cockroach myotropic and cricket diuretic activity in in vitro tissue assays (Nachman and Holman, 1991; Nachman et al., 2003) and for bioluminescence response in CHO-K1 cells expressing kinin receptors (Holmes et al., 2003; Pietrantonio et al., 2005; Taneja-Bageshwar et al., 2006). Both the tissue assays and the receptor expressing system revealed that the C-terminal amide of the insect kinin is important for their activity. Activity of the insect kinin core was completely lost when the C-terminal amide was replaced with the negatively charged acid moiety (Nachman et al., 1995; Taneja-Bageshwar et al., 2006). Activity was also completely lost in these assay systems when either Phε or Trp was replaced with Ala, confirming the importance of these two key positions (Taneja-Bageshwar et al., 2006). However, the variable position 2 tolerates a wide range of chemical characteristics, from acidic to basic residues, and from hydrophilic to hydrophobic, although highest potencies in Malpighian tubule diuretic activity were observed in acidic to basic residues, and from hydrophilic to hydrophobic, with highest potencies in Malpighian tubule fluid secretion assays and receptor expressing systems were observed with aromatic residues at this position (Nachman and Holman, 1991; Roberts et al., 1997; Taneja-Bageshwar et al., 2006). Based on these observations the plausible receptor interaction model positions the side chains of Phε and Trp toward the same region where they interact with the receptor, and away from the side chain position 2.

In vivo, kinins are subjected to rapid biological degradation. Experimentally, the angiotensin-converting enzyme (ACE) from the housefly can cleave the insect kinin primary hydrolysis site and neprilysin (NEP) can cleave both the primary and secondary hydrolysis sites (Cornell et al., 1995; Lamango et al., 1996; Nachman et al., 2002, 1997a,b, 1990; Roberts et al., 1997). Therefore, pseudopeptides with enhanced resistance to peptidases that retain biological activity on ‘insect kinin’ receptors of arthropod vectors at a potency that matches native or control peptide agonists are needed. Towards this purpose, incorporation of a single α-amino isobutyric acid (Aib) into the third position of the insect kinin active core effectively protects the primary hydrolysis site from tissue-bound peptidase (Nachman et al., 1997a,b, 2002; Taneja-Bageshwar et al., 2006). Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site further enhances biostability (Nachman et al., 2002).

The availability of kinin receptors from the southern cattle tick, *Rhipicephalus (Boophilus) microplus* (Holmes et al., 2000, 2003), and the dengue vector, the mosquito *Aedes aegypti* (Pietrantonio et al., 2005), stably expressed in CHO-K1 cells, allows the opportunity to evaluate a new series of kinin analogs featuring enhanced biostability. These analogs that incorporate sterically bulky α,α-disubstituted amino acid residues, such as α-amino isobutyric acid (Aib), adjacent to both primary and secondary peptidase hydrolysis sites were evaluated through a functional calcium bioluminescence assay on the two recombinant receptors. Six such analogs were synthesized in which the third position (5 or P), adjacent to the primary peptidase hydrolysis site, was replaced with Aib. In these analogs, a position adjacent to the secondary peptidase hydrolysis site was also replaced with either an α,α-disubstituted amino acid or a β-amino acid that leads to an enhancement of biostability (see Fig. 1). These analogs are:

- K-Aib-2 [α MeF][F][Aib]WGa
- K-Aib-3 Ac-[Aib][F][F][Aib]WGa
- K-Aib-4 Ac-[Aib][3F][F][Aib]WGa
- K-Aib-5 [Aib][RFF][Aib]WGa
- K-Aib-6 [Aib-Aib-Aib-Aib][RFF][Aib]WGa

where K stands for Kinin analog.

### 2. Materials and methods

#### 2.1. Analog synthesis and purification

Analogs were synthesized on an ABI 433A peptide synthesizer with a modified FastMoc 0.25 procedure using an Fmoc-strategy starting from Rink amide resin (Novabiochem, San Diego, CA, 0.5 mm/g). The Fmoc protecting group was removed by 20% 4-methyl piperidin in DMF (Dimethyl formamide). A 4-fold excess of the respective Fmoc-amino acids was activated in situ using HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (1 eq.)/HOBT (1-hydroxybenzotriazole) (1 eq.) in NMP (N-methylpyrrolidone) or HATU (2-(7-Aza-1H-Benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate) (1 eq.)/HOAt (1-hydroxy-7-azabenzotriazole) (1 eq.) in NMP for Aib and the amino acid immediately following it in the sequence. The coupling reactions were base catalyzed with DIPEA (N,N-diisopropyl-ethylamine) (4 eq.) The amino acid side-chain protecting groups were PMC for Arginine and Boc for Tryptophan. The analogs were cleaved from the resin with side-chain protection by treatment with TFA (Trifluoroacetic acid);H2O:TIS (Triisopropylsilane) (95.5:2.5:2.5 v/v/v) for 1.5 h. The solvents were evaporated by vacuum centrifugation and the analogs were desalted on a Waters C18 Sep Pak cartridge (Milford, MA) in preparation for purification by HPLC.

The analogs were purified on a Waters Delta-Pak C18 reverse-phase column (8 × 100 mm, 15 μm particle size, 100 Å pore size) with a Waters 510 HPLC system with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous TFA; Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Initial conditions were 10% B followed by a linear increase to 90% B over 40 min; flow rate, 2 ml/min. Delta-Pak C18 retention times: K-Aib-1, [Aib][F-F][Aib]WGa, 15.0 min.; K-Aib-2, [α MeF][F][Aib]WGa, 16.0 min.; K-Aib-3, Ac-[Aib][F][F][Aib]WGa, 16.4 min.; K-Aib-4, Ac-[Aib][3F][F][Aib]WGa, 17.0 min.; K-Aib-5, [Aib][RFF][Aib]WGa, 14.0 min.; K-Aib-6, [Aib-Aib-Aib-Aib][RFF][Aib]WGa, 16.4 min. The analogs were further purified on a Waters Protein Pak 1125 column (7.8 × 300 mm). Conditions: isocratic using 80% acetonitrile containing 0.1% TFA; flow rate, 2 ml/min. Waters Protein Pak retention times: K-Aib-1, 5.0 min.; K-Aib-2, 5.9 min.; K-Aib-3, 4.5 min.; K-Aib-4, 6.0 min.; K-Aib-5, 6.0 min.; K-Aib-6, 6.0 min. Amino acid analysis was carried out under previously reported conditions (Nachman et al., 2004) to quantify the analogs and to confirm identity:

- K-Aib-1: F[1.0], F[1.0], G[0.7];
- K-Aib-2: F[1.0], F[1.0], G[0.9];
- K-Aib-3: R[0.9], F[1.0], F[1.0], G[0.8];
- K-Aib-4: R[0.9], F[1.0], F[1.0], G[0.9];
Receptor cloning, transfection and selection of single clonal cell lines expressing the kinin receptors from the southern cattle tick, *B. microplus* (AF228521) (leukokinin-like receptor) and the yellow fever mosquito, *A. aegypti* (AY596453) was reported previously (Holmes et al., 2000, 2003; Pietrantonio et al., 2005). The CHO-K1 cell lines expressing, respectively, the tick receptor, BmLK3 and mosquito insect kinin receptor, E10 (Pietrantonio et al., 2005), were maintained in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum (Equitech Bio, Kerrville, TX) with 400 mg/ml GENETICIN at 37 °C and 5% CO₂.

### 2.2. Cell lines

The enzyme degradation assays were performed on those three analogs that were most potent on both the receptors. The assays were performed as reported previously (Zubrak et al., 2007; Cornett et al., 1995; Lamango et al., 1996). In short, peptide analogs (10 μM) were incubated with either angiotensin-converting enzyme (ANCE) (0.5–2 ng), leucine aminopeptidase (Sigma–Aldrich, Type VI; 0.3–10 ng) or human neprilysin (10–100 ng) in 0.1 M Hepes buffer, pH 7.0 (total volume, 20 μl) at 37 °C. Reactions were terminated by the addition 5 μl of 8% TFA and in preparation for HPLC analysis the volume was increased to 260 μl by the addition of 0.1% TFA. Percent hydrolysis of each peptide was determined by using HPLC to measure the amount of the parent peak remaining after incubation. Results are the mean of three individual assays. HPLC was performed using a Jupiter 5 μm column (C18, 250 mm in length × 4.5 mm, internal diameter) and a linear solvent gradient of 24% rising to 50% of acetonitrile in 0.1% TFA over 20 min at flow rate of 1 ml/min.

### 2.3. Analysis of activity of Aib-containing analogs of insect kinin peptide by a Ca²⁺ bioluminescence plate assay

The functional analysis of Aib-containing insect kinin analogs with stably transformed CHO-K1 cells expressing the tick or mosquito receptor was performed by an intracellular calcium bioluminescence assay as described previously (Pietrantonio et al., 2005; Taneja-Bageshwar et al., 2006). This assay uses a photoprotein isolated from jelly fish (*Aequorea victoria*), composed of an apoprotein (apoaequorin) and a prosthetic group coelenterazine; holoprotein aequorin is formed in the presence of oxygen. When aequorin comes in contact with calcium, it undergoes a conformational change, forming up the calcium binding sites on the protein. This releases oxygen that in turn oxidizes coelenterazine to excited coelenteramide. When excited coelenteramide relaxes to the ground state it emits light at 469 nm (Mithofer and Mazars, 2002). Transient transfection with aequorin was as described previously (Taneja-Bageshwar et al., 2006, 2008a,b). The CHO-K1 cells expressing the kinin receptors were grown in F12K media containing 10% fetal bovine serum and 400 mg/ml GENETICIN® to about 90% confluency in T-25 flasks at 37 °C and 5% CO₂. Cells were trypsinized and seeded in each well of 6-well tissue culture plate at a density of 2 × 10⁵ cells/100 μl drops of bathing fluid with the following composition (in mM/l): NaCl, 150; NaHCO₃, 1.8; KCl, 3.4; CaCl₂, 1.7; MgSO₄, 4.1; glucose, 5; HEPES (N-2-hydroxyethylpiperazine-N₂-ethanesulfonic acid), 25; pH adjusted to 7.1 with 1 mol/l NaOH. The control rate of secretion was measured over 30 min after which test compounds were added to the bathing fluid dissolved in 1 μl of saline at 10-times the required concentration. Stimulated rates of secretion were then measured over two 15 min periods and the average rate of secretion (in μl/min) expressed as a percentage of the control rate.

### 2.4. Enzyme degradation assays

Malpighian tubules were removed from adult females (*Aedes aegypti*) at 3–10 days post-emergence. Tubule secretion assays were performed as described by Clark et al. (1998), but using 9 μl drops of bathing fluid with the following composition (in mmol/l): NaCl, 150; NaHCO₃, 1.8; KCl, 3.4; CaCl₂, 1.7; MgSO₄, 4.1; glucose, 5; HEPES (N-2-hydroxyethylpiperazine-N₂-ethanesulfonic acid), 25; pH adjusted to 7.1 with 1 mol/l NaOH. The control rate of secretion was measured over 30 min after which test compounds were added to the bathing fluid dissolved in 1 μl of saline at 10-times the required concentration. Stimulated rates of secretion were then measured over two 15 min periods and the average rate of secretion (in μl/min) expressed as a percentage of the control rate.

### 3. Results

Effect of substitution of α-amino isobutyric acid on the activity of insect kinin C-terminal pentapeptide core by calcium bioluminescence plate assay

Aequorin-based functional calcium bioluminescence plate assay was used to study the effect of Aib substitution on the activity of insect kinin C-terminal pentapeptide core FFSWGa. Six Aib analogs of the pentapeptide core were synthesized and tested on stable tick (BmLK3 cell line) and mosquito insect kinin receptor (E10 cell line) expressing CHO-K1 cell lines. All the analogs were tested from 1 to 10 μM final concentration.

On both tick and mosquito receptors, all six analogs were active. On tick receptor, analog K-Aib-1, [Aib]FF[Aib]WGa was the most

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**Footnotes:**

1. 2×10⁵ cells/100 μl
2. 37 °C
3. 5% CO₂
4. 469 nm
5. 8% TFA
6. 0.1% TFA
7. 250 mm in length × 4.5 mm, internal diameter
active and K-Aib-6, [Aib-Aib-Aib-Aib]RFF[Aib]WGa was the least active analog. All the analogs were as efficacious as the positive control, FFFSWGa, in eliciting the response but varied in their potencies (Fig. 2 and Table 2). Determination of EC50 values revealed the order of potency as K-Aib-1, EC50 = 49 nM > K-Aib-2, [xMeF][Aib]WGa, EC50 = 111 nM > K-Aib-3, Ac-R[Aib]FF[Aib]WGa, EC50 = 112 nM > K-Aib-4, Ac-R[F][Aib]WGa, EC50 = 211 nM > FFFSWGa, EC50 = 271 nM > K-Aib-5, [Aib][RF][Aib]WGa, EC50 = 299 nM > K-Aib-6, EC50 = 624 nM (Fig. 2). Statistical analysis showed analog K-Aib-1 to be statistically more potent than the control agonist FFFSWGa, and analogs K-Aib-1, K-Aib-3 and K-Aib-2 to be statistically more potent than analog K-Aib-6. The analog K-Aib-1 is more than 5-fold more potent than the control, FFFSWGa.

On the mosquito receptor, the most and least active analogs were the same as for the tick receptor; analog K-Aib-1 was the most potent and analog K-Aib-6 was the least potent of all six analogs tested. All the analogs were as efficacious as the positive control, FFFSWGa, but varied in their potencies. Determination of EC50 values revealed the order of potency as K-Aib-1, EC50 = 76 nM > K-Aib-2, [xMeF][Aib]WGa, EC50 = 289 nM > K-Aib-4, Ac-R[F][Aib]WGa, EC50 = 411 nM > FFFSWGa, EC50 = 625 nM > K-Aib-5, [Aib][RF][Aib]WGa, EC50 = 814 nM (Fig. 3, Table 2). Statistical analysis showed analog K-Aib-1 to be significantly different from analogs K-Aib-3, K-Aib-4, K-Aib-5, FFFSWGa, and K-Aib-6; and was also significantly different from Aedes kinin-II (data not shown). The order of activity for these analogs on the mosquito receptor deviated from that observed for the tick receptor only in that the order of potency for analogs K-Aib-3 and K-Aib-4 were switched. Analog K-Aib-1 was found to be 8-fold more potent than control analog, FFFSWGa, and also more potent than the native Aedes kinin-II, EC50 = 164 nM (Table 2) (Taneja-Bageshwar et al., 2008b).

3.1. Enzyme degradation assays

A comparison of the rates of hydrolysis of a select group of the Aib-containing insect kinin analogs with the standard agonist FFFSWGa indicates that all are significantly more stable (see Table 1). Challenged with the enzyme neprilysin, analogs K-Aib-1, K-Aib-2 and K-Aib-4 are 17-, 5- and 10-fold more resistant than the standard kinin sequence. The same analogs proved to be 24-, 45- and 9-fold more resistant to hydrolysis by the enzyme angiotensin-converting enzyme (ANCE), respectively. When challenged with the Leucine amino peptidase (also known as aminopeptidase M), the analog K-Aib-4 was 95-fold less susceptible to hydrolysis.

3.2. Malpighian tubule secretion assay

The in vitro diuretic assays measuring Aedes Malpighian tubule fluid secretion rates demonstrated that analog K-Aib-1, is more potent than the control, FFFSWGa and as active as native Aedes kinin 1, NSKYVSKQKFYSWG a at 0.1 nM (Fig. 4).

Table 1

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<th>Substrate</th>
<th>Enzyme rate of hydrolysis (pmol/h/ng of enzyme)</th>
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<td>K-Aib-4</td>
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* Rates are expressed as the mean of at least three assays with a standard error of less than 10%.

b LAP Leucine aminopeptidase is also called aminopeptidase M and N and is a general aminopeptidase, but prefers hydrophobic side chains at position 1.
Previous studies demonstrate that in addition to hemolymph aminopeptidases, the insect kinins are hydrolyzed by tissue-bound peptidases at both a primary and secondary site in the peptide chain. The primary hydrolysis-susceptible site lies within the insect kinin C-terminal pentapeptide core region between the Ser$^3$ and Pro$^3$ residues. A secondary site is found just outside of the core region at the peptide bond N-terminal to Phe$^1$ (Fig. 1). Furthermore, it has been established that the peptidase angiotensin-converting enzyme (ACE or ANCE) cleaves insect kinin peptides at both the primary and secondary sites. The peptidase nephrilysin cleaves the insect kinins at the primary hydrolysis site. Replacement of Ser$^3$ (or Pro$^3$) with an unnatural, sterically bulky residue Aib leads to analogs that not only mimic a critical β-turn conformation but also blocks tissue-bound peptidase, ANCE, and nephrilysin hydrolysis at the primary site. Previous studies in cell lines expressing the tick and mosquito receptors indicated that the core Aib insect kinin analog FF[Aib]WGa retains significant potency, demonstrating more potency than the control agonist FFFSWGa in tick receptor cell lines and approaching the potency of FFFSWGa in mosquito receptor cell lines (Taneja-Bageshwar et al., 2006). However, while this core Aib analog protects the primary hydrolysis-susceptible site, it cannot protect the secondary site in extended analogs. Notably, the N-terminus of this short analog is vulnerable to hydrolysis by aminopeptidases, which also leads to inactivation (Fig. 1). Furthermore, this analog is significantly less potent than the native Aedes kinins, as the mosquito receptor prefers analogs that are extended beyond the C-terminal pentapeptide core. Extended insect kinin analogs would also require additional protection from endopeptidase attack at the secondary site. For these reasons, we prepared a series of biostable Aib-containing analogs that incorporate a second modification (a second Aib residue) to the residue N-terminal to Phe$^1$ of the core that has been shown to protect the secondary hydrolysis-susceptible site (Fig. 1). For instance, although a non-protected insect kinin was degraded within 1 h by tissue-bound peptidases, the disubstituted Aib kinin analog [Aib][Aib]FS[Aib]WGa was found to be completely impervious even up to 4 h, at which time the experiment was terminated (Nachman et al., 2002). In addition, each of the analogs features a sequence that either terminates in a bulky Aib residue or is capped at the N-terminus with an acetyl group to prevent hydrolysis by aminopeptidases. In this study, experiments with ANCE, nephrilysin, and Leucine aminopeptidase (aminopeptidase M) indicate that K-Aib-1, K-Aib-2 and K-Aib-4 demonstrate a highly significant increase in resistance to hydrolysis as compared with the standard kinin FFFSWGa (Table 1). The analog K-Aib-4 was 95-fold less susceptible to hydrolysis by aminopeptidase. This result was expected, as it is capped with an acetyl group on the N-terminus. Interestingly, uncapped analogs K-Aib-1 and K-Aib-2 also demonstrated resistance to hydrolysis by the aminopeptidase, with rates of hydrolysis 32-fold and 244-fold lower, respectively, than the standard insect kinin sequence. The marked resistance to aminopeptidase hydrolysis is likely due to the steric hindrance of the α,α-disubstituted nature of the amino acids Aib and α-MePhe located at the N-terminus.

This series of Aib analogs was tested on tick and mosquito receptor expressing cell lines. An analysis of the biostable Aib analogs showed that all of them elicit a very strong bioluminescence response in both tick and mosquito receptor expressing cell lines. Analog K-Aib-1, [Aib][FF][Aib]WGa was found to be the most active, demonstrating a statistically significant higher potency to the control agonist FFFSWGa in both receptor systems. In the mosquito this double Aib analog proved to be much more potent than the single Aib analog and was statistically different from the control, FFFSWGa. Indeed, the EC$_{50}$ of analog K-Aib-1 indicated that it was eight times more potent than the control agonist and, remarkably, more than 3-fold more active than the natural Aedes kinin-II, EC$_{50}$ = 164 nM (Taneja-Bageshwar et al., 2008b), representing a milestone as the first such biostable analog to either match and/or exceed the activity of insect kinins native to mosquitoes or any other arthropod pest. Analog K-Aib-1 proved to be greatly superior to a previously reported insect kinin analog incorporating

![Fig. 4.](image-url)
two β-amino acids to protect the primary and secondary proteolytic sites. The β-amino acid analog demonstrated a higher EC₅₀ value indicating it was 2-fold less potent than the control agonist in the tick receptor and at least 4-fold less potent than native insect kinins in the mosquito receptor (Taneja-Bageshwar et al., 2008b). Furthermore, this disubstituted β-amino acid analog demonstrated only 65% of the maximal response of insect kinins, in contrast with the 100% maximal response observed for analog K-Aib-1 in assays with both the arthropod receptor expressing cell lines.

Three other Aib analogs in this study, K-Aib-2 ([xMe]FF-[Aib]WGa), K-Aib-3 (Ac-R[Aib]FF[Aib]WGa), and K-Aib-4 (Ac-R[p][FF][Aib]WGa) had EC₅₀ values of 111 ± 30, 112 ± 40, and 211 ± 30 nM, respectively, in the mosquito receptor (Table 2). In contrast, analog K-Aib-6 ([Aib]-[Aib]-[Aib]-RF-[Aib]WGa) containing 4 Aib residues appended to the N-terminus of the insect kinin hexapeptide core, proved to be the least active of this series of six Aib-containing analogs in both receptors. The string of bulky Aib residues present in the N-terminal region of this analog likely interferes with optimal interaction of the analog with the two arthropod kinin receptors. When tested for Malpighian tubule diuretic activity, analog K-Aib-1 proved to be as active as native Aedes kinin I in the mosquito receptor (Table 2). In contrast, analog K-Aib-6 ([Aib]-[Aib]-[Aib]-RF-[Aib]WGa) containing 4 Aib residues appended to the N-terminus of the insect kinin hexapeptide core, proved to be the least active of this series of six Aib-containing analogs in both receptors. The string of bulky Aib residues present in the N-terminal region of this analog likely interferes with optimal interaction of the analog with the two arthropod kinin receptors. When tested for Malpighian tubule diuretic activity, analog K-Aib-1 proved to be as active as native Aedes kinin I in the mosquito receptor (Table 2). In contrast, analog K-Aib-6 ([Aib]-[Aib]-[Aib]-RF-[Aib]WGa) containing 4 Aib residues appended to the N-terminus of the insect kinin hexapeptide core, proved to be the least active of this series of six Aib-containing analogs in both receptors. The string of bulky Aib residues present in the N-terminal region of this analog likely interferes with optimal interaction of the analog with the two arthropod kinin receptors. When tested for Malpighian tubule diuretic activity, analog K-Aib-1 proved to be as active as native Aedes kinin I in the mosquito receptor (Table 2). In contrast, analog K-Aib-6 ([Aib]-[Aib]-[Aib]-RF-[Aib]WGa) containing 4 Aib residues appended to the N-terminus of the insect kinin hexapeptide core, proved to be the least active of this series of six Aib-containing analogs in both receptors. The string of bulky Aib residues present in the N-terminal region of this analog likely interferes with optimal interaction of the analog with the two arthropod kinin receptors. When tested for Malpighian tubule diuretic activity, analog K-Aib-1 proved to be as active as native Aedes kinin I in the mosquito receptor (Table 2).


