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Mosquito-Derived Anophelin Sulfoproteins Are Potent Antithrombotics

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Supporting Information

ABSTRACT: The anophelins are small protein thrombin inhibitors that are produced in the salivary glands of the *Anopheles* mosquito to fulfill a vital role in blood feeding. A bioinformatic analysis of anophelin sequences revealed the presence of conserved tyrosine residues in an acidic environment that were predicted to be post-translationally sulfated *in vivo*. To test this prediction, insect cell expression of two anophelin proteins, from *Anopheles albimanus* and *Anopheles gambiae*, was performed, followed by analysis by mass spectrometry, which showed heterogeneous sulfation at the predicted sites. Homogeneously sulfated variants of the two proteins were subsequently generated by chemical synthesis via a one-pot ligation−desulfurization strategy. Tyrosine sulfation of the anophelins was shown to significantly enhance the thrombin inhibitory activity, with a doubly sulfated variant of the anophelin from *A. albimanus* exhibiting a 100-fold increase in potency compared with the unmodified homologue. Sulfated anophelins were also shown to exhibit potent *in vivo* anticoagulant and antithrombotic activity.

INTRODUCTION

Hematophagous invertebrates have evolved highly effective mechanisms to facilitate the acquisition of a blood meal via the production of exquisitely potent and selective inhibitors of host coagulation factors, in particular the serine proteinase thrombin.[1,2] These organism-evolved molecules have been reported to possess reduced toxicity and immunogenicity, privileged properties that are often difficult to design or predict in small-molecule thrombin inhibitors. It is therefore not surprising that molecules derived from blood feeding organisms have found utility in therapy, with the archetypal anticoagulant hirudin (from the medicinal leech *Hirudo medicinalis*) and structural analogues of this protein being used clinically for a range of thromboembolic disorders and postsurgery.[3]

One feature of these natural thrombin-inhibiting molecules that is often overlooked is the potential presence of post-translational modifications and the effect that these may have on the activity and/or stability of the native peptides and proteins. Interestingly, despite the fact that recombinant hirudin used clinically does not bear any modifications, the native leech protein was first identified with a tyrosine sulfate modification, which was shown to impart a ca. 10-fold improvement in thrombin inhibitory activity.[4] We have recently discovered an important role of tyrosine sulfation for the thrombin inhibitory activity of two saliva-derived thrombin inhibitors, madanin-1 and chimadanin, from the bush tick *Haemaphysalis longicornis*.[5] These two inhibitors were shown to exert their activity by binding to the active site and exosite II of human thrombin, with sulfation at two conserved tyrosine residues providing significant improvement in the thrombin inhibitory activity. This improved potency was attributed to enhanced binding of the inhibitor to exosite II of thrombin mediated by electrostatic interactions between the sulfated tyrosine residues and conserved basic residues on the enzyme, as identified through X-ray crystallography.[5] On the basis of these findings, we now hypothesize that tyrosine sulfation could be a ubiquitous modification of salivary proteins from other

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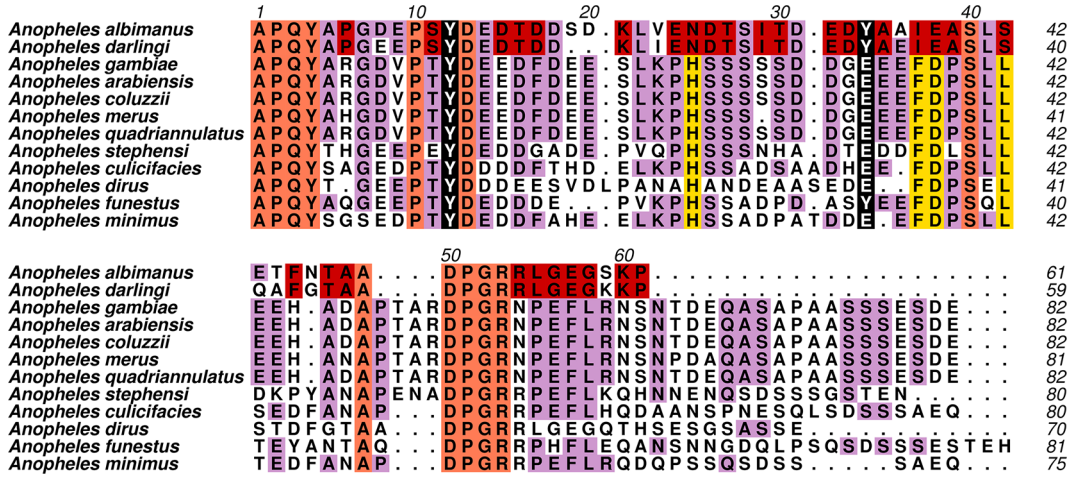

Figure 1. Sequence alignment of members of the anophelin family showing strictly conserved residues (salmon), widely conserved amino acids (purple), conserved residues in the "new-world" (red) or "old-world" (yellow) anophelins, and potential sulfated tyrosine (Tyr or Y) sites (black).[6] NB: The C-terminal sulfation site (position 34 in A^{Aa}) is not strictly conserved and a negatively charged glutamate residue can instead be found at this position in anophelins from old-world mosquitoes.[6]

hematophagous organisms that could serve as a general mechanism for modulating anticoagulant activity during feeding.

The anophelins are an interesting family of thrombin-inhibiting proteins produced in the salivary glands of the *Anopheles* malaria mosquito vector.[6,7] To date, the genomes of 16 *Anopheles* mosquitoes have been reported (Figure 1),[8] leading to the identification of a number of anophelin protein homologues in "old-world" mosquitoes, e.g., *Anopheles funestus*, *Anopheles gambiae*, and *Anopheles stephensi*, as well as "new-world" mosquitoes such as *Anopheles albimanus* and *Anopheles darlingi*.[6,7] Anophelin proteins that are recombinantly expressed in *Escherichia coli* have previously been shown to exhibit potent inhibitory activity against human thrombin via a binding mode distinct from that of other proteinaceous thrombin inhibitors that have been identified to date. In contrast to hirudin, where the N-terminus blocks the active site of thrombin and the C-terminus binds to exosite I, the anophelins bind in the opposite orientation, with the C-terminal portion occupying the active site (thus disrupting the catalytic triad) while the N-terminus extends onto exosite I.[6,9] Guided by sequence analysis of a number of other peptides and proteins that are known to be post-translationally sulfated, we used a bioinformatics approach to predict that each of the proteins within the anophelin family possessed one or two conserved tyrosine residues that would be candidates for sulfation by tyrosylprotein sulfotransferase (TPST) enzymes *in vivo*. Specifically, the anophelins possess conserved tyrosine residues embedded within highly acidic stretches within their amino acid sequences, which is a predictor for post-translational sulfation by TPSTs.[10−13] While to date tyrosine sulfation has not been suggested or identified as a modification of mosquito salivary proteins, this analysis provided the impetus to test the hypothesis that the anophelins are natively sulfated and that this modification would directly affect the inhibitory activity of the proteins against thrombin. To test this hypothesis, we chose to investigate an "old-world" mosquito and a "new-world" mosquito as examples in order to probe the effect of tyrosine sulfation on the inhibition of thrombin by the family of anophelin proteins. For this purpose, we selected anophelins from *A. gambiae* and *A. albimanus* (herein denoted as A^{Ag} and A^{Aa}, respectively) that exhibited the most potent inhibition of thrombin in unmodified recombinant form from each family.[6] In this study, we demonstrate that sulfation of A^{Aa} and A^{Ag} occurs in insect cells (by baculovirus-assisted recombinant expression) at the sites predicted in our bioinformatics analysis. We also demonstrate an efficient synthetic method to access homogeneously sulfated A^{Aa} and A^{Ag} through the use of ligation−desulfurization chemistry. Importantly, these synthetic sulfoproteins were shown to be significantly more potent thrombin inhibitors than the unmodified counterparts and possessed potent antithrombotic activity in an *in vivo* thrombosis model.

RESULTS

Anophelins Are Sulfated in Insect Cells. Access to homogeneous samples of post-translationally modified proteins is challenging, as extremely low quantities of the proteins can typically be isolated from the natural source, in this case the salivary glands of the *Anopheles* mosquito. Typical prokaryotic expression systems lack the TPST enzymatic machinery to generate appropriately post-translationally modified molecules.[13] Therefore, we first expressed A^{Aa} and A^{Ag} in a eukaryotic system to investigate whether the proteins could be sulfated *in vivo*. For this purpose, we used *Trichoplusia ni* insect cells as a model of the *Anopheles* mosquito. Specifically, codon-optimized sequences encoding A^{Aa} and A^{Ag} were designed as N-terminal fusions with the honeybee mellitin signal sequence in order to direct the recombinant proteins to the secretory pathway. Following expression, the cell medium containing the secreted proteins was analyzed by nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC−MS/MS). Analysis of insect-cell-expressed A^{Aa} showed a heterogeneous population of sulfated protein with approximately 0.8% monosulfated and 41% disulfated variants (see Figure 2A and the Supporting Information, Table 1). MS/MS analysis of full length A^{Aa} with complementary fragmentation approaches, including higher-collisional dissociation (HCD) and electron transfer dissociation (ETD), confirmed the presence of sulfation via neutral loss but was unable to localize the sites of modification (Figure 2B). A tryptic digest of A^{Aa} revealed an N-terminal trypsin fragment

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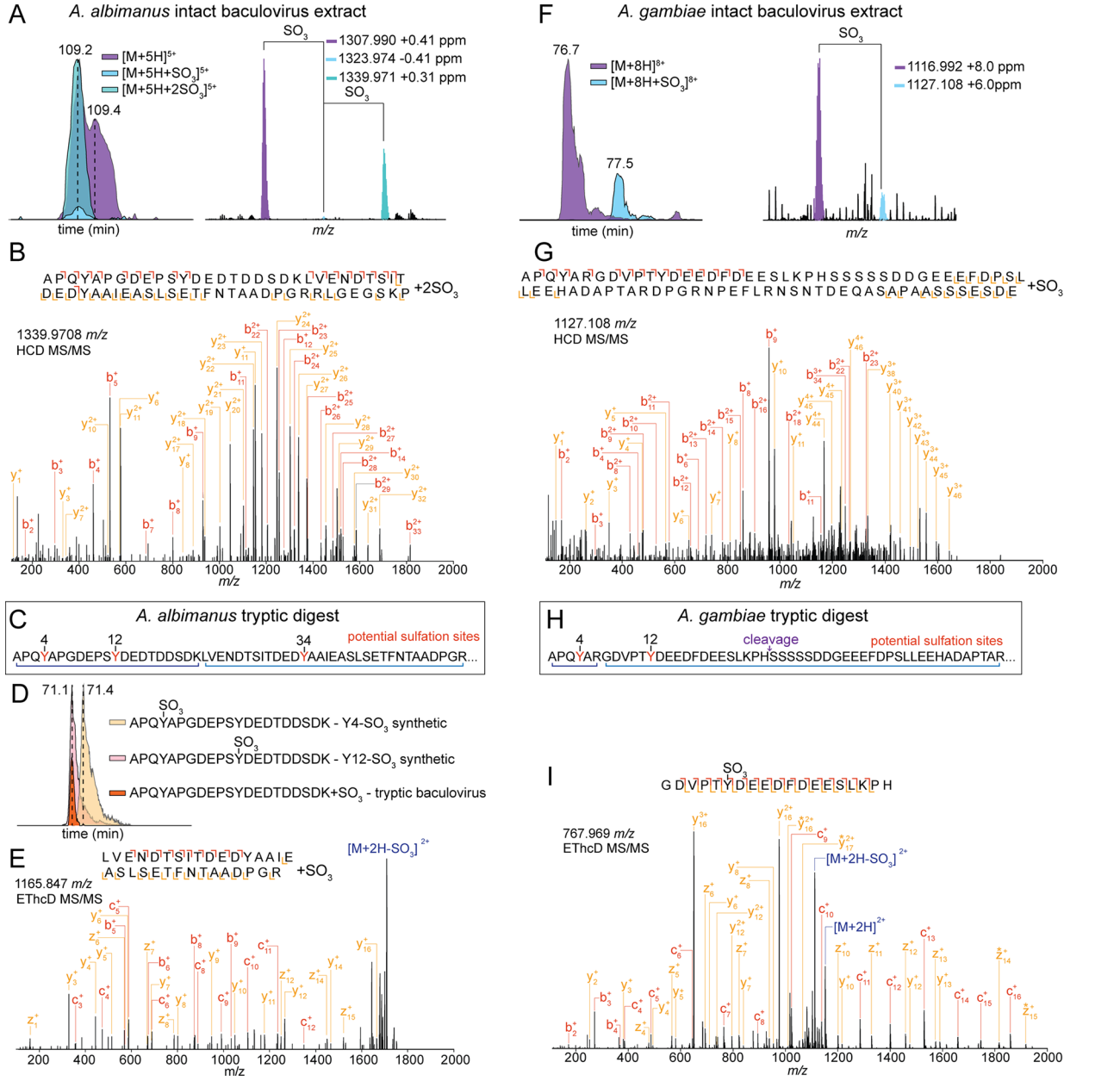

Figure 2. Analysis of A^{Aa} and A^{Ag} produced in *Trichoplusia ni* insect cells by nanoLC−MS/MS. (A) $[M + 5H]^{5+}$ extracted ion chromatograms and average precursor MS1 spectrum of full-length A^{Aa} in the unmodified, mono-, and disulfated isoforms. (B) HCD MS/MS of disulfated A^{Aa} $[M + 5H + 2SO_3]^{5+}$. (C) *In silico* tryptic digestion of A^{Aa} highlighting the potential sites of tyrosine sulfation. (D) Extracted ion chromatograms of synthetic and trypsin-digested monosulfated peptide APQYAPGDEPSYDEDTDDSDK of A^{Aa}. (E) EThcD MS/MS of the monosulfated peptide LVENDTSITDEDYAAIEASLSETFNTAADPGR of A^{Aa}. (F) $[M + 8H]^{8+}$ extracted ion chromatograms and average precursor MS1 spectrum of full-length A^{Ag} in the unmodified and monosulfated isoforms. (G) HCD MS/MS of disulfated A^{Ag} $[M + 8H + 2SO_3]^{8+}$. (H) *In silico* tryptic digestion of A^{Ag} highlighting the potential sites of tyrosine sulfation. (I) EThcD MS/MS of the monosulfated peptide GDVPTYDEEDFDEESLKPH of A^{Ag} (* indicates ions that contain SO_3^-).

(APQYAPGDEPSYDEDTDDSDK) possessing two tyrosine residues (Tyr4 and Tyr12, underlined) in addition to a second fragment (LVENDTSITDEDYAAIEASLSETFNTAADPGR) containing a single tyrosine residue (Tyr34, underlined) (Figure 2C). In order to pinpoint the site of sulfation, we synthesized two monosulfated tryptic peptides with sulfation at Tyr4 and Tyr12 (see the Supporting Information). By comparing the retention time to those of the trypsinized fragments from the insect cell expression, we were able to demonstrate that Tyr12 was (as predicted) the site of sulfation on A^{Aa} (Figure 2D). Analysis of the medium from the A^{Ag} expression by nanoLC−MS revealed a heterogeneous mixture of a protein with approximately 20% monosulfation together with the unsulfated form, which was confirmed by MS/MS analysis (see Figure 2F,G and the Supporting Information, Table 2). It should be noted that in the case of A^{Ag} a glutamate residue is found in the position equivalent to Tyr34 in A^{Aa}, similar to many other anophelins from old-world mosquitoes (Figure 1), precluding the possibility of sulfation at this site. As with A^{Aa}, two tyrosine residues (Tyr4 and Tyr12) are present within the N-terminal region, but the presence of Arg at position 6 of this protein enabled the generation of two tryptic

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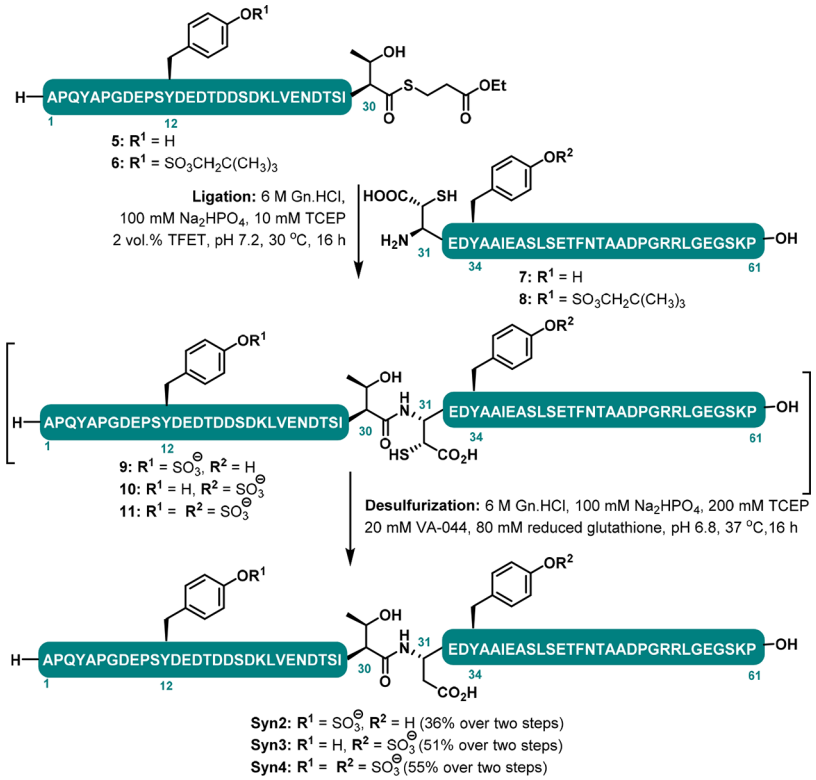
Scheme 1. Synthesis of Homogeneously Sulfated A^{Aa} Variants Syn2−4 via One-Pot Ligation−Desulfurization Chemistry



peptides in addition to minor amounts of an endogenous cleavage event at His25 (Figure 2H). MS/MS analysis of the fragment GVDPTYDEEDFDEESLKPH confirmed the presence of sulfation and was able to unequivocally localize the modification to Tyr12 (Figure 2I).

Having established that A^{Aa} and A^{Ag} can be sulfated *in vivo* (albeit as heterogeneous mixtures), we next sought to investigate the effect of a given tyrosine sulfate modification on thrombin inhibitory activity, which necessitated access to homogeneously sulfated variants of the anophelins. Toward this end, we initially envisaged the use of amber-stop codon suppression,[14] a methodology that has been used by Schultz and co-workers for the production of sulfated hirudin.[15,16] Importantly, this technology enables ribosomal incorporation of sulfated tyrosine at a genetically encoded site using a mutated tRNA/tRNA synthetase pair and therefore removes the heterogeneity of enzymatic TPST-catalyzed sulfation observed in the insect cell expression system. Because *in vivo* expression with sulfotyrosine requires unusually high concentrations of the unnatural amino acid[17] (presumably because of poor uptake by the cells), we chose to access the sulfated A^{Aa} and A^{Ag} through cell-free expression with amber codon incorporation of the sulfated tyrosine residues at the specific sites shown to be sulfated in the baculovirus system. Briefly, A^{Aa} and A^{Ag} were expressed *in vitro* supplemented with sulfotyrosine, optimized suppressor-tRNA,[18] and purified sulfotyrosyl-tRNA synthetase[17,19] or polyspecific *p*-cyanophenylalanyl-tRNA synthetase. The anophelin constructs were fused N-terminally to His_6-tagged ubiquitin, which permitted affinity purification of the expressed protein and liberation of the native sequence through proteolytic cleavage with deubiquitinase[20] (see the Supporting Information for methods and characterization data). By means of this cell-free expression strategy, a homogeneous variant of the monosulfated A^{Ag} **Cfe1** (displaying sulfation at Tyr12) was generated, together with the truncated polypeptide in which the ribosomal synthesis stopped at the amber codon. **Cfe1** could be easily separated from the truncated polypeptide via reversed-phase HPLC to generate pure monosulfated **Cfe1**. Unfortunately, attempts to express doubly sulfated A^{Aa} were unsuccessful. Nonetheless, with the sulfated A^{Ag} **Cfe1** in hand, we next assessed the effect of sulfation at Tyr12 on the inhibitory activity against human α-thrombin by measuring the rate of thrombin-catalyzed cleavage of the chromogenic substrate Tos-Gly-Pro-Arg-*p*-nitroanilide in the presence of **Cfe1** (see the Supporting Information). Importantly, we were able to demonstrate that **Cfe1** was a significantly more potent thrombin inhibitor (K_i = 1.13 ± 0.21 pM) than the unsulfated counterpart (K_i = 5.50 ± 1.26 pM),[9] thus validating our hypothesis that sulfation is capable of modulating the thrombin inhibitory activity of anophelins. However, as a result of the small quantities of A^{Ag} produced (300 μg from a 10 mL cell-free reaction) coupled with the fact that in our hands doubly sulfated A^{Aa} could not be accessed through this methodology, we sought an alternative means to access the entire family of differentially sulfated variants of the two proteins. For this purpose, we turned to a chemical synthesis approach as a means of preparing the entire family of sulfoforms for both A^{Aa} and A^{Ag} in sufficient quantities for in depth *in vitro* and *in vivo* studies.

Chemical Synthesis of Homogeneously Sulfated A^{Aa} and A^{Ag} Proteins. **Syn1−Syn4** represented the differentially sulfated A^{Aa} and A^{Ag} targets for chemical synthesis. As both A^{Aa} and A^{Ag} exceed the typical polypeptide length limit for preparation by Fmoc-strategy solid-phase peptide synthesis (SPPS) (~40 residues), it was envisioned that these could be accessed via convergent peptide ligation chemistry. Specifically, our synthetic strategy centered on the use of the native chemical ligation[21]−desulfurization chemistry that has recently

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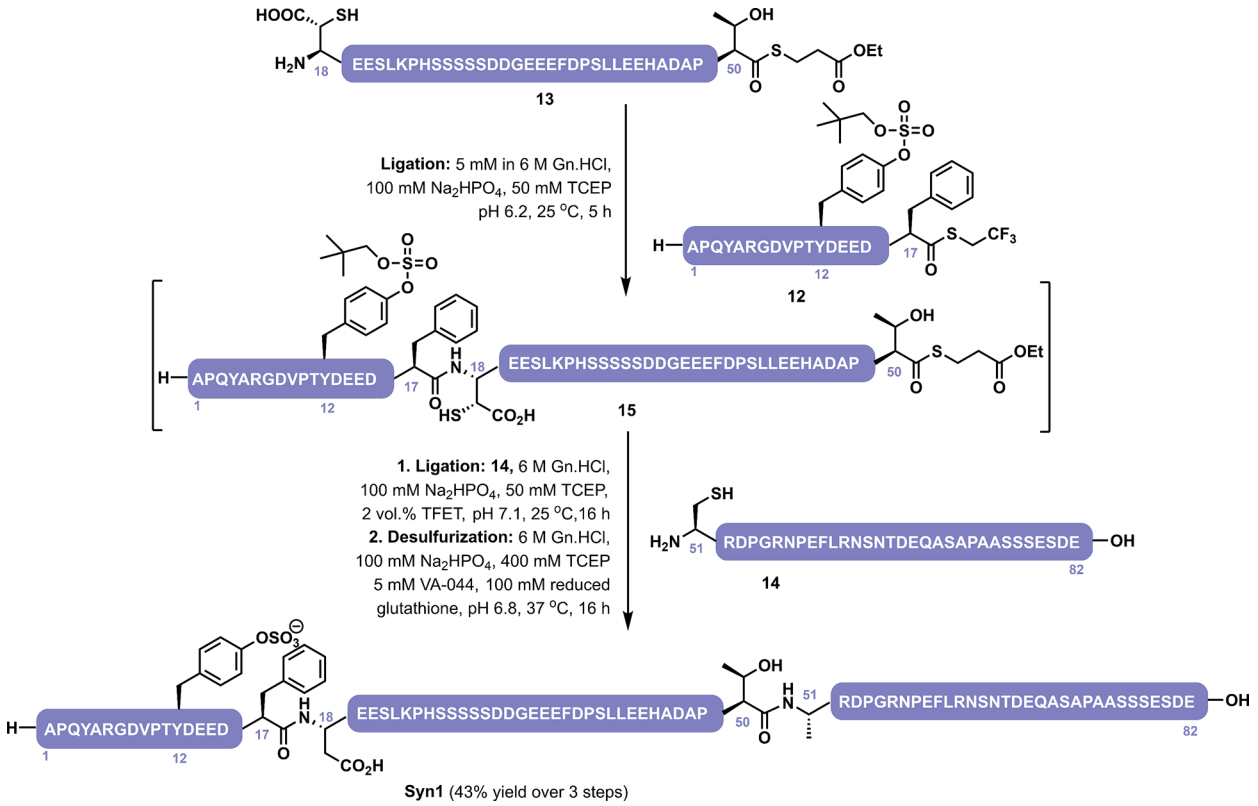
Scheme 2. Synthesis of Sulfated A^{Ag} Syn1 via a One-Pot Kinetically Controlled Ligation−Desulfurization in the N- to C-Terminal Direction



been developed for a range of thiol-derived amino acids.[22−24] We began by designing a synthetic strategy that would enable access to the three possible sulfated variants of the 61-residue A^{Aa}. Toward this end, we envisaged disconnection near the middle of the sequence between Thr30 and Asp31, which would enable assembly through ligation−desulfurization chemistry at β-thiol aspartate.[25] We conceived that the sulfoprotein library could be assembled from two N-terminal peptide thioester fragments (A^{Aa}1−30), **5** and **6**, with tyrosine or sulfated tyrosine at Tyr12, respectively, and two C-terminal fragments (A^{Aa}31−61) bearing a β-thiol aspartate[25] moiety at Asp-31 and tyrosine (**7**) or sulfated tyrosine (**8**) at Tyr34 (Scheme 1). The (sulfo)peptide fragments **5**−**8** were readily synthesized by Fmoc-strategy SPPS and purified to homogeneity via reversed-phase HPLC (see the Supporting Information for synthetic details and characterization data).

With the target fragments in hand, the A^{Aa} sulfoforms (**Syn2**−**4**) were next assembled using a one-pot ligation−desulfurization strategy. Specifically, peptide thioester **5** or sulfopeptide thioester **6** and β-thiol aspartate-containing peptide **7** or sulfopeptide **8** were reacted in aqueous denaturing buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 10 mM TCEP, pH 7.2) with the addition of trifluoroethanethiol (TFET)[26] to accelerate the ligation reactions through the *in situ* formation of a C-terminal TFET thioester from the unreactive thioester in **5** and **6**. Under these conditions, each of the ligation reactions was complete within 16 h as judged by HPLC−MS analysis (see the Supporting Information). Interestingly, the ligation conditions also resulted in concomitant deprotection of the neopentyl groups to afford the free tyrosine sulfate esters, thereby affording the intermediate proteins **9**−**11**. These were not isolated, but rather, *in situ* treatment with the water-soluble radical initiator VA-044[24] in the presence of the reductant TCEP and a hydrogen atom source (reduced glutathione) effected clean desulfurization of the β-thiol aspartate at the ligation junction to afford the desired target sulfoproteins **Syn2**−**4** in crude form. Following a single reversed-phase HPLC purification (using 0.1 M ammonium formate and acetonitrile as eluents to prevent acidolysis of the fragile tyrosine sulfate ester[13]), this one-pot ligation−desulfurization sequence provided synthetic A^{Aa} proteins with sulfation at Tyr12 (**Syn2**), Tyr34 (**Syn3**), and both Tyr12 and 34 (**Syn4**) in good yields over the ligation and desulfurization steps.

Having successfully prepared the three possible sulfoforms of A^{Aa}, we next focused on the preparation of the longer (82-residue) A^{Ag} by chemical synthesis, in this case a singly sulfated variant at Tyr12 (**Syn1**; *vide supra*). In this case, the increased length of the polypeptide required the ligation-based assembly of three suitably functionalized peptide fragments. We envisaged that this could be achieved in a one-pot reaction using a kinetically controlled ligation of three fragments in the N- to C-terminal direction[26,27] followed by *in situ* desulfurization. Specifically, we chose to disconnect the sequence between Phe17 and Asp18 and between Thr50 and Ala51. This strategy gave rise to three target fragments for synthesis: A^{Ag}(1−17) **12** bearing a neopentyl-protected sulfate functionality at Tyr12 and a reactive *S*-trifluoroethyl thioester on the C-terminus, A^{Ag}(18−50) **13** containing a β-thiol aspartate moiety on the N-terminus and functionalized with an unreactive *S*-propionate thioester on the C-terminus, and A^{Ag}(51−82) **14** possessing a cysteine residue in place of the native Ala51 on the N-terminus. Each of these fragments was successfully prepared via Fmoc-strategy SPPS (see the Supporting Information for synthetic methods and characterization). The one-pot synthesis of **Syn1** began

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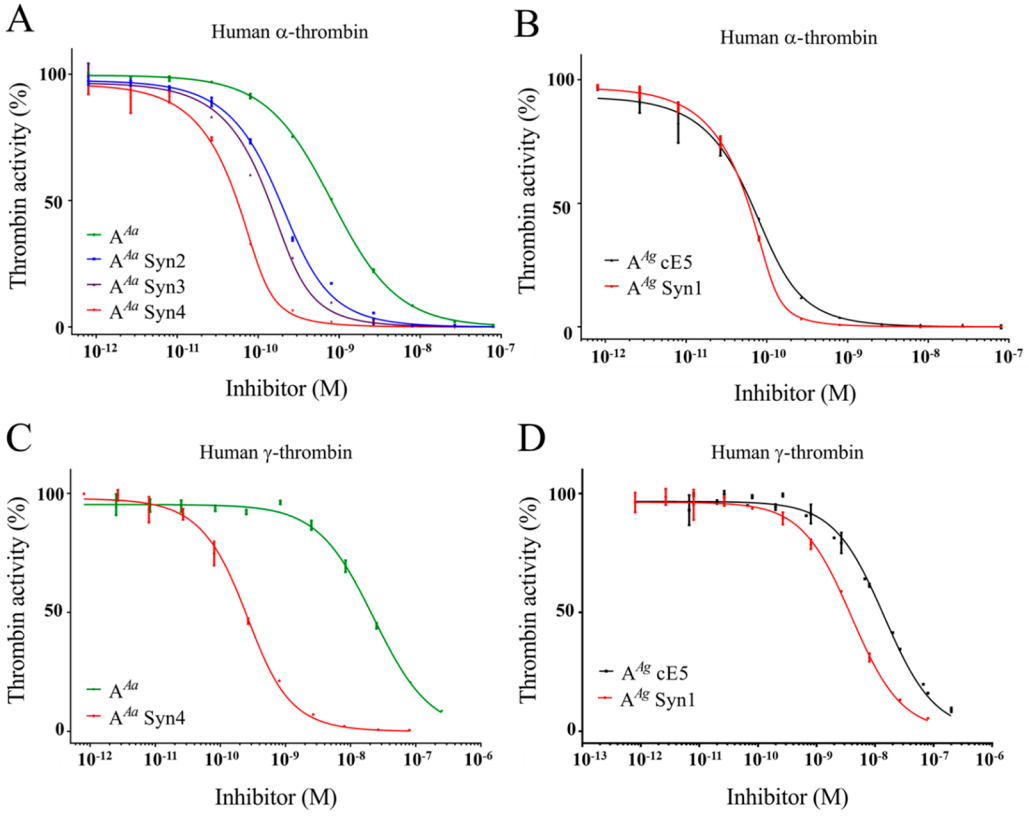

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Inhibitor	K_i Human α-thrombin (pM)	TT (s)	K_i Human γ-thrombin (nM)
A^{Aa}	128.3 ± 2.0; R^2 = 0.999	37	6.1 ± 0.3; R^2 = 0.995
A^{Aa} **Syn2**	16.7 ± 1.2; R^2 = 0.998	61.5	—
A^{Aa} **Syn3**	7.7 ± 1.1; R^2 = 0.995	62	—
A^{Aa} **Syn4**	1.7 ± 0.6; R^2 = 0.997	72.5	0.041 ± 0.003; R^2 = 0.996
A^{Ag}	5.5 ± 1.3[9]	64	3.8 ± 0.2[9]
A^{Ag} **Syn1**	1.1 ± 0.2; R^2 = 0.999	>140	1.05 ± 0.06; R^2 = 0.995

Figure 3. Tyrosine sulfation of A^{Aa} and A^{Ag} significantly improves thrombin inhibition and prolongs blood coagulation *in vitro*. (A, B) Dose response curves for the inhibition of α-thrombin activity by increasing concentrations of (A) unsulfated A^{Aa} and synthetic sulfated variants **Syn2−4** and (B) recombinant A^{Ag} (**cE5**)[9] and synthetic sulfated variant **Syn1**. (C, D) Dose response curves for the inhibition of γ-thrombin activity by increasing concentrations of (C) unsulfated A^{Aa} and doubly sulfated variant **Syn4** and (D) recombinant A^{Ag} (**cE5**)[9] and synthetic sulfated variant **Syn1**. (E) Inhibition constants (K_i) for A^{Aa} and A^{Ag} sulfoforms, determined by fitting the inhibited steady-state velocity data to the Morrison model. The given K_i values ± SEM are representative of two independent experiments. Also shown are the thrombin times (TTs), measuring the clotting time of human plasma in the presence of different A^{Aa} and A^{Ag} sulfoforms (at 5 nM). In the absence of inhibitor, the TT was 21.3 s (mean of four replicates), and TT values were not evaluated past 140 s. The TT values reported are means of two independent determinations. Errors are depicted as standard deviations from two independent measurements.

with the chemoselective ligation between reactive sulfopeptide thioester **12** and bifunctional peptide fragment **13**, which was carried out under standard buffer conditions (6 M Gn·HCl, 100 mM Na_2HPO_4, 10 mM TCEP, pH 6.2) in the absence of an external thiol additive and reached completion within 5 h as determined by HPLC−MS to afford ligation product **15** (Scheme 2; see the Supporting Information for the crude data). It should be noted that the shorter reaction time led to only partial (25%) loss of the neopentyl ester from the sulfated tyrosine residue. The ligation product was not isolated at this stage, but rather, TFET was added to the crude ligation reaction to activate the unreactive alkyl thioester on the C-terminus of **15** to generate the reactive *S*-trifluoroethyl thioester, followed by the addition of fragment **14** bearing an N-terminal cysteine residue to facilitate native chemical ligation. In this case, the ligation reaction required 16 h to reach completion and occurred with concomitant deprotection of all of the remaining neopentyl ester. All that remained for the completion of the synthesis was desulfurization of the non-native β-thiol aspartate and cysteine residues to Asp18 and Ala51, respectively. The crude reaction mixture was therefore treated with VA-044, TCEP, and reduced glutathione at 37 °C, and after 16 h the reaction had reached completion as judged by HPLC−MS analysis to afford the A^{Ag} sulfoprotein **Syn1** in

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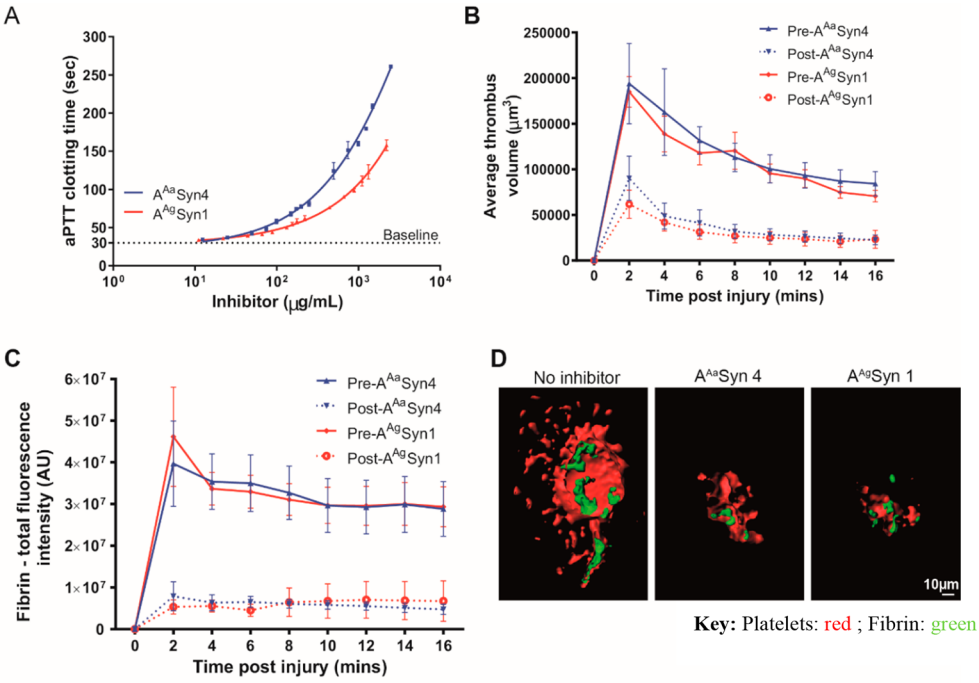

Figure 4. Effect of thrombin inhibition *in vitro* and antithrombotic effect of A^{Aa} **Syn4** and A^{Ag} **Syn1** in a needle injury thrombus model. (A) Dose response curves of activated partial thromboplastin time (aPTT) in pooled C57Bl/6 plasma for **Syn4** and **Syn1**. (B, C) Time courses of changes in (B) platelet thrombus volume and (C) fibrin total fluorescence intensity over 16 min pre- and post-injection of **Syn4** (blue) and **Syn1** (red). (D) Representative confocal fluorescence images depicting platelet thrombus and fibrin formation on injured endothelium with no inhibitor, **Syn4**, and **Syn1**. Data points represent mean ± SEM of three to five independent pooled plasma samples for the aPTT assay and three independent animals with four to seven injuries each for needle injury experiments, with hirudin as a positive control (see the Supporting Information).

crude form. It should be noted that in this case the concentration of VA-044 was reduced (5 mM vs 20 mM for **Syn2**−**4**) to prevent the formation of VA-044 adducts, which were observed when higher concentrations of the radical initiator were employed. Following reversed-phase HPLC purification, **Syn1** bearing a sulfotyrosine residue at residue Tyr12 was isolated in 43% isolated yield following the one-pot process that included two ligations and a double desulfurization step.

Sulfation Modulates the Thrombin Inhibitory Activity of the Anophelins. Having successfully prepared sulfated A^{Ag} (**Syn1**) and the three sulfated variants of A^{Aa} (**Syn2**−**4**), we next assessed the *in vitro* inhibition of human α-thrombin by the synthetic sulfoproteins to evaluate the effect of tyrosine sulfation on the activity. The inhibitory potencies of **Syn1** and **Syn 4** were also assessed against human γ-thrombin, which has a disrupted exosite I. Thus, screening against both α- and γ-thrombin was used to provide an indirect measure of the mode of binding of the inhibitors to thrombin, i.e., whether the anophelins may exhibit enhanced exosite II binding when sulfated.[28,29] Inhibition of the amidolytic activities of α- and γ-thrombin by **Syn1**−**4** was assessed using the kinetic assay described above by measuring proteolytic cleavage of the chromogenic substrate Tos-Gly-Pro-Arg-*p*-nitroanilide (see Figure 3). As expected, on the basis of the data obtained for **Cfe1**, Tyr12 sulfation of A^{Ag} in **Syn1** led to a mild enhancement in the thrombin inhibitory activity (K_i = 1.12 ± 0.23 pM) compared with the unsulfated A^{Ag} counterpart (K_i = 5.50 ± 1.26 pM) (Figure 3B,E).[9] A more dramatic effect was observed when sulfation was introduced to A^{Aa} in **Syn2**−**4** (Figure 3A,E). Specifically, sulfation at Tyr12 in **Syn2** led to a 7-fold improvement in α-thrombin inhibition (K_i = 16.7 ± 1.2 pM for **Syn2** vs K_i = 128.3 ± 2.0 pM for unsulfated A^{Aa}). Monosulfation at Tyr34 in **Syn3** provided a more pronounced improvement in activity over the unmodified A^{Aa} (K_i = 7.7 ± 1.1 pM). However, sulfation at both Tyr12 and Tyr34 in **Syn4** led to a synergistic improvement in activity with a K_i of 1.7 ± 0.6 pM. Interestingly, while unsulfated A^{Ag} and sulfated **Syn1** lost 2 and 3 orders of magnitude activity against γ-thrombin (Figure 3D,E), strongly suggesting an inhibitory mode involving exosite I binding, unsulfated A^{Aa} and doubly sulfated **Syn4** only lost 48−24-fold activity against γ-thrombin (Figure 3C,E). This may suggest that A^{Aa} might overcome the decrease in affinity brought about by the disruption of exosite I by establishing an additional interaction with exosite II.[6] Having shown that sulfation of the anophelins led to an improvement in thrombin inhibitory activity *in vitro*, we next assessed how the sulfated variants affected the time for thrombin-catalyzed blood coagulation using a clinical thrombin time (TT) assay. Gratifyingly, when used at 5 nM concentration, both doubly sulfated A^{Aa} (**Syn4**, TT = 72.5 s) and sulfated A^{Ag} (**Syn1**, TT ≥ 140 s) led to a significant increase in thrombin time over the unmodified counterparts (37 and 64 s for unmodified A^{Aa} and A^{Ag}, respectively).

Anophelin Sulfoproteins Possess Potent Antithrombotic Activity *in Vivo*. Having shown that sulfation of A^{Aa} and A^{Ag} significantly improved the thrombin inhibitory potency and anticoagulant activity *in vitro*, we next investigated the *in vivo* antithrombotic activity of the most potent synthetic variants, **Syn1** and **Syn4**. We initially confirmed that sulfated A^{Aa} and A^{Ag} retained potent inhibitory activity against mouse thrombin by assessing the impact of these inhibitors on the *in vitro* mouse activated partial thromboplastin time (aPTT), a measure of the time taken for clotting to occur via the intrinsic pathway. **Syn1**

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and **Syn4** prolonged the aPTT in a concentration-dependent manner (Figure 4A) over a similar dose range required to inhibit human thrombin. To determine the dose requirements of **Syn1** and **Syn4** to produce an anticoagulant effect *in vivo*, we performed *ex vivo* aPTT assays on mouse plasma post-i.v. injection of **Syn1**, **Syn4**, or hirudin as a control. **Syn1** and **Syn4** each prolonged the aPTT in a concentration-dependent manner (see the Supporting Information). The increase in aPTT correlated well with the prolongation in tail bleeding time (see the Supporting Information), confirming the ability of these peptides to counter the hemostatic function of thrombin *in vivo*. On the basis of the *ex vivo* aPTT data, we employed therapeutically relevant doses of each inhibitor (typically a 2−3-fold prolongation of the aPTT, i.e., 25.4×10^{-2} μmol/kg for A^{Aa} **Syn4** and 44.3×10^{-2} μmol/kg for A^{Ag} **Syn1**) to assess their antithrombotic potentials *in vivo*. For these studies, we employed a localized needle injury model in the mesenteric venous circulation of mice that leads to the formation of highly reproducible mural thrombi composed of platelets and fibrin, which can be visualized via Alexa 649 αGP1bβ and Alexa 546 αfibrin, respectively.[30] Treatment with the doubly sulfated A^{Aa} **Syn4** and monosulfated A^{Ag} **Syn1** resulted in major reductions in platelet thrombus volume (Figure 4B) that coincided with reduced formation of fibrin polymers (Figure 4C), culminating in significant reduction of the total thrombus size in both treatment groups (Figure 4D; see the Supporting Information for raw data). Importantly, this potent *in vivo* antithrombotic activity of sulfated A^{Aa} and A^{Ag} suggests that these molecules may prove useful as leads for the development of effective direct thrombin inhibitors that operate through a novel binding mode compared with those currently used in the clinic. Studies toward this end will be the focus of future work within our laboratories.

DISCUSSION

Hematophagous organisms produce a suite of potent inhibitors of the blood coagulation cascade to facilitate blood feeding activities. A number of thrombin-inhibiting peptides and proteins have recently been identified in the sialome of a variety of blood feeders, including leeches, ticks, and flies. Post-translational modifications made to these proteins have largely been overlooked, presumably because of the paucity of material that can be isolated and characterized from these small organisms. Previous biochemical characterization of the mosquito-derived anophelins,[6,7] similar to tick salivary proteins,[31] relied on recombinant inhibitors expressed in prokaryotic systems that do not possess the requisite machinery to install PTMs specific to secreted eukaryotic proteins. It is therefore likely that the thrombin inhibitory activity and, by extension, the anticoagulant and antithrombotic potency have been underestimated compared with the activity of the proteins produced natively in the salivary glands. A key finding that post-translational sulfation of tyrosine residues on specific leech and tick proteins significantly improves the thrombin inhibitory activity by 1−3 orders of magnitude exemplifies the importance of post-translational modifications for the modulation of bioactivity.[5,32]

Tyrosine sulfation has yet to be observed on salivary proteins from mosquitoes, a possibility that we explored in this work. A bioinformatic approach was employed to predict the presence of this post-translational modification on the anophelins, the thrombin-inhibiting proteins from the *Anopheles* mosquito. Expression of an anophelin from the "new-world" mosquito *A. albimanus* (A^{Aa}) and from the "old-world" mosquito *A. gambiae* (A^{Ag}) in insect cells followed by nanoLC−MS/MS analysis unequivocally demonstrated that these proteins can be sulfated at the predicted tyrosine sites in eukaryotic cells, albeit as heterogeneous mixtures. Small quantities of pure monosulfated A^{Ag} (**Cfe1**) could be generated in a cell-free expression system via amber stop-codon suppression technology, and this material was used to show that sulfation improved the thrombin inhibitory activity of the protein. While the cell-free expression method served as a useful screening tool in this study, the low yields, coupled with the inability to access A^{Aa} bearing two sulfation sites, led to exploration of an alternative means to access differentially sulfated variants of A^{Aa} and A^{Ag} proteins, namely, total chemical synthesis. By means of a one-pot native chemical ligation−desulfurization manifold, the three homogeneously sulfated homologues of A^{Aa} (**Syn2−4**) and sulfated A^{Ag} (**Syn1**) were generated in multimilligram quantities in good overall yields, enabling the role of sulfation on thrombin inhibitory and anticoagulant activity to be assessed. Sulfation of A^{Aa} at residues Tyr12 and Tyr34 (**Syn4**) showed the most dramatic effect, with a 100-fold increase in thrombin inhibition (K_i = 1.7 pM), while A^{Ag} sulfated at Tyr12 (**Syn1**) led to a 5-fold improvement in activity (K_i = 1.1 pM). The two sulfated anophelins showed differing inhibitory activities against γ-thrombin, which may suggest that the proteins can also associate with exosite II of thrombin.

Both **Syn1** and **Syn4** exhibited potent anticoagulant activity in a clinical thrombin time assay, prolonged aPTT in a dose-dependent manner *ex vivo*, and were able to abrogate clot formation in an *in vivo* needle injury model of thrombosis. These results lay the foundation for the study of these molecules, as well as sulfated anticoagulants from other bloodfeeders, as novel antithrombotic leads. Indeed, our findings now provide the impetus to investigate salivary proteins from other species of mosquito with a view to discovering potent bioactive molecules whose activity is modulated by tyrosine sulfation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00612.

Thrombin inhibition data, ligation traces, *ex vivo* aPTT data, tail bleeding data, needle injury data, and experimental procedures (PDF)

Supporting Information, Table 1 Anophelin from *Anopheles albimanus* (UniProtKB - Q9NJS1) peptides identified by nanoLC-MS/MS following expression in *Trichoplusia ni* insect cells (XLSX)

Supporting Information, Table 2 Anophelin from *Anopheles gambiae* (UniProtKB - Q7Q3R9) peptides identified by nanoLC-MS/MS following expression in *Trichoplusia ni* insect cells (XLSX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Corral- Rodríguez, M. Á.; Macedo-Ribeiro, S.; Pereira, P. J. B.; Fuentes-Prior, P. Leech-derived thrombin inhibitors: from structures to mechanisms to clinical applications. *J. Med. Chem.* **2010**, *53* (10), 3847−3861.

(2) Corral-Rodríguez, M. Á.; Macedo-Ribeiro, S.; Pereira, P. J. B.; Fuentes-Prior, P. Tick-derived Kunitz-type inhibitors as antihemostatic factors. *Insect Biochem. Mol. Biol.* **2009**, *39* (9), 579−595.

(3) Sohn, J. H.; Kang, H. A.; Rao, K. J.; Kim, C. H.; Choi, E. S.; Chung, B. H.; Rhee, S. K. Current status of the anticoagulant hirudin: its biotechnological production and clinical practice. *Appl. Microbiol. Biotechnol.* **2001**, *57* (5), 606−613.

(4) Stone, S. R.; Hofsteenge, J. Kinetics of the inhibition of thrombin by hirudin. *Biochemistry* **1986**, *25* (16), 4622−4628.

(5) Thompson, R. E.; Liu, X.; Ripoll-Rozada, J.; Alonso-García, N.; Parker, B. L.; Pereira, P. J. B.; Payne, R. J. Tyrosine sulfation modulates activity of tick-derived thrombin inhibitors. *Nat. Chem.* **2017**, *9*, 909−917.

(6) Figueiredo, A. C.; de Sanctis, D.; Gutiérrez-Gallego, R.; Cereija, T. B.; Macedo-Ribeiro, S.; Fuentes-Prior, P.; Pereira, P. J. B. Unique thrombin inhibition mechanism by anophelin, an anticoagulant from the malaria vector. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (52), E3649−E3658.

(7) Valenzuela, J. G.; Francischetti, I. M. B.; Ribeiro, J. M. C. Purification, cloning, and synthesis of a novel salivary anti-thrombin from the mosquito *Anopheles albimanus*. *Biochemistry* **1999**, *38* (34), 11209−11215.

(8) Neafsey, D. E.; Waterhouse, R. M.; Abai, M. R.; Aganezov, S. S.; Alekseyev, M. A.; Allen, J. E.; Amon, J.; Arcà, B.; Arensburger, P.; Artemov, G.; et al. Highly evolvable malaria vectors: the genomes of 16 *Anopheles* mosquitoes. *Science* **2015**, *347* (6217), 1258522.

(9) Pirone, L.; Ripoll-Rozada, J.; Leone, M.; Ronca, R.; Lombardo, F.; Fiorentino, G.; Andersen, J. F.; Pereira, P. J. B.; Arcà, B.; Pedone, E. Functional analyses yield detailed insight into the mechanism of thrombin inhibition by the antihemostatic salivary protein cE5 from *Anopheles gambiae*. *J. Biol. Chem.* **2017**, *292* (30), 12632−12642.

(10) Huang, S. Y.; Shi, S. P.; Qiu, J. D.; Sun, X. Y.; Suo, S. B.; Liang, R. P. PredSulSite: prediction of protein tyrosine sulfation sites with multiple features and analysis. *Anal. Biochem.* **2012**, *428* (1), 16−23.

(11) Niu, S.; Huang, T.; Feng, K.; Cai, Y.; Li, Y. Prediction of tyrosine sulfation with mRMR feature selection and analysis. *J. Proteome Res.* **2010**, *9* (12), 6490−6497.

(12) Monigatti, F.; Gasteiger, E.; Bairoch, A.; Jung, E. The Sulfinator: predicting tyrosine sulfation sites in protein sequences. *Bioinformatics* **2002**, *18* (5), 769−770.

(13) Stone, M. J.; Payne, R. J. Homogeneous sulfopeptides and sulfoproteins: synthetic approaches and applications to characterize the effects of tyrosine sulfation on biochemical function. *Acc. Chem. Res.* **2015**, *48* (8), 2251−2261.

(14) Wang, L.; Brock, A.; Herberich, B.; Schultz, P. G. Expanding the genetic code of *Escherichia coli*. *Science* **2001**, *292* (5516), 498−500.

(15) Liu, C. C.; Schultz, P. G. Adding new chemistries to the genetic code. *Annu. Rev. Biochem.* **2010**, *79*, 413−444.

(16) Wang, L.; Schultz, P. G. A general approach for the generation of orthogonal tRNAs. *Chem. Biol.* **2001**, *8* (9), 883−890.

(17) Liu, C. C.; Schultz, P. G. Recombinant expression of selectively sulfated proteins in *Escherichia coli*. *Nat. Biotechnol.* **2006**, *24* (11), 1436−1440.

(18) Young, T. S.; Ahmad, I.; Yin, J. A.; Schultz, P. G. An enhanced system for unnatural amino acid mutagenesis in *E. coli*. *J. Mol. Biol.* **2010**, *395* (2), 361−374.

(19) Young, D. D.; Young, T. S.; Jahnz, M.; Ahmad, I.; Spraggon, G.; Schultz, P. G. An evolved aminoacyl-tRNA synthetase with atypical polysubstrate specificity. *Biochemistry* **2011**, *50* (11), 1894−1900.

(20) Catanzariti, A. M.; Soboleva, T. A.; Jans, D. A.; Board, P. G.; Baker, R. T. An efficient system for high-level expression and easy purification of authentic recombinant proteins. *Protein Sci.* **2004**, *13* (5), 1331−1339.

(21) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. Synthesis of proteins by native chemical ligation. *Science* **1994**, *266* (5186), 776−779.

(22) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. Native chemical ligation at valine: a contribution to peptide and glycopeptide synthesis. *Angew. Chem., Int. Ed.* **2008**, *47* (44), 8521−8524.

(23) Malins, L. R.; Cergol, K. M.; Payne, R. J. Peptide ligation−desulfurization chemistry at arginine. *ChemBioChem* **2013**, *14* (5), 559−563.

(24) Wan, Q.; Danishefsky, S. J. Free-radical-based, specific desulfurization of cysteine: a powerful advance in the synthesis of polypeptides and glycopolypeptides. *Angew. Chem., Int. Ed.* **2007**, *46* (48), 9248−9252.

(25) Thompson, R. E.; Chan, B.; Radom, L.; Jolliffe, K. A.; Payne, R. J. Chemoselective peptide ligation−desulfurization at aspartate. *Angew. Chem., Int. Ed.* **2013**, *52* (37), 9723−9727.

(26) Thompson, R. E.; Liu, X.; Alonso-Garcia, N.; Pereira, P. J. B.; Jolliffe, K. A.; Payne, R. J. Trifluoroethanethiol: an additive for efficient one-pot peptide ligation−desulfurization chemistry. *J. Am. Chem. Soc.* **2014**, *136* (23), 8161−8164.

(27) Bang, D.; Pentelute, B. L.; Kent, S. B. H. Kinetically controlled ligation for the convergent chemical synthesis of proteins. *Angew. Chem., Int. Ed.* **2006**, *45* (24), 3985−3988.

(28) Chang, T. L.; Feinman, R. D.; Landis, B. H.; Fenton, J. W. Antithrombin reactions with α- and γ-thrombins. *Biochemistry* **1979**, *18* (1), 113−119.

(29) Ascenzi, P.; Amiconi, G.; Coletta, M.; Lupidi, G.; Menegatti, E.; Onesti, S.; Bolognesi, M. Binding of hirudin to α, β and γ-thrombin. A comparative kinetic and thermodynamic study. *J. Mol. Biol.* **1992**, *225* (1), 177−184.

(30) Kaplan, Z. S.; Zarpellon, A.; Alwis, I.; Yuan, Y.; McFadyen, J.; Ghasemzadeh, M.; Schoenwaelder, S. M.; Ruggeri, Z. M.; Jackson, S. P. Thrombin-dependent intravascular leukocyte trafficking regulated by fibrin and the platelet receptors GPIb and PAR4. *Nat. Commun.* **2015**, *6*, 7835.

(31) Figueiredo, A. C.; de Sanctis, D.; Pereira, P. J. B. The tick-derived anticoagulant Madanin is processed by thrombin and factor Xa. *PLoS One* **2013**, *8* (8), e71866.

(32) Hsieh, Y. S. Y.; Wijeyewickrema, L. C.; Wilkinson, B. L.; Pike, R. N.; Payne, R. J. Total synthesis of homogeneous variants of hirudin P6: a post-translationally modified anti-thrombotic leech-derived protein. *Angew. Chem., Int. Ed.* **2014**, *53* (15), 3947−3951.

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