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# Effect of Sulfotyrosine and Negatively Charged Amino Acid of Leech-Derived Peptides on Binding and Inhibitory Activity Against Thrombin

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Hirudins, natural sulfo(glyco)proteins, are clinical anticoagulants that directly inhibit thrombin, a key coagulation factor. Their potent thrombin inhibition primarily results from antagonistic interactions with both the catalytic and non-catalytic sites of thrombin. Hirudins often feature sulfate moieties on Tyr residues in their anionic C-terminus region, enabling strong interactions with thrombin exosite-I and effectively blocking its engagement with fibrinogen. Although sulfotyrosines have been identified in various hirudin variants, the precise relationship between sulfotyrosine and the number of negatively charged amino acids within the anionic-rich C-terminus peptide

domain for the binding of thrombin has remained elusive. By using Fmoc-SPPS, hirudin dodecapeptides homologous to the C-terminus of hirudin variants from various leech species were successfully synthesized, and the effect of sulfotyrosine and the number of negatively charged amino acids on hirudin-thrombin interactions was investigated. Our findings did not reveal any synergistic effect between an increasing number of sulfotyrosines or negatively charged amino acids and their inhibitory activity on thrombin or fibrinolysis in the assays, despite a higher binding level toward thrombin in the sulfated dodecapeptide Hnip\_Hirudin was observed in SPR analysis.

## Introduction

Hirudin, originally isolated from the salivary glands of medicinal leeches, is a powerful anticoagulant which falls under the direct thrombin inhibitor (DTI) category.<sup>[1]</sup> Hirudin forms a strong and nearly irreversible binding with thrombin, facilitated by both hydrophobic and ionic forces. This interaction effectively hinders thrombin from cleaving fibrinogen, thereby inhibiting the formation of fibrin.<sup>[2]</sup> In contrast to other anticoagulants, hirudin possesses the ability to inhibit thrombin activity independently, without the cofactor antithrombin III.<sup>[3]</sup> Numer-

ous subtypes of hirudin have been discovered throughout the ages, among which the most well-studied is the hirudin variant isolated from *Hirudo medicinalis*.<sup>[1,4]</sup> The significant common trait between all hirudin subtypes is their core structure, which is closely related to hirudin's strong inhibitory activity toward thrombin.<sup>[5]</sup> The N-terminus domain of hirudin, particularly the first three amino acid residues and the intramolecular disulfide bonds located at Cys6-Cys14, Cys16-Cys28 and Cys22-Cys39, contributes to the binding and inhibition of the thrombin active site and hence blocks thrombin amidolytic activity.<sup>[2b,6]</sup> On the other end of hirudin, the C-terminus is often referred to as an

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“acid tail”, a domain rich in negatively charged amino acid residues, which contributes approximately one-third of the overall binding strength between hirudin and thrombin.<sup>[7]</sup> In combination with the presence of sulfated tyrosine, this C-terminus domain targets the thrombin cationic fibrinogen recognition site (exosite-1)<sup>[8]</sup> by means of both ionic and hydrophobic interactions.<sup>[1,2b]</sup>

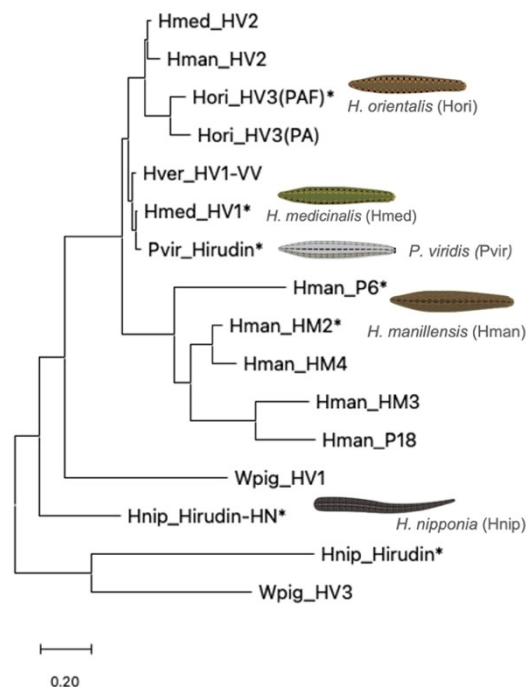
Although the natural sulfoform hirudin is a powerful thrombin inhibitor, its research and application have been constrained due to its limited production. Previous studies have provided evidence that the natural form of hirudin can strongly inhibit thrombin with an inhibition constant ( $K_i$ ) of approximately 25 fM toward thrombin. In contrast, recombinantly produced therapeutic forms of hirudin without tyrosine sulfate, such as lepirudin and desirudin, exhibit  $K_i$  values of approximately 300 fM.<sup>[9]</sup> Despite many strategies have been attempted to enhance the expression of recombinant hirudin, thrombin  $K_i$  remains inferior to that of natural hirudin, primarily due to the absence of the sulfated tyrosine at site 63, which is located in the anion-rich C-terminus region.<sup>[9a,10]</sup> The presence of this sulfated Tyr63 can bring about a more than 10-fold increase in the inhibitory activity toward thrombin,<sup>[9a]</sup> based on its ionic interaction with thrombin Lys81.<sup>[10a]</sup> Bivalirudin (Angiomax® or hirulog) is a synthetic 20-amino acid, bivalent hirudin analogue that displays approximately 2 nM of  $K_i$  value, approximately 800 times weaker inhibitory activity than hirudin. This reversible thrombin inhibitor connects an active-site binding N-terminus segment of tetrapeptide (D-Phe-Pro-Arg-Pro to C-terminus dodecapeptide (ddp)) derived from natural hirudin variant 1 (residues 53–64) via four glycine residues as linkers.<sup>[11]</sup>

As one of the most common post-translational modifications (PTMs) of hirudin, tyrosine sulfation plays a critical role in numerous biological processes, such as hemostasis, hormonal regulation, molecular recognition, and immune regulation.<sup>[12]</sup> In earlier studies on the C-terminus of hirudin P6 from the leech *Hirudinaria manillensis*, it was discovered that tyrosine sulfation plays a more essential role than O-glycosylation in thrombin binding and fibrinogenolytic inhibition.<sup>[13]</sup> Inspired by these discoveries, we were intrigued to gain further knowledge regarding the relationship between thrombin and both the C-terminus anionic region as well as the tyrosine sulfation located at the C-terminus end of hirudin. Previously, a synthetic N-acetylated C-terminus dodecapeptide (residues 53–64) of hirudin with the sequence Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO<sub>3</sub>)-Leu-OH, also known as hirugen, has been reported as a thrombin competitive inhibitor targeting its fibrinogen binding site.<sup>[14]</sup> The recent study of hirudin variant 1 (HV1) once again underscores the significance of tyrosine sulfation, highlighting its crucial role in enhancing the tight fit of Phe56 within the hydrophobic binding pocket of thrombin.<sup>[15]</sup> In addition, hirudin variant 1 from the leech *H. medicinalis*<sup>[4,16]</sup> and hirudin P6 from the leech *H. manillensis*<sup>[13,17]</sup> were the only two hirudin peptides among the hirudin family that have been studied upon their thrombin binding ability in their sulfated form. To further expand our knowledge of this, our aim is to study the different hirudin variants naturally secreted by different leech species by synthesizing chemically and modifying

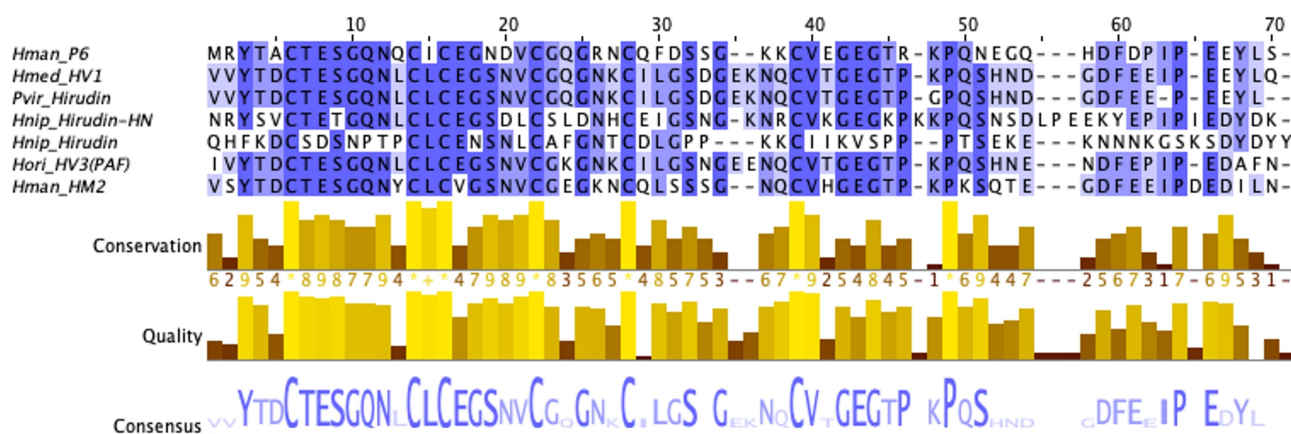
various C-terminus dodecapeptides with tyrosine sulfation and later comparing their thrombin binding and fibrinolytic activities. Seven hirudin variants that have previously been reported to have activity against thrombin were selected as candidates to be included in this study.<sup>[18]</sup>

## Results and Discussion

The phylogenetic tree was constructed with their sequence without a signal peptide using *H. medicinalis* hirudin variant 1, the well-studied hirudin variant, and other hirudin homologs with sequence identities ranging from 29% to 99% (Figure 1). Based on the phylogeny, we chose seven synthetic candidates within distinct hirudin subclades. These selections were made with both strategic considerations, aiming to represent hirudin subtypes and to decipher the subtle differences in hirudin peptide compositions. Among the chosen candidates, the hirudin variant from *Poecilobdella viridis* is the most closely related to Hmed\_HV1 with 95% identity (Figure 2). In contrast, *Hirudo orientalis* hirudin variant HV3 (PAF) and *H. manillensis* hirudin variants HM2 and P6 displayed identities of 83%, 68%, and 60%, respectively (Figure 2). Notably, two hirudins derived from *Hirudo nipponia* isoforms, Hnip\_Hirudin-HN and Hnip\_Hirudin, exhibit the lowest sequence identities (53% and 29%, respectively); however, these variants display strong thrombin inhibitory activity, akin to other characterized hirudin variants.<sup>[18a,d,e]</sup>



**Figure 1.** Phylogeny of Hirudin variants of *H. manillensis* (Hman), *H. medicinalis* (Hmed), *P. viridis* (Pvir), *H. nipponia* (Hnip), *H. orientalis* (Hori), *H. verbena* (Hver), and *W. pigra* (Wpig). The phylogeny was obtained using the Maximum Likelihood method in MEGA7. The star sign\* indicates the seven candidates in our study.



**Figure 2.** Protein sequence alignment of hirudin variants Hman\_P6, Hmed\_HV1, Pvir\_Hirudin, Hnip\_Hirudin-HN, Hnip\_Hirudin, Hori\_HV3(PAF), and Hman\_HM2. The alignment was conducted with the MUSCLE method in Jalview software (ver 2.11.2.6). Hman: *H. manillensis*; Hmed: *H. medicinalis*; Pvir: *P. viridis*; Hnip: *H. nipponia*; and Hori: *H. orientalis*.

The selection of these candidates was driven by a distinct pattern evident in the sequence alignment (Figure 2). Except for the *H. nipponia* variant, all other candidates demonstrated a pronounced similarity in the N-terminus region while simultaneously displaying marked dissimilarity in the region of our particular interest, the C-terminus segment. This choice was guided by the objective of exploring the evolutionary divergence and biochemical variation within hirudin variants. In addition to their phylogeny, we considered the diversity in the number of tyrosine sulfation sites and the distribution of negatively charged amino acid residues as key factors informing our selection process (Table 1).

### Synthesis of hirudin dodecapeptides

Dodecapeptides (13–19, 30–34) with the number of negatively charged amino acid residues varying from two to six and the number of tyrosine sulfation(s) ranging from zero to three (Table 1) were successfully synthesized. After purification by preparative HPLC, we found that ddps 13–19 exhibited a significantly higher overall yield (40–72%) than their sulfated

counterparts (30–34) (7–32%). This difference in yield may be attributed to the use of a lower equivalent (1.2 equiv.) of the cassette Fmoc-Tyr(SO<sub>3</sub>nP)-OH (1) in coupling chemistry. The deprotection process for the triple neopentyl protective groups on ddp 34 was also undertaken in 1 M NH<sub>4</sub>OAc over 96 h to ensure complete removal. Interestingly, in our preliminary experiment, we noted that ddp 34 derived from Hnip\_Hirudin, featuring three sulfotyrosine residues, was partially hydrolysed during storage, even when it was stored at temperatures as low as –80 °C with argon. Consequently, we decided to maintain the neopentyl group (29) and proceed with deprotection before embarking on the investigation of hirudin peptide binding and the evaluation of peptidolytic activity on thrombin via thrombin assays.

### The binding affinity of hirudin dodecapeptides

Having successfully synthesized 12 hirudin dodecapeptides, our subsequent exploration focused on examining how sulfoesters and carboxylate anions influence the peptide sequence's impact on binding to thrombin and its peptidolytic activity. We

**Table 1.** The synthetic dodecapeptide (ddp) derived from five hirudin variants. The 12 chosen synthetic hirudin ddp congeners 13–19 and 30–34 were given abbreviated names based on their species origins, such as Hman (from *H. manillensis*), Hmed (for *H. medicinalis*), Pvir (for *P. viridis*), Hnip (for *H. nipponia*), and Hori (for *H. orientalis*).

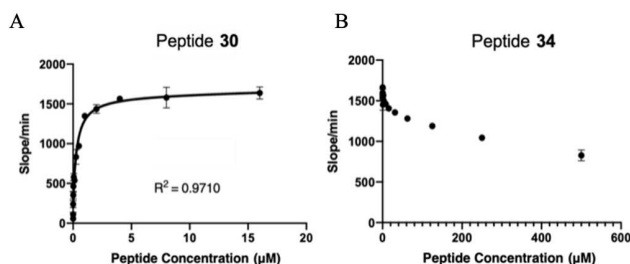
ddp No.	Abbreviated name	Sequence	Anionic Glu + Asp	Tyr-SO <sub>3</sub> <sup>-</sup>
13 <sup>[a]</sup> /30 <sup>[b]</sup>	ddp_Hman_P6	His-Asp-Phe-Asp-Pro-Ile-Pro-Glu-Glu-Tyr-Leu-Ser	4	1
14 <sup>[a]</sup> /31 <sup>[b]</sup>	ddp_Hmed_HV1	Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln	5	1
15 <sup>[a]</sup> /32 <sup>[b]</sup>	ddp_Pvir_Hirudin	Asn-Asp-Gly-Asp-Phe-Glu-Glu-Pro-Glu-Glu-Tyr-Leu	6	1
16 <sup>[a]</sup> /33 <sup>[b]</sup>	ddp_Hnip_Hirudin-HN	Lys-Tyr-Glu-Pro-Ile-Pro-Ile-Glu-Asp-Tyr-Asp-Lys	4	2
17 <sup>[a]</sup> /34 <sup>[b]</sup>	ddp_Hnip_Hirudin	Asn-Asn-Lys-Gly-Ser-Lys-Ser-Asp-Tyr-Asp-Tyr-Tyr	2	3
18 <sup>[a]</sup>	ddp_Hori_HV3(PAF)	Asn-Asp-Phe-Asp-Pro-Ile-Pro-Glu-Asp-Ala-Phe-Asn	4	0
19 <sup>[a]</sup>	ddp_Hman_HM2	Asp-Phe-Glu-Glu-Ile-Pro-Asp-Glu-Asp-Ile-Leu-Asn	6	0

[a] Unsulfated hirudin dodecapeptide. [b] Sulfated hirudin dodecapeptide.

conducted these investigations using a fluorogenic tripeptide substrate, Z-Gly-Pro-Arg-NHMec (NHMeC = 7-amido-4-methylcoumarin). The synthesized Hmed\_HV1 peptides **14** (unsulfated) and **31** (sulfated) served as positive controls among the hirudin dodecapeptides, as the binding and inhibitory activity on thrombin of these sequences have been well studied previously.<sup>[2b,19]</sup> Its sulfated form **31** exhibited a 10-fold increase in the binding affinity for thrombin ( $K_d = 158$  nM) in comparison to unsulfated **14** (Table 2). However, identical  $K_d$  values between unsulfated ddp **13** and sulfated ddp **30** were unexpected. Hori\_HV3 (PAF) ddp **18** and Hman\_HM2 ddp **19** exhibited low binding affinity toward thrombin. Moreover, we were not able to obtain valid  $K_d$  values for Pvir\_Hirudin peptides **15** and **32**, which have high sequence similarity with Hmed\_HV1. The sulfated forms of Hnip\_Hirudin-HN **33** and Hnip\_Hirudin peptides **17** and **34** presented similar results. The raw data of these peptides were demonstrated without subtraction of the initial velocity of thrombin alone, indicating that the inhibition of thrombin activity took place with the binding of these peptides, especially in the sulfated ddps **32**, **33**, and **34** (Table 2, Figure 3B).

**Table 2.** Dissociation constants ( $K_d$ ) for thrombin and inhibitory activity of fibrin formation by synthetic dodecapeptides **13–19** (unsulfated) and **30–34** (sulfated). The abbreviations of US represent unsulfated peptide; S, sulfated peptide; P6, Hman\_P6; HV1, Hmed\_HV1; Pvir, Pvir\_Hirudin; Hnip(HN), Hnip\_Hirudin-HN; Hnip, Hnip\_Hirudin; Hori, Hori\_HV3(PAF); HM2, Hman\_HM2. ND: not detected, NA: no activity.

	Hirudin ddp		$K_d$ [nM]	$IC_{50}$ [ $\mu$ M]
<b>Unsulfated ddp</b>	<b>13</b>	P6-US	285	0.5
	<b>14</b>	HV1-US	3,255	2.4
	<b>15</b>	Pvir-US	ND	57
	<b>16</b>	Hnip(HN)-US	38,070	40
	<b>17</b>	Hnip-US	ND	NA
	<b>18</b>	Hori-US	10,330	15
	<b>19</b>	HM2-US	11,520	29
<b>Sulfated ddp</b>	<b>30</b>	P6-S	299	0.2
	<b>31</b>	HV1-S	158	0.5
	<b>32</b>	Pvir-S	ND	260
	<b>33</b>	Hnip(HN)-S	ND	63
	<b>34</b>	Hnip-S	ND	301



**Figure 3.** The representation of thrombin activity against Z-Gly-Pro-Arg-AMC in the presence of increasing concentrations of sulfated Hman\_P6 dodecapeptide **30** (A) and sulfated Hnip\_Hirudin dodecapeptide **34** (B).

## Fibrinolytic activity of hirudin dodecapeptides

The fibrinolytic activity of dodecapeptides was evaluated using a turbidimetric clotting assay. Notably, only the dodecapeptides from Hmed\_HV1 and Hman\_P6 exhibited near-nanomolar inhibition of fibrinogen cleavage. Sulfated ddp Hmed\_HV1 **31**, with an  $IC_{50}$  value of  $0.4 \mu$ M, is five-folds more potent than unsulfated ddp Hmed\_HV1 **14** (Table 2). The sulfated ddp Hman\_P6 **30**, with an  $IC_{50}$  value of  $0.2 \mu$ M, demonstrated twice the potency in inhibition compared to unsulfated ddp Hman\_P6 **13**. Interestingly, ddp Hnip-Hirudin **34**, featuring three sulfoesters, displayed  $301 \mu$ M inhibitory activity against fibrinogenolysis, whereas no activity was observed in unsulfated ddp Hnip-Hirudin **17** (Table 2). Despite having three sulfoesters, **34** appears that its inhibitory activity of fibrin formation is not significantly superior to most synthetic dodecapeptides, and **30** and **31** derived from Hman\_P6 and Hmed\_HV1, respectively, with single tyrosine sulfate are still the best inhibitors in the fibrinogenolysis assay.

Taken together, these results suggested that the presence of sulfation and the number of negatively charged groups of the hirudin C-terminus sequence do not synergistically improve its binding affinity to thrombin. In addition, rather than inhibiting the cleavage activity of the thrombin substrate, ddps Hman\_P6 **13** and **30**, Hmed\_HV1 **14** and **31**, Hnip\_Hirudin-HN **16**, Hori\_HV3(PAF) **18**, and Hman\_HM2 **19** increased the peptidolytic activity of thrombin. Contrary to some literature suggesting that the C-terminus of HV1 inhibits thrombin peptidolytic activity, it is important to note that other hirudin C-terminally derived peptides have also shown an increase in thrombin peptidolytic activity.<sup>[13–14,20]</sup>

Looking into the amino acid sequence in the C-terminus regio-pattern to thrombin binding, which is primarily characterized by salt bridges and hydrophobic interactions.<sup>[2b,19]</sup> Remarkably, despite a high sequence similarity of 95% between HV1 and Pvir, the sole distinction lies in a single amino acid, Ile. However, this seemingly minor variation results in a nearly 24-fold difference in fibrinolytic activity (HV1 **14** to Pvir **15**), indicating the substantial contribution of the lipophilic side chain of Ile to hydrophobic binding interactions.<sup>[2b]</sup> Furthermore, the observation of an 11-fold increase in fibrinolytic activity in P6 **13** to HV1 **14** may be attributed to the substitution of anionic Glu with the lipophilic Pro residue, which exhibits heightened efficacy following the introduction of proline.<sup>[21]</sup>

## Probing hirudin dodecapeptides-thrombin interactions with SPR

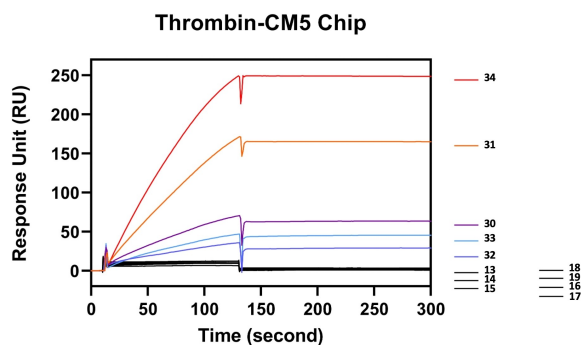
To further investigate the interactions between hirudin dodecapeptides and thrombin, we conducted a surface plasmon resonance (SPR) analysis, which can directly detect the binding between synthetic dodecapeptides and thrombin. Similar to thrombin assays, we have observed that most peptides exhibit very large  $K_d$  values, indicating weak binding between the peptides and thrombin. Nonetheless, the maximum Responsive Unit (RU) values are valuable parameters for the relative

comparative studies, as a higher RU indicates a larger number of peptides binding to the sensor surface. This implies a greater binding capacity or a more substantial interaction. The RU value does unequivocally illustrate the importance of tyrosine sulfation in augmenting the binding levels of hirudin dodecapeptides to thrombin, as depicted in Figure 4 and Figure S1–7. Among the dodecapeptides tested, Hnip\_Hirudin peptide **34**, featuring three sulfotyrosine residues, displayed the highest RU (Figure 4), followed by ddp Hmed\_HV1 **31** and ddp Hman\_P6 **30**, with a single sulfotyrosine residue. Notably, among the five sulfopeptides synthesized, Hnip\_Hirudin-HN **33**, which contains two sulfotyrosine residues, exhibits the lower binding level. The unsulfated form of Hnip\_Hirudin ddp **17** displays lowest RU (Figure S1). This implies a potential positive correlation between the number of sulfated tyrosine residues and binding ability toward thrombin. However, this correlation is not absolute, as Hnip\_Hirudin-HN **33**, with two tyrosine residues, shows lower RU than **30** and **31**.

We also studied seven chosen unsulfated hirudin dodecapeptides, each differing in the number of negatively charged amino acid residues (comprising aspartic acid- Asp (D) and glutamic acid- Glu (E)), and we noticed negligible variations in their binding levels (Figure S1). Based on SPR findings, two significant conclusions can be drawn. 1) It is clear that the tyrosine sulfate is important for the thrombin binding, 2) An increased number of negatively charged groups in dodecapeptides does not lead to a synergistic improvement in thrombin binding.

## Conclusions

In summary, this study successfully synthesized twelve C-terminus hirudin dodecapeptides (**13–19**, **30–34**) derived from various leech species using Fmoc-SPPS with a preassembled cassette (1). These peptides exhibited distinct characteristics, including varying numbers of negatively charged amino acid residues and sulfotyrosines, allowing us to interrogate their impact on thrombin activity for the first time. Interestingly, our findings did not reveal any synergistic effect between an increasing number of sulfated tyrosine residues or negatively



**Figure 4.** The binding levels of twelve synthetic hirudin dodecapeptides with thrombin were evaluated using surface plasmon resonance (SPR). Notably, the strongest RU toward thrombin was observed with the 400  $\mu\text{M}$  concentration of sulfated ddp Hnip\_Hirudin **34**, as illustrated in the figure.

charged amino acids and the inhibitory activity on thrombin or fibrinolysis in the assays. Despite observing a higher binding level toward thrombin in ddp **34** during SPR analysis, it became evident that hirudin dodecapeptides derived from hirudin variants with a higher number of negative charges in carboxylate moieties and tyrosine sulfate residues, does not demonstrate increased potency. Sulfated ddp Hman\_P6 **30** and ddp Hmed\_HV1 **31** remain the most promising candidates by a significant margin.

Natural hirudin variants exhibit anticoagulant properties that offer potential therapeutic applications for diverse thrombotic disorders. Through our systematic analysis of synthetic peptides derived from seven hirudin variants, we have gained valuable insights into how post-translational modifications and negatively charged amino acids impact hirudin's interaction with thrombin. This newfound knowledge holds the potential to refine the efficacy of recombinant hirudins and synthetic hirulogs in the future.

## Experimental Section

### The phylogeny and sequence alignment of the hirudin family

The hirudin variants were acquired from the National Center for Biotechnology Information (NCBI) database, followed by phylogenetic tree construction by the maximum likelihood method in MEGA7<sup>[22]</sup> and sequence alignment via Jalview ver 2.11.2.6 software. For the phylogenetic study, we incorporated hirudin variants hirudin variant HV2 (3HTC\_I), HM2 (P81492.2), HM3 (APA20847.1), HM4 (APA20848.1), P6 (P28512.1), and P18 (P26631.1) from *H. manillensis*;<sup>[23]</sup> hirudin variant HV1 (P01050.1) and hirudin variant HV2 (ALA14576.1) from *H. medicinalis*; hirudin variant (P84590.1) from *Poecilobdella viridi*; hirudin variant HV1-VV (APA20831.1) from *Hirudo verbena*; hirudin variant (QIA62024.1) and HN (QDZ37419.1) from *Hirudo nipponia*;<sup>[24]</sup> hirudin variant HV3(PA) (APA20834.1) and HV3 (PAF) (APA20857.1) from *Hirudo orientalis*;<sup>[25]</sup> and hirudin variant HV1 (USH09346.1) and HV3 (USH09356.1) from *Whitmania pigra*. We selected seven hirudin variants from five leech species, Hmed\_HV1, Pvir\_Hirudin, Hnip\_Hirudin-HN and Hnip\_Hirudin, Hori\_HV3 (PAF), and Hman\_HM2 and Hman\_P6, as synthetic dodecapeptide candidates. The 12 synthetic hirudin ddp congeners **13–19** and **30–34** were selected based on the different numbers of sulfated tyrosine(s) and different numbers of negatively charged amino acid residues in the C-terminus regions of hirudin.

### Synthesis of unsulfated hirudin dodecapeptides 13–19

The preparation of unsulfated ddps **13–19** was conducted through Fmoc-strategy solid phase peptide synthesis (Fmoc-SPPS). The synthesis began with Rink amide resin LL (100  $\mu\text{mol}$ ), underwent iterative Fmoc-SPPS to obtain fully assembled resin-bound ddps **6–12**, and subsequently conducted acidolytic cleavage (90:5:5 v/v/v TFA/*i*Pr<sub>3</sub>SiH/H<sub>2</sub>O) of peptide from the resin concomitantly with side-chain protection removal to provide desired unsulfated ddps **13–19** in 40–72% overall yield based on the original resin loading (38 linear steps) after purification by reverse-phase HPLC.

### Synthesis of sulfated hirudin dodecapeptides 30–34

With the aim to synthesize hirudin dodecapeptides incorporating tyrosine sulfation by Fmoc solid-phase peptide synthesis (Fmoc-

SPPS), a building block with sulfated tyrosine is needed. However, the lability of tyrosine *O*-sulfate in acidic conditions leads to the need for a protective group on the sulfated tyrosine. Neopentyl (nP) ester, one of the most commonly used protecting groups of sulfonate ester, was therefore chosen due to its great stability in both acidic and basic conditions, as well as the mild conditions for deprotecting the neopentyl protecting group.<sup>[26]</sup> The cassette Fmoc-Tyr(SO<sub>3</sub>nP)-OH (1) was synthesized and then incorporated into sulfopeptide synthesis via standard Fmoc-SPPS. Three steps were conducted for the preparation of Fmoc-Tyr(SO<sub>3</sub>nP)-OH (1), with modified procedures.<sup>[26a]</sup> Sulfate monoester (5) was obtained by treating commercially available Boc-Tyr-OtBu (2) with neopentyl chlorosulfate (4), which was pre-synthesized from fresh sulfonyl chloride (3) and neopentyl alcohol. TFA (95%) was utilized to deprotect the *N*-terminal Boc group and *C*-terminal *tert*-butyl ester, followed by Fmoc-OSu in the presence of 10% Na<sub>2</sub>CO<sub>3(aq)</sub> to afford the precious building block Fmoc-Tyr(SO<sub>3</sub>nP)-OH (1) in 66% yield (See Scheme S1).

The neopentyl-protected sulfopeptides 25–29 were synthesized from Rink amide resin LL (50 μmol) using standard Fmoc-strategy SPPS. Sulfated tyrosine was coupled with a slight excess of pre-synthesized Fmoc-Tyr(SO<sub>3</sub>nP)-OH 1 (1.2 equiv.) and the coupling agent 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluoro-phosphate (HATU). After fully assembling resin-bound sulfated ddps 20–24, the resin was cleaved simultaneously with side-chain protection removal via acidic cocktail (90:5:5 v/v/v, TFA/*i*Pr<sub>3</sub>SiH/H<sub>2</sub>O), followed by purification by reverse-phase HPLC to afford neopentyl-protected sulfopeptides 25–29 in 21–50% yield. The purified neopentyl-protected sulfopeptides 25–28 were then treated with 0.1 M NH<sub>4</sub>OAc (2 mL) and incubated at 37 °C for 16 h. During the process of deprotecting the neopentyl group, we found that the removal of the neopentyl group was extremely difficult for the neopentyl-protected Hnip\_Hirudin sulfated peptide 29 with 0.1 M NH<sub>4</sub>OAc<sub>(aq)</sub>, which consists of three neopentyl-protected sulfated tyrosine. However, by increasing the aqueous NH<sub>4</sub>OAc concentration to 1 M and prolonging the reaction time from 16 h to 96 h, we eventually removed all three neopentyl protection groups and moved on to the next step. The resulting unprotected sulfopeptides were then purified with reverse-phase HPLC, followed by repetitive lyophilization until a constant dry weight was achieved to obtain final sulfated ddps 30–34 in 7–32% overall yield based on the original resin loading (39 linear steps). (See Scheme S2 for the synthesis of Hnip\_Hirudin 34).

### Measurement of the affinity of dodecapeptide for thrombin

The *K<sub>d</sub>* values, which indicate the binding affinities of ddps 13–19 and 30–34, were determined through an analysis of their impact on the cleavage of a peptide substrate by the enzyme. Human thrombin at a final concentration of 20 pM was incubated with 0–500 μM of 13–19 and 30–34 for 15 min at 37 °C. The buffer that was used in the assay contained 0.05 M Tris, pH 7.8, 0.1 M NaCl, and 0.1% w/v PEG6000. The activity of the enzyme was measured by determining the rate of cleavage of the substrate Z-Gly-Pro-Arg-AMC (Z-Gly-Pro-Arg 7-amido-4-methylcoumarin hydrobromide) (Sigma Aldrich) using a BMG Fluorostar plate reader with an excitation filter of 370 nm and an emission filter of 460 nm. The initial velocity for thrombin alone was subtracted from the values for thrombin with the addition of the various peptides, and the resulting values were plotted against the concentration of the analogue peptides. These data were fitted using non-linear regression on GraphPad Prism to the following equation for one site-total binding:  $Y = B_{\max} \times X / (K_d + X)$ . The *B<sub>max</sub>* value represents the maximal stimulation of thrombin activity. The experiment was performed in triplicate.

### Competition assay of thrombin fibrinolytic activity of dodecapeptides derived from hirudin variants

Competition for the secondary fibrinogen-binding site (Exosite I) on thrombin was measured by a turbidimetric clotting assay. Human thrombin (Haematologic Technologies, Essex Junction, VT, USA) at a concentration of 200 pM was incubated at 37 °C with ddps 13–19, 30–34 [0–500 μM] in 0.05 M Tris, pH 7.8, 0.1 M NaCl, and 0.1% (w/v) PEG6000 for 30 min prior to the addition of 5 μM fibrinogen (Sigma Aldrich). Fibrin formation was followed by measuring the optical turbidity at 405 nm for 30 min at 37 °C. The values for absorbance at 405 nm were then plotted against the concentration of peptide, and the slopes were fitted by non-linear regression on GraphPad Prism to the sigmoidal dose response formula.

### Analysis of thrombin-peptide interaction by surface plasmon resonance

The binding ability of dodecapeptide samples to thrombin protein was determined using a Biacore T200 surface plasmon resonance instrument (GE Healthcare, Chicago, IL, US). Thrombin (Merck, Darmstadt, Germany) was diluted to 100 μg/mL in 10 mM sodium acetate buffer (pH 4.0), injected over 420 s at 10 μL/min with approximately 4,000 RU and immobilized on a CM5 sensor chip using an amine coupling kit (Cytiva, Uppsala, Sweden). The diluted peptide samples in PBS running buffer were injected into the flow channels at a flow rate of 10 μL/min for 120 s at 25 °C. The obtained signals were subtracted from the reference channel that had not been coated with Thrombin. The binding curves were plotted in a resonance unit against a time sensorgram.

### Supporting Information

The Supporting Information comprises several sections: detailed synthetic methods and analytical data (Section 1 and 2), data for the thrombin assay (Section 3), surface plasmon resonance (SPR) data (Section 4; Figure S1–S7), and NMR spectra (Section 5).

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### Conflict of Interests

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** hirudin · negatively charged amino acid · sulfotyrosine · thrombin

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