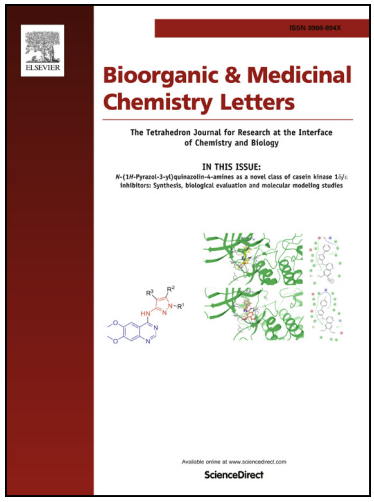

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Synthesis and Biological Evaluation of a New Series of Cinnamic Acid Amide Derivatives as Potent Haemostatic Agents Containing a 2-Aminothiazole Substructure

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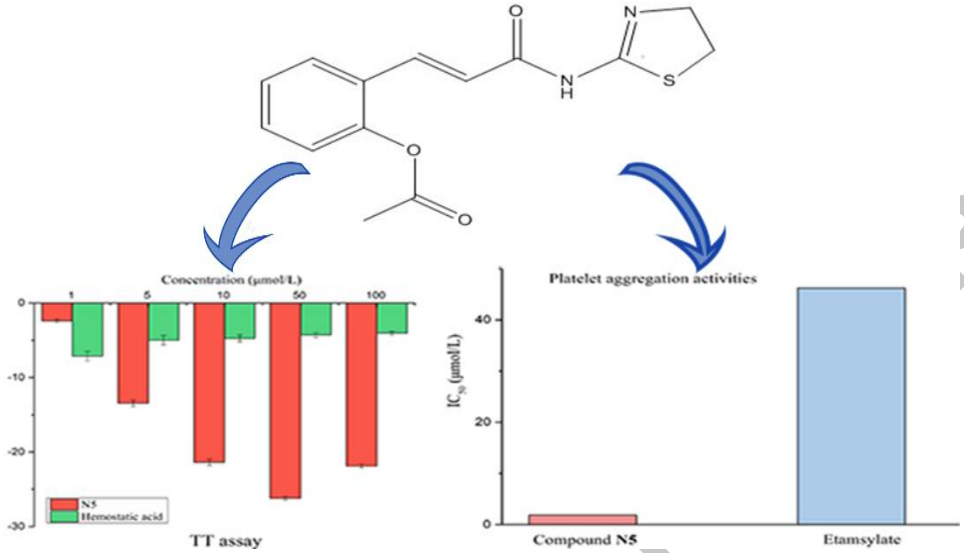
Graphical Abstract

This study demonstrates the use of new compound **N5** with potential characteristics for the development of drugs combining coagulant and platelet activities.



ABSTRACT

Ten new cinnamic acid derivatives containing a 2-aminothiazole substructure were designed and synthesized. This series of compounds exhibited good thermostabilities as demonstrated by thermogravimetric analysis. In coagulation assays (prothrombin time, activated partial thromboplastin time and thrombin time) *in vitro*, most compounds demonstrated excellent activities to promote blood coagulation. Among the studied series, compounds **N1**, **N4**, **N5** and **W5** exhibited a significant coagulation activity. Further studies indicated that compound **N5** (IC_{50} = 1.87 μmol/L) displayed the most suitable efficacy of promoting platelet aggregation than the clinically used haemostatic drug etamsylate (IC_{50} = 46.22 μmol/L). Furthermore, the relationship between the functional groups of the compounds and the corresponding blood coagulant activity was explored in this study.

Keywords: Cinnamic acid derivatives; 2-aminothiazole; Crystal structure; Platelet aggregation; Coagulant activity

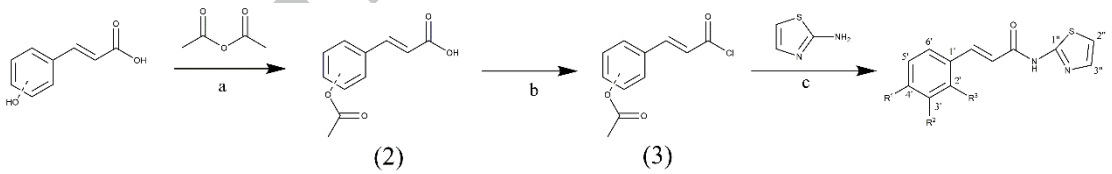
To date, excessive blood loss during surgery still remains a critical operative complication in patients undergoing major surgery, potentially increasing the risk of postoperative morbidity and mortality.[1] Excessive blood loss generally results in prolonged hospital stays, delayed rehabilitation and increased mortality.[2] In surgical procedures, transfusion of homologous blood components is often associated with infectious and immunologic risks as well as increased mortality. Therefore, blood-saving strategies are essential to reduce the amount of homologous blood used in transfusion.[3] In 1988, a Lancet editorial article described that the use of drugs can reduce surgical blood loss.[4] For this reason, improving the efficacy of haemostatic agents represents one of the direct routes to reduce bleeding during surgery. A variety of medications have been studied as haemostatic agents. As such, newer but expensive to produce medications such as aprotinin, etamsylate and hemostatic acid have been studied in recent years.[5] However, studies[6] by Mangno *et al.* have shown that drugs such as aprotinin exhibit considerable safety risks with the clinical usage to be reevaluated. Therefore, the search for safer and more efficient hemostatic drugs still represents a crucial, albeit unmet, scientific goal.

Normal haemostatic mechanisms involve normal functions of blood vessels, platelets and blood coagulation. Blood coagulation represents an important part of haemostasis in which the wall of damaged blood vessels is covered by a platelet and fibrin-containing clot to reduce bleeding and begin damage repair of the vessel.[7, 8] Simultaneously, the extrinsic coagulation pathway is activated. Hence, an inexpensive drug with high efficiency of haemostatic activity through more than two pathways as mentioned above would offer significant advantages. Natural products and/or structures stemming from natural product are highly significant for the development process of new drugs.[9, 10] Compounds based on cinnamic acid represent natural extracts with a variety of biological activities and can be found in a large number of Chinese herbal medicines.[11] Cinnamic acid derivatives are reported to possess various biologically relevant activities such as antioxidant,[12] anti-inflammatory,[13] anti-malarial,[14] anti-tumor,[15] anti-diabetic,[16] anti-tubercular[17] and anti-cancer[18]

activities. Moreover, the coagulation activity of this compound species was evaluated in our previously reported study.[19] Using cinnamic acid as a lead compound, we carried out further structural modifications and studied the effects. According to a patent publication[20] by Shiota *et al.*, 2-aminothiazole was used to synthesize a drug composition that promotes thrombocytosis.

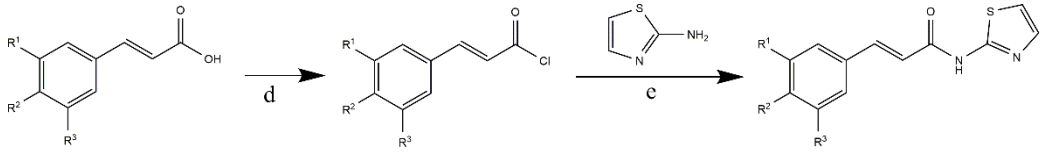
According to the combination principle of drug design, two different compounds were combined via a covalent bond in anticipation of a synergistic effect of each individual compound. Hence, we designed ten new cinnamic acid derivatives with an amide group and 2-aminothiazole structure to evaluate the blood coagulation activity. We determined the thermodynamic stability of the compounds using thermogravimetry for the evaluation of drug stability. Based on the haemostatic mechanism,[8] the blood clotting activities of the above referenced compounds were evaluated by *in vitro* experiments of a plasma coagulation assay (prothrombin time, activated partial thromboplastin time and thrombin time) and platelet aggregation determination. The relationship between structure and the corresponding coagulant activity was also discussed based on the obtained test results.

The new cinnamic acid derivatives **N1**-**N5** (cf. Scheme 1), **W1**-**W5** (cf. Scheme 2 and 3) were synthesized and further purified by reverse phase C18 silica gel column chromatography (mobile phase A: methanol; mobile phase B: water. The isocratic system was A:B = 55%:45%). The detailed description of the experimental information, corresponding yields, melting points, characteristic IR, NMR, MS and HPLC purity data are provided in the Supporting information section.



N1:R^1 = -OOCCH3, R^2 = -OCH_3, R^3 = -H **N2**:R^1 = -$OOCCH_3$, R^2 = -$OOCCH_3$, R^3 = -H **N3**:R^1 = -$OOCCH_3$, R^2 = -H, R^3 = -H
N4:R^1 = -H, R^2 = -$OOCCH_3$, R^3 = -H **N5**:R^1 = -H, R^2 = -H, R^3 = -OCH_3

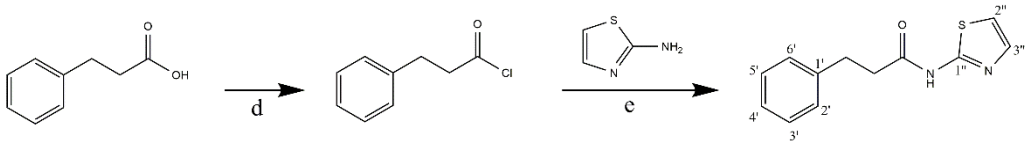
Scheme 1 Reagents and conditions: (a) AA/Py, 120 °C, 99-100%. (b) $SOCl_2$, 70 °C, 100%. (c) NaOAc, pH = 6-7, 0-5°C to rt, 80-95%.



(4)

W1:R^1 = -H, R^2 = -H, R^3 = -H **W3**:R^1 = -OCH_3, R^2 = -H, R^3 = -H
W4:R^1 = -OCH_3, R^2 = -OCH_3, R^3 = -H **W5**:R^1 = -OCH_3, R^2 = -OCH_3, R^3 = -OCH_3

Scheme 2 Reagents and conditions: (d) $SOCl_2$/DMF, 70 °C, 100%. (e) NaOAc, pH = 5-6, 0 °C to rt, 72-95%.



Scheme 3 Reagents and conditions: (d) $SOCl_2$/DMF, 70 °C, 100%. (e) NaOAc, pH = 5-6, 0 °C to rt, 91.2%.

Single crystals of compounds N1, N3, W1 and W2 (CCDC1534821-1534824) [21] were grown from *N*,*N*-dimethylformamide or dimethyl sulfoxide and the corresponding structures were confirmed by single crystal X-ray diffraction analysis (cf. Figure 1). Coupled with the integrals in the NMR spectra, X-ray diffraction analysis provided the crystal structures for the four compounds **N1**, **N3**, **W1** and **W2**. The crystal lattices of compounds **N1**, **W1** and **W2** belong to the monoclinic system but exhibit different space groups. The crystal lattices of compound **N3** belong to the orthorhombic system, containing two (*E*)-4-(3-oxo-3-(thiazol-2-ylamino)prop-1-en-1-yl)phenyl acetate molecules. Selected bond lengths (Å) and angles (°) are listed in Tables 1 and 2, a detailed description of the method is provided in the Supporting information section.

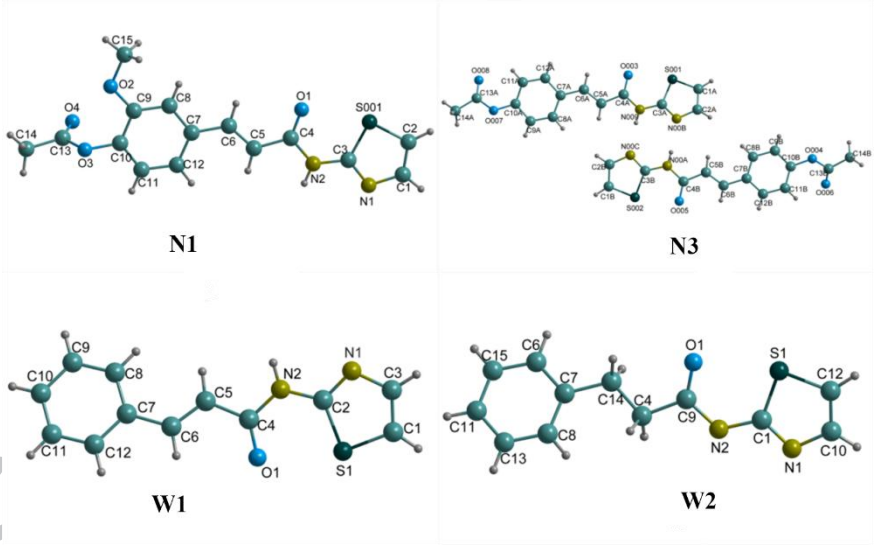


Fig. 1 Ball-and-stick structures of compounds **N1**, **N3**, **W1** and **W2**.

Table 1 Selected bond lengths (Å) for compounds **N1**, **N3**, **W1** and **W2**

Compound **N1**		Compound **N3**		Compound **W1**		Compound **W2**	
N(2)-C(4)	1.3762(18)	N(009)-C(4A)	1.3749(19)	N(2)-C(2)	1.3756(17)	N(2)-C(1)	1.394(3)
O(1)-C(4)	1.2279(17)	O(003)-C(4A)	1.2277(19)	N(2)-C(4)	1.3745(17)	N(2)-C(9)	1.397(2)
O(2)-C(15)	1.4395(19)	O(007)-C(10A)	1.3995(18)	O(1)-C(4)	1.2178(17)	O(1)-C(9)	1.221(3)
O(3)-C(13)	1.3670(17)	O(007)-C(13A)	1.3674(19)	C(4)-C(5)	1.474(2)	C(4)-C(9)	1.510(4)
O(4)-C(13)	1.1962(19)	O(008)-C(13A)	1.190(2)	C(5)-C(6)	1.3265(19)	C(4)-C(14)	1.470(4)
O(3)-C(10)	1.3945(17)						
O(2)-C(9)	1.3586(18)						

Table 2 Selected bond lengths (Å) and angles (°) for compounds **N1**, **N3**, **W1** and **W2**

Compound **N1**		Compound **N3**		Compound **W1**		Compound **W2**	
O(1)-C(4)-N(2)	121.37(13)	O(003)-C(4A)-N(009)	121.11(14)	C(4)-N(2)-C(2)	123.09(12)	C(9)-N(2)-C(1)	122.5(2)
O(1)-C(4)-C(5)	124.71(13)	O(003)-C(4A)-C(5A)	124.23(14)	O(1)-C(4)-N(2)	121.20(13)	O(1)-C(9)-N(2)	122.0(3)
C(9)-O(2)-C(15)	117.17(12)	C(13A)-O(007)-C10A	122.94(12)	O(1)-C(4)-C(5)	124.23(13)	O(1)-C(9)-C(4)	123.4(2)
C(13)-O(3)-C(10)	117.04(11)	O(008)-C(13A)-O(007)	124.18(15)	N(1)-C(2)-N(2)	121.30(12)	N(2)-C(9)-C(4)	114.6(2)
O(4)-C(13)-O(3)	122.53(13)	O(008)-C(13A)-C(14A)	126.16(15)	N(2)-C(2)-S(1)	123.34(10)	C(14)-C(4)-C(9)	113.2(2)
O(4)-C(13)-C(14)	127.62(13)			C(6)-C(5)-C(4)	120.72(13)		

The mass losses and the heat flux of the cinnamic acid amide derivatives upon changing the temperatures are shown in Figures 2 and 3 and a detailed description of the method is provided in the Supporting information section. Compound **W2** started to decompose at 157 °C while others decomposed above 200 °C as determined by decreasing quality characteristics. It can be inferred that the thermal stability of compound **W2** decreases with the decrease of the degree of unsaturation. Thermogravimetric analysis indicated that these compounds exhibit a suitable thermal stability at 150 °C. Therefore, these compounds are appropriate for further screening as coagulant drugs.

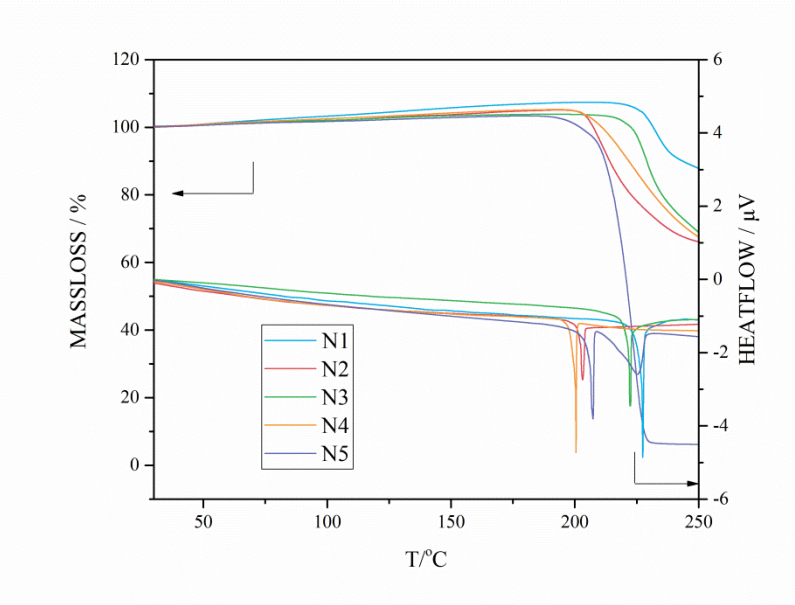


Fig. 2 Thermogravimetric curves and heatflow curves of **N1**-**N5** recorded at a constant heating rate. The mass loss is expressed as the fraction M/M_0 of the mass at temperature T to the initial mass of the samples equlibrated at laboratory atmosphere.

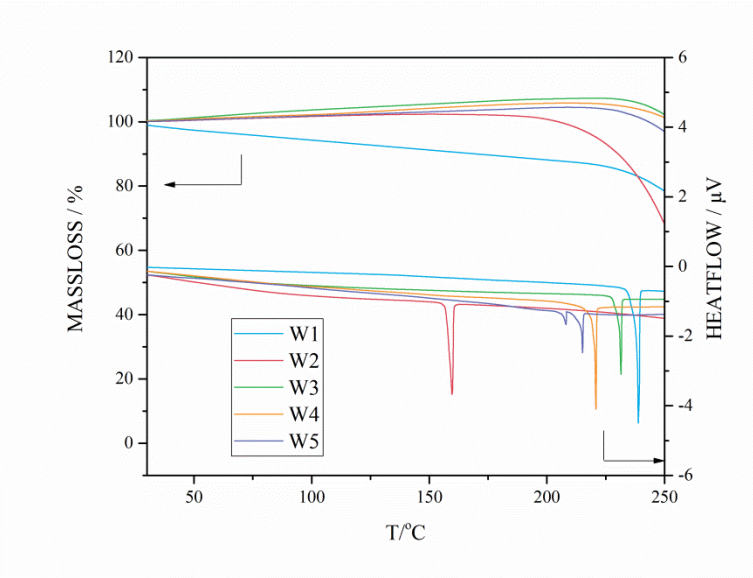


Fig. 3 Thermogravimetric curves and heatflow curves of **W1**-**W5** recorded at a constant heating rate. The mass loss is expressed as the fraction M/M_0 of the mass at temperature T to the initial mass of the samples equlibrated at laboratory atmosphere.

To investigate the coagulant activity effects of the 10 compounds (**N1**-**N5**, **W1**-**W5**), we conducted assays of PT (prothrombin time), APTT (activated partial thromboplastin time), TT (thrombin time) and platelet aggregation activities. PT is an ideal and commonly used screening assay for the detection of extrinsic coagulation systems,[22] whereas APTT is a test of the intrinsic coagulation activity. The latter may be a rough detection of coagulation factor II, V, VII and X activity.[22, 23] Note that a prolonged PT may suggest the inhibition of the extrinsic and/or the common coagulation pathway, whereas a prolonged APTT may suggest the inhibition of the intrinsic and/or the common pathway.[24] TT is a screening test for determining the thrombin activity and the ability of converting plasma fibrin into fibrin.[25] In particular, TT reflects the conversion time of fibrinogen to fibrin. A shortened TT may represent an increase in coagulatable fibrinogen or an increase in its activity.[26] The coagulation rate (R_{xx}) was used to evaluate the haemostasis activity. Negative values of drugs may represent haemostasis activity and smaller values may indicate that improved clotting effects of the compounds are demonstrated. Overall, platelet aggregation represents a vastly complex process. Its activation is mediated primarily through platelet adhesion at the site of injury as well as the action of endogenous agonists such as ADP (adenosine diphosphate), collage, and thrombin, followed by the release of TXA_2, which acts as an amplifying factor.[27] Activation is enhanced by the formation and liberation of TXA_2, the secretion of ADP and serotonin from the platelet dense granules, and the exposure of a procoagulant surface that promoted the generation of thrombin.[27] These agonists stimulate platelet aggregation via specific receptors on the platelet membrane.[28] All results of PT, APTT, TT assay can be found listed in Table 3-5, and a detailed description of the method is provided in the Supporting information section. The results of the ADP induced platelet aggregation assay in human platelet-rich plasma (PRP) using the Born's method[29] are listed in Table 6.

Table 3 R_{PT} (%) values of the synthesized compounds

Compound	Concentration (μmol/L)				
	1	5	10	50	100
Haemostatic acid	1.15 ± 0.28	1.05 ± 0.18	0.73 ± 0.48	0.63 ± 0.28	0.73 ± 0.18
2-aminothiazole	-6.63 ± 0.23***	-4.91 ± 0.65***	-2.84 ± 0.39**	-5.17 ± 0.52***	-5.43 ± 0.65***
N1	-1.98 ± 0.23***	-2.24 ± 0.31***	-4.39 ± 0.41***	-5.77 ± 0.22***	-7.32 ± 0.45***
N2	-4.30 ± 0.18***	-4.09 ± 0.38***	-1.36 ± 0.69	-1.68 ± 0.46**	-3.88 ± 0.36***
N3	-3.20 ± 0.62***	-4.40 ± 0.17***	-5.29 ± 0.17***	-4.30 ± 0.66***	1.20 ± 0.40
N4	-0.10 ± 0.58	-1.05 ± 0.10*	-0.73 ± 0.10	-0.42 ± 0.28	-0.84 ± 0.31
N5	-1.68 ± 0.10**	-3.25 ± 0.28***	-4.62 ± 0.46***	-2.20 ± 0.56***	-1.78 ± 0.55*
W1	-5.69 ± 0.61***	-7.09 ± 0.35***	-5.59 ± 0.17***	-4.40 ± 0.17***	-3.70 ± 0.20***
W2	-0.56 ± 0.52	-2.44 ± 0.49***	-3.19 ± 0.34***	-2.81 ± 0.25***	0.00 ± 0.50
W3	-1.51 ± 0.57**	-2.27 ± 0.09***	0.00 ± 0.49	-0.75 ± 0.50	-6.75 ± 0.19***
W4	-1.70 ± 0.25**	0.28 ± 0.41	-0.22 ± 0.16	-1.49 ± 0.25**	-2.99 ± 0.34***
W5	0.56 ± 0.34	-0.95 ± 0.57*	3.22 ± 0.25*	0.55 ± 0.25	2.55 ± 0.25

Data represent mean ± SEM. n = 4.

* P < 0.05 vs. haemostatic acid.

** P < 0.01 vs. haemostatic acid.

*** P < 0.001 vs. haemostatic acid.

Table 4 R_{APTT} (%) values of the synthesized compounds

Compound	Concentration (μmol/L)				
	1	5	10	50	100
Haemostatic acid	-6.37 ± 0.23	-7.74 ± 0.11	-7.28 ± 0.30	-6.83 ± 0.39	-5.23 ± 0.46
2-aminothiazole	-3.71 ± 0.49***	-2.00 ± 0.25***	-0.43 ± 0.29***	0.00 ± 0.51***	2.53 ± 0.29***
N1	-1.14 ± 0.25***	-2.57 ± 0.38***	-3.42 ± 0.38***	-6.28 ± 0.25	-1.85 ± 0.39**
N2	-2.94 ± 0.11***	-6.00 ± 0.33	-2.94 ± 0.23***	-2.04 ± 0.60***	0.00 ± 0.57***
N3	-1.68 ± 0.52***	-3.10 ± 0.22***	-3.88 ± 0.22***	-2.71 ± 0.22***	-2.45 ± 0.52*
N4	-4.64 ± 0.41*	-6.57 ± 0.20	-10.19 ± 0.60***	-9.06 ± 0.49	-8.61 ± 0.20**
N5	-5.55 ± 0.00	-9.51 ± 0.30	-8.61 ± 0.20	-8.15 ± 0.34	-6.12 ± 0.11
W1	-4.78 ± 0.52	-5.81 ± 0.22*	-9.95 ± 0.47**	-6.72 ± 0.47	-2.71 ± 0.38
W2	1.00 ± 0.38***	-2.42 ± 0.14***	-1.85 ± 0.25***	-1.56 ± 0.57***	0.28 ± 0.65***
W3	-1.28 ± 0.28***	-2.70 ± 0.34***	0.14 ± 0.28***	0.28 ± 0.65***	-0.71 ± 0.33***
W4	-0.14 ± 0.37***	-6.47 ± 0.48	-1.41 ± 0.32***	-0.56 ± 0.38***	1.97 ± 0.37***
W5	0.56 ± 0.37***	1.55 ± 0.51***	2.25 ± 0.14***	1.96 ± 0.37***	1.69 ± 0.24***

Data represent mean ± SEM. n = 4.

* P < 0.05 vs. haemostatic acid.

** P < 0.01 vs. haemostatic acid.

*** P < 0.001 vs. haemostatic acid.

Table 5 R_{TT} (%) values of the synthesized compounds

Compound	Concentration (μmol/L)				
	1	5	10	50	100
Haemostatic acid	-7.13 ± 0.63	-4.99 ± 0.63	-4.75 ± 0.48	-4.28 ± 0.36	-4.04 ± 0.24
2-aminothiazole	-3.53 ± 0.23	-5.06 ± 0.22	-6.47 ± 0.61	-5.41 ± 0.20	-3.53 ± 0.62
N1	-5.76 ± 0.33	-7.88 ± 0.20**	-13.06 ± 0.31***	-12.35 ± 0.42***	-11.06 ± 0.35***
N2	-13.94 ± 0.31***	-30.77 ± 0.33***	-28.13 ± 0.64***	-27.89 ± 0.42***	-15.63 ± 0.00***
N3	-11.45 ± 0.38*	-14.54 ± 0.22***	-22.03 ± 0.38***	-21.15 ± 0.58***	-6.39 ± 0.22
N4	-5.05 ± 0.24	-7.21 ± 0.24*	-15.39 ± 0.24***	-19.95 ± 0.00***	-21.64 ± 0.24***
N5	-2.40 ± 0.24*	-13.46 ± 0.43***	-21.39 ± 0.42***	-26.20 ± 0.24***	-21.88 ± 0.24***
W1	-7.93 ± 0.44	-10.79 ± 0.38***	-11.23 ± 0.22***	-13.66 ± 0.39***	-9.69 ± 0.48*
W2	-3.15 ± 0.48	-4.60 ± 0.48	-5..33 ± 0.64	-7.02 ± 0.84*	-7.93 ± 0.44***
W3	-6.54 ± 0.64	-7.51 ± 0.24*	-8.23 ± 0.24***	-13.80 ± 0.24***	-16.95 ± 0.24***
W4	-14.62 ± 0.24***	-16.04 ± 0.24***	-22.41 ± 0.62***	-23.82 ± 0.47***	-14.62 ± 0.47***
W5	-29.01 ± 0.24***	-22.17 ± 0.41***	-10.14 ± 0.41***	-21.70 ± 0.45***	-50.24 ± 0.62***

Data represent mean ± SEM. n = 4.

* P < 0.05 vs. haemostatic acid.

** P < 0.01 vs. haemostatic acid.

*** P < 0.001 vs. haemostatic acid.

Table 6 In vitro activities on platelet aggregation of the synthesized compounds

Compound	IC_{50}[a] (μmol/L)	Compound	IC_{50}[a] (μmol/L)
Etamsylate	46.22	2-Aminothiazole	201.96
N1	25.46	**W1**	36.57
N2	75.63	**W2**	45.11
N3	58.12	**W3**	73.71
N4	35.47	**W4**	54.54
N5	1.87	**W5**	18.09

[a] Concentration required reducing ADP-induced human platelet aggregation response by 50%. The IC_{50} values are expressed as the average of at least three determinations.

Compound **N5** showed the highest efficacy as a platelet aggregation promoter and the highest IC_{50} value (1.87 μmol/L) in the micromolar range (1 μmol/L). Compounds **W5**, **N1**, **N4**, **W1** and **W2** (IC_{50} = 18.09, 25.46, 35.47, 36.57 and 45.11 μmol/L) all demonstrated a better haemostatic activity than etamsylate (positive control, IC_{50} = 46.22 μmol/L, Table 6). Simultaneously, compound **N5** significantly shortened the TT (p <0.05 or p < 0.001, Table 5), while shortening the APTT and PT to a certain extent. Compound **N1** exhibited the lowest PT value (R_{PT} = -7.32 ± 0.45 at 100 μmol/L, Table 3), a significantly shortened TT (p <0.01 or p < 0.001, Table 5) and exhibited an excellent platelet aggregation activity compared to the positive control. Compound **N1** also shortened APTT and PT to some extent. The haemostatic mechanism is most likely due to compounds **N1** and **N5** promoting the fibrinogen conversion to fibrin in the plasma while promoting platelet adhesion, stimulating the formation and the release of agonist ADP-activated TXA_2 to aggregate platelets.

However, the intrinsic and extrinsic pathways were found to be less affected. The results of compound **N5** further highlight the important effect of the chemical group in 1-position of the *N*-(thiazol-2-yl)cinnamamide scaffold on the platelet activity. Generally, the mechanisms of stimulation-induced platelet aggregation are different at the beginning of the aggregation process: one correlates to the activation of a specific receptor, and another one deals with the massive entry of external calcium into the platelet (in the case of ionomycin).[30] Both pathways have some common features: an increase in the level of cytosolic calcium, the binding of fibrinogen to its receptor and the phosphorylation of certain proteins.[31] These points lead us to believe that the mode of action of compound **N5** focused on one of these key steps in the process of platelet aggregation. The significantly reduced TT values of compound **N5** indicate that the compound does not feature the required structural elements to block the fibrinogen receptor. However, in this study the effects of cytoplasmic calcium levels and phosphorylation status on platelet protein have not been studied but the mechanism of this action is subject to continuous research in our laboratory.

Although compound **N4** exhibited a minimum APTT value (R_{APTT} = -10.19 ± 0.60, Table 4), it also significantly shortened the TT value ($p < 0.05$ or $p < 0.001$, cf. Table 5) and promoted platelet aggregation compared to the positive control etamsylate. However, a rare effect on PT could be observed. This in turn suggests that compound **N4** achieved the desired haemostatic results primarily through thrombin-mediated fibrin formation and partly by activating intrinsic pathways of blood coagulation and promoting platelet aggregation. However, hardly any haemostatic effects were found to be due to activating extrinsic pathways of blood coagulation.

Compound **W5** exhibited the lowest TT value (R_{TT} = -50.24 ± 0.62, Table 5) and was found to be effective in promoting platelet aggregation relative to the positive control etamsylate while increasing PT and APTT to a certain extent. This in turn indicates that compound **W5** mainly achieved a haemostatic effect through stimulating fibrinogen or increasing fibrin and activation of platelet aggregation. However, coagulation was not found to be caused by intrinsic and extrinsic pathways.

In combination with the experimental studies of the 10 compounds, preliminary SARs analysis showed that the condensation of acids and amine to form amides could shorten PT, APTT and TT, and promote platelet aggregation. The addition of a methoxy functional group could shorten the TT and promote platelet aggregation. The formyloxy group in ortho- or para-position of the phenyl functionality could shorten not only the TT but also the PT to a certain extend. Changing the double bonds to a single bond on the branch could extend PT, APTT and TT and inhibit platelet aggregation. However, the number of methoxy groups or the position of the formyl group on the benzene ring has been found to have little effect on APTT.

In conclusion, the structure-activity relationships of a series of studied potent haemostatic agents demonstrated that the introduction of carboxyl groups to amino groups decreased PT, APTT, TT and promoted platelet aggregation; the position and

the quantity of methoxy and formyloxy groups also influenced the PT, APTT, TT and platelet aggregation activities of the cinnamic acid amide derivatives containing thiazoles. In addition, the introduction of methoxy and formyloxy groups resulted in an improved platelet aggregation effect compared to the mere conversion of carboxyl groups into amide groups. However, further work is necessary to better define the relationship between the functional groups of the compounds and the blood coagulation activity. This understanding may facilitate the design of chemical compounds exhibiting a higher potency to serve as procoagulant and will further provide information on the exploitation and utilization of amide compounds containing thiazole as a procoagulant for bleeding treatments during surgery. Finally, this work demonstrated that compound **N5** features potential characteristics for the development of drugs combining both coagulant and platelet effects.

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