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A Chromogenic and Fluorogenic Peptide Substrate for the Highly Sensitive Detection of Proteases in Biological Matrices

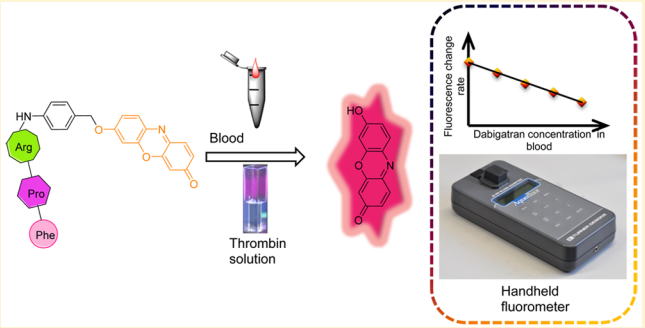
Dumitru Arian,[†] Job Harenberg,[‡] and Roland Krämer*[,†]

[†]Anorganisch-Chemisches Institut, Universität Heidelberg, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany

[‡]Medical Faculty Mannheim, Universität Heidelberg, Maybachstrasse 14, 68169 Mannheim, Germany

Supporting Information

ABSTRACT: The synthesis and application of a novel type of chromogenic and fluorogenic substrate for protease detection is described. The outstanding performance of the tripeptide substrates is exemplified by specific fluorescence detection of thrombin and factor Xa at only 500 fM concentration. The substrate is also applicable to the sensitive detection of the thrombin inhibitor dabigatran in human plasma and whole blood samples, highlighting its potential for a point-of-care test for instant monitoring the blood levels of this blockbuster anticoagulant drug in specific clinical situations.



INTRODUCTION

There are over 500 proteases which account for approximately 2% of all proteins in the human body. As one of the largest and most important groups of enzymes, they are involved in many physiological processes, including protein digestion and turnover, blood clotting, apoptosis, hormone activation, fertilization, and growth differentiation. An ill-regulated protease activity is associated with many diseases. Therefore, there is a strong demand for the development of sensitive protease assays for proteomic research, disease diagnosis, and drug development and monitoring.[1,2] In addition, triggered protease activation has been applied in cascade-like amplification schemes for the highly sensitive detection of various analytes.[3,4] The majority of commercial kits for detection of protease activity include chromogenic (*p*-nitroanilides) or fluorogenic (7-amido-4-methylcoumarines, rhodamine) substrates that release a colored or fluorescent compound upon selective cleavage by the target protease (Figure 1). Fluorogenic substrates provide a much more sensitive read out. Design strategies for fluorogenic probes have been reported.[5] Up to now, latent fluorophores (pro-fluorophores) are some of the most widely used tools in visualization of biologically relevant molecules (H_2O_2, NO, sulfite, O_3, $^1O_2$, etc.)[6−10] and enzyme activities (esterases, *β*-galactosidase, proteases, ribonucleases).[11−15]

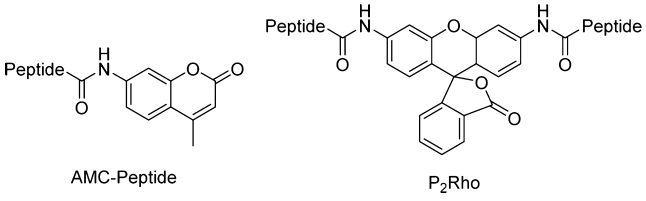


Figure 1. Fluorogenic protease substrates.

We present here novel chromogenic and fluorogenic protease substrates based on resorufin, a highly colored and highly fluorescent, red-emissive dye. Resorufin-based substrates often provide significantly greater sensitivity in fluorescence-based assays due to lower biological background absorbance and fluorescence.[7,8,12,16] It is a longer wavelength dye (ex 570/em 585 nm) with high quantum yield (φ = 0.75) and extinction coefficient (ε = 60.000 M^{-1} cm^{-1}). Relatively low pK_a of resorufin (~6.0) permits continuous measurement of enzymatic activity at physiological pH. Application of the substrates is exemplified by the highly sensitive detection of the coagulation proteases thrombin and factor Xa as well as of the thrombin inhibitor dabigatran in human plasma and whole blood. Point-of-care testing of direct oral anticoagulants, including the blockbuster drugs dabigatran and rivaroxaban, is an unmet need.[17,18] Such a test may facilitate medical decisions for acute therapeutic interventions, such as application of a recently approved dabigatran-specific antidote idarucizumab.

RESULTS AND DISCUSSION

Probe Design. Successful probes for biomolecular applications need to fulfill several requirements: increase in emission intensity upon reaction with the enzyme, efficiency, and stability. We chose the model of a self-cleavable linker as spacer between peptide substrate and the fluorescent label. Katzenellenbogen reported for the first time the prodrug linker *p*-aminobenzyl alcohol (PABA).[19] It made it possible to easily conjugate peptides to different dyes and drugs.[14,20,21] The

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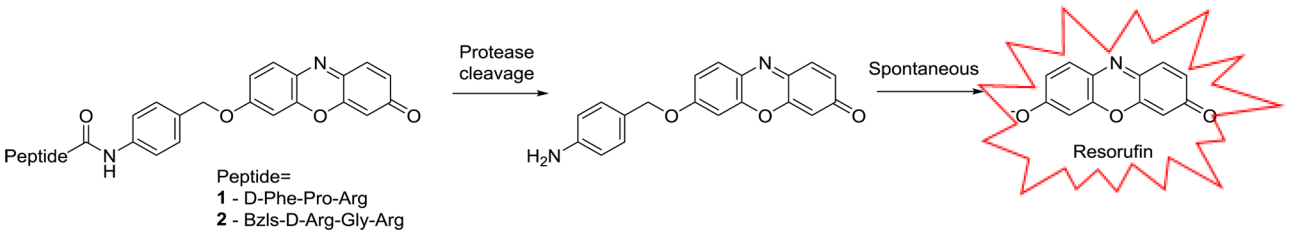
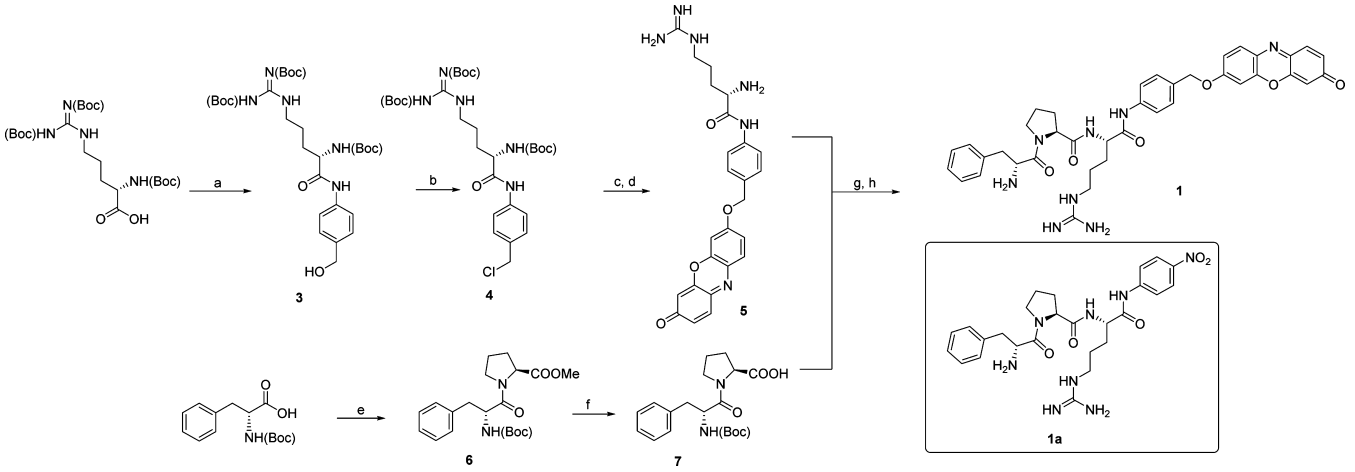


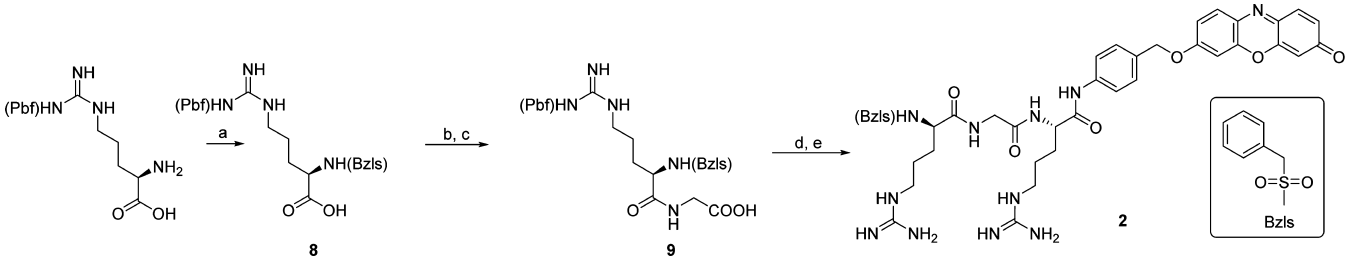
Figure 2. Activation of pro-fluorophore by proteolytic enzymes.

Scheme 1. Synthesis of the Fluorogenic Peptide 1[a]



[a]Reagents and conditions: (a) PABA, TBTU, DIEA, DMF, rt, 12 h (93%); (b) cyanuric chloride, DMSO, rt, 1 h (30%); (c) resorufin, K_2CO_3, DMF, rt, 12 h (95%); (d) TFA−DCM 1:1, rt, 3 h (88%); (e) L-proline methyl ester, TBTU, DIEA, DMF, rt, 12 h (73%); (f) THF−H_2O, NaOH, 0 °C, 3 h (79%); (g) TBTU, DIEA, DMF, rt, 12 h; (h) TFA−DCM 1:1, rt, 1 h (60% over 2 steps).

Scheme 2. Synthesis of the Fluorogenic Peptide 2[a]



[a]Reagents and conditions: (a) NaOH, Bzls-Cl, Et_3N, acetone−water (27%); (b) NHS−ester, NHS, DCC; (c) DME, $NaHCO_3$, glycine (19%); (d) **5**, TBTU, DIEA, DMF, rt, 12 h; (e) TFA−DCM 1:1, rt, 3 h (10% over 2 steps).

spacer is also beneficial to prevent steric hindrance around the cleavage site. Resorufin will serve as a fluorescent reporter molecule, featuring good water solubility, long emission wavelength, and very efficient quenching via 7-hydroxy substitution (Figure 2).

We synthesized the prodrug-inspired substrates for thrombin (D-Phe-Pro-Arg-PABA-resorufin) and factor Xa (Bzls-D-Arg-Gly-Arg-PABA-resorufin) and evaluated the efficiency of their activation (Figure 2).

Synthesis. The synthesis of the building block **5**, which was used for both enzyme substrates started from Boc-Arg(Boc)$_2$-OH (Scheme 1). It coupled to PABA under standard conditions, affording the benzylic alcohol **3** in almost quantitative yield. The chlorination of the alcohol was complicated by the acid-labile Boc groups. Commonly used chlorination reagents ($SOCl_2/Et_3N$, CCl_4/Ph_3P) failed to deliver the desired compound. Even MsCl/Et_3N, a mild chlorination reagent,[22] afforded **4** in unsatisfactory yields (9−15%). The best results were obtained with cyanuric chloride/DMSO mixture, which gave **4** in 30% yield.[23] It is worthwhile mentioning that 0.5 equiv of cyanuric chloride was optimal; more reagent leads to decrease of isolated product. Alkylation of resorufin[12] and Boc-deprotection proceeded smoothly, yielding the conjugate **5** in good yield. As shown in Scheme 1, the synthesis of the second building block started with the coupling of Boc-D-Phe-OH with H-Pro-OMe to provide the methyl ester **6** in good yield. The ester was hydrolyzed in THF/H_2O mixture with NaOH at 0 °C, providing the free dipeptide **7** in 79% yield. The coupling of dipeptide **7** and building block **5** worked better with TBTU as activator than with other agents. The reaction afforded better yields, and the product could be easily isolated from the byproducts. The Boc-deprotection with TFA/DCM mixture and C18 silica chromatography afforded **1** as TFA salt in good yields.

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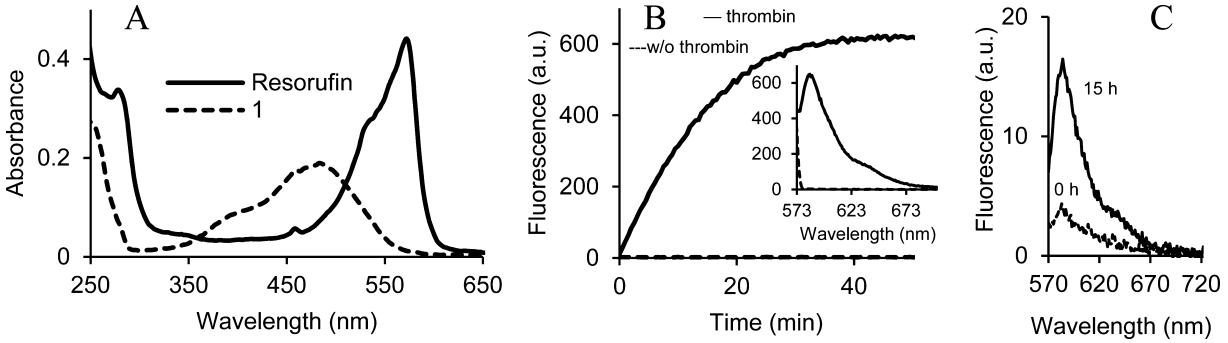

Figure 3. (A) Absorption spectra of resorufin and fluorogenic substrate **1**. (B) Enzymatic hydrolysis of **1** (5 μM) in the presence (100 pM) and in the absence of thrombin (λ_{ex} = 570 nm; λ_{em} = 583 nm) (inset: emission spectra recorded after 60 min). (C) Stability of **1** in Tris buffer pH 8.3, at rt.

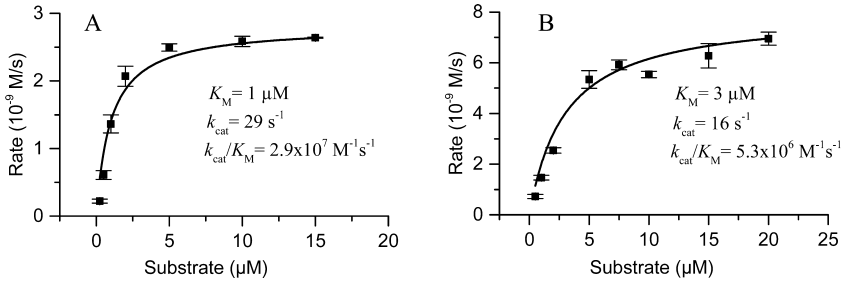

Figure 4. Kinetic parameters obtained for thrombin substrate **1** (A) and factor Xa substrate **2** (B).

The factor Xa substrate **2** was synthesized similarly (Scheme 2). H-D-Arg(Pbf)-OH was first protected with benzylsulfonyl chloride (Bzls-Cl). The coupling with glycine via NHS−ester afforded dipeptide **9**, which was further coupled to the building block **5** following the same procedure as that used for the preparation of **1**. The total yield of **2** is substantially lower if compared to compound **1**. The critical step is the final deprotection with TFA, where longer reaction time is required in order to completely remove the Pbf protecting group.

Substrate 1 Properties. We first investigated the photophysical properties of substrate **1** as well as its enzymatic conversion to fluorescent product resorufin. Compound **1** displays a blue-shift in the absorption spectra (~90 nm) relative to resorufin (Figure 3A). It also has a negligible emission, if exited either at its maxima (480 nm) or at resorufin maxima (570 nm). Additionally, no spontaneous hydrolysis is observed during incubation with thrombin buffer (Tris pH 8.3), indicating high stability of the conjugate (Figure 3C). Thrombin-induced substrate hydrolysis gives rise to ~300-fold increase in fluorescence, which demonstrates that quenching of resorufin upon *O*-alkylation is extremely efficient (Figure 3B).

Kinetic Measurements. Commercial substrate **1a**, depicted in the Scheme 1, was used as reference in the kinetic measurements. The kinetic values K_M, k_{cat}, and k_{cat}/K_M were established for the fluorogenic peptide **1** using human thrombin. To compare **1** and **1a**, we measured the absorption of the released chromophores during the enzymatic reaction (570 nm for resorufin and 405 nm for *p*-nitroaniline). We were very pleased to find out that thrombin turnover of **1** was not affected, suggesting that the PABA is a suitable linker that places the fluorophore away from the active site (literature kinetic parameters for **1a**[24] K_M = 3 μM; k_{cat}/K_M = 0.93 × 10^7 M^{-1} s^{-1}). We also determined the same parameters for **1** by measuring the emission of resorufin at 583 nm and found similar values to the ones obtained from absorption (Figure 4A). The factor Xa substrate **2** had a surprisingly low K_M value, relatively good turnover number, and very good catalytic efficiency (Figure 4B). The conjugate with the same peptide sequence, but having *p*-nitroanilide as reporter, is a poorer substrate (K_M = 40 μM; k_{cat}/K_M = 2.7 × 10^6 M^{-1} s^{-1}).[25] To our knowledge, compound **2** performs better than the best synthetic factor Xa substrate so far reported in the literature.[24,25]

Specificity of 1 for Thrombin and the Limit of Detection. Compound **1** (5 μM) was incubated in the presence of thrombin (100 pM) and some possibly interfering proteases and proteins, like trypsin, factor Xa, myoglobin, cytochrome C, and BSA (100 pM). The increase of fluorescence in response to factor Xa, myoglobin, cytochrome C, and BSA was negligible (Figure 5). Only trypsin hydrolyses

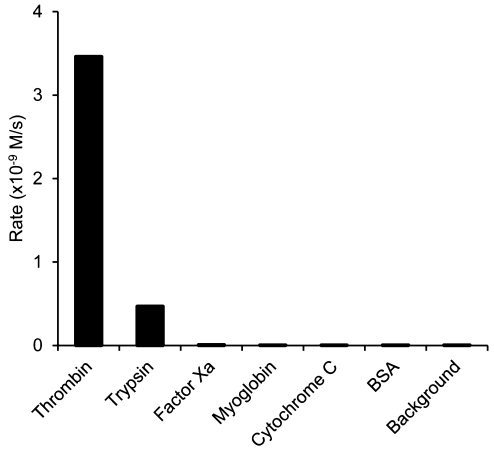

Figure 5. Specificity of **1** for thrombin. The graph shows the hydrolysis rate of substrate in the presence of the corresponding enzymes (each 100 pM).

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our substrate but at a slower rate compared to thrombin (7.5-fold more selective for thrombin over trypsin). Additionally, the assay allows the detection of thrombin at the concentration as low as 0.5 pM (Figure 6). To our knowledge, the sensitivity of

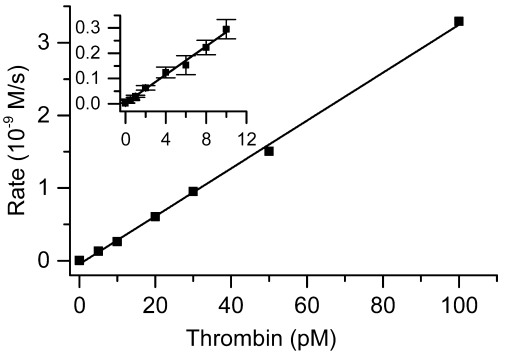

Figure 6. Detection of thrombin in buffer solution (LOD 0.5 pM).

our assay is way lower than many of the optical-based methods for detection of thrombin, reported in the literature.[26−28] The detection range from 0.5 to 100 pM was linear. There are methods which offer an order of magnitude better sensitivity, but they are way more complex than our assay.[29−31] A commercial assay can detect 1 pM thrombin by fishing it out of plasma sample using microwells coated with a DNA-aptamer.[32] The AMC-based substrate is subsequently converted by thrombin to fluorescent product after the enrichment step. Our fluorescence assay allowed the detection of thrombin at similar concentrations without any enrichment step.

Detection of Anticoagulant Dabigatran in Human Plasma and Whole Blood. The thrombin inhibitor dabigatran is commonly used anticoagulant in the clinic.[33] While routine monitoring of dabigatran is not recommended, the determination of its blood level in specific situations (such as bleeding complications, emergency, adherence to therapy) and/or patient populations (such as the elderly, renal impairment) may increase drug safety. Specific assays for dabigatran have not been established along with drug development, and further clinical trials are required to determine the relation of assay results to bleeding or thrombotic complications.[34,35] In many laboratories, only qualitative coagulation-based tests are available such as prothrombin time (PT) assay or the activated partial thromboplastin time (APTT) assay. Unfortunately, these tests often give false-negative results.[36,37] Other coagulation-based tests, such as diluted thrombin clotting time (Hemoclot assay), may have similar limitations in patients.

Next, we describe our assay that uses **1** for quantification of dabigatran in plasma and, most importantly, in whole blood, as a key step in the development of a point-of-care test. First, we tested the substrate **1** in human plasma. Human plasma was spiked with dabigatran (25−500 ng/mL) and added to a thrombin solution. After incubation for 5 min, the substrate **1** was added and the fluorescence increased at 585 nm as measured over time. We were able to construct a calibration curve, which can be used to determine the dabigatran concentration in an unknown sample (Figure 7A). The results can even be visualized by naked eye under an UV lamp.

Finally, we have constructed a similar calibration curve but using whole blood instead of plasma. The experimental procedure is similar to the one with plasma. Fresh blood portions (20 μL) were spiked with dabigatran solution (2 μL) and added to thrombin (2 mL) in a single-use fluorescence plastic cuvette. After 5 min preincubation at room temperature, the fluorogenic substrate was added and the fluorescence change was monitored using a portable fluorescence device. We were very pleased to find out that the rate of the enzymatic reaction decreases linearly with the increasing dabigatran concentration (Figure 7B).

CONCLUSIONS

In this study, we describe the synthesis and the application of a new thrombin substrate **1** based on three modules: resorufin fluorophore, self-cleavable PABA linker, and recognition tripeptide. Similarly, a new factor Xa substrate **2** was synthesized.

The new substrates are chemically stable toward spontaneous hydrolysis. Fluorogenic peptides **1** and **2** do not lose their specificity for thrombin and factor Xa correspondingly if compared to the *p*-nitroanilide analogues. Furthermore, **1** and **2** are both chromogenic and fluorogenic substrates: upon reaction with the enzyme, a more than 300-fold fluorescence increase is observed; simultaneously we observe a color change from yellow to purple. We also show that compound **1** is 7.5 times more specific for thrombin if compared to trypsin and 400 times more specific for thrombin if compared to factor Xa. We were able to detect as low as 0.5 pM thrombin in buffer solution using the substrate **1**. The sensitivity of our probe is higher than most of the aptamer-based methods reported in the

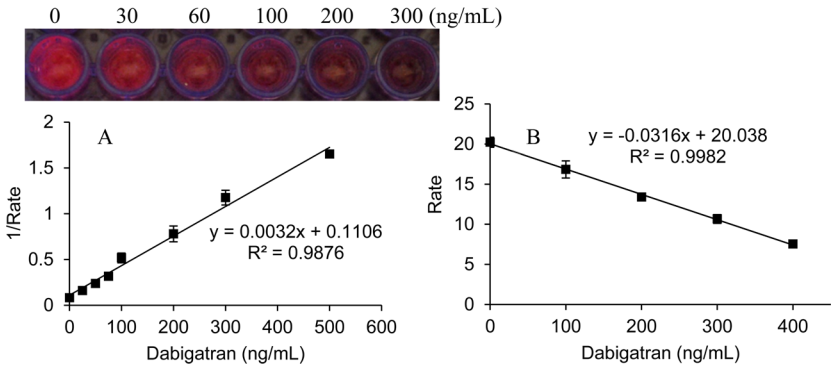

Figure 7. Calibration curves for determination of dabigatran concentration in human plasma (A) and whole blood (B). The picture of the well plate after 20 min incubation (increasing dabigatran concentration in ng/mL from left to right). Rate represents resorufin fluorescence change over time. Experiments were carried out in triplicate.

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literature. Taking advantage of its high selectivity and sensitivity, we applied our fluorogenic substrate **1** for quantification of a commonly used thrombin inhibitor dabigatran in the therapeutic range (27−411 ng/mL) in plasma and whole blood. Compound **2** might be used similarly for detection of the important factor Xa inhibitor rivaroxaban. We also adapted our whole blood assay for use at the point of care. To our knowledge, this is the first fluorogenic assay which can measure directly the dabigatran concentration without separating the red blood cells.

EXPERIMENTAL SECTION

General Methods. Proton and carbon NMR spectra were acquired on Bruker Avance III 600 spectrometer. High resolution mass spectra (HR-ESI) were recorded on Bruker ICR APEX-QE mass spectrometer. UV−vis spectra were measured on spectrophotometer Shimadzu UV-2600 and the emission spectra on a Varian Cary Eclipse and a portable fluorimeter Aquafluor from Turner Designs. Thin layer chromatography (TLC) and silica gel column chromatography were performed using Polygram Sil G/UV_{254} sheets and silica gel 60 Å 40−63 μM (Macherey-Nagel), respectively. Polygoprep 60−50 C18 silica was used for reversed phase chromatography. HPLC of target compounds was performed on a Shimadzu system (diode array detector) using a 250 mm × 4 mm Macherey-Nagel Nucleosil column for analytics and 250 mm × 10 mm for preparative with 5 μm C18 silica stationary phase. The mobile phase represented 0.1% TFA in water (solution A) and acetonitrile (solution B) with flow rate at 1 or 4 mL/min and gradient 30 min/90%. All other reagents and solvents were used as purchased without further purification.

*D-Phe-Pro-Arg-PAB-Resorufin·2TFA (**1**).* Dipeptide **7** (7.2 mg, 0.02 mmol) and TBTU (8 mg, 0.025 mmol) were dissolved in dry DMF (0.2 mL), followed by addition of DIEA (6.5 mg, 8.3 μL, 0.05 mmol). The resulted solution was stirred for 15 min under argon at rt. Resorufin conjugate **5** (10 mg, 0.014 mmol) was dissolved in DMF (0.1 mL), DIEA (3.6 mg, 4.9 μL, 0.028 mmol) was added, and the resulted mixture was added to the activated dipeptide. The reaction was stirred overnight at rt. DMF was evaporated under vacuum, and the residue was dissolved in a mixture of acetonitrile−water 2:3 (1 mL) which also contained 0.1% TFA. The red-colored solution was passed through a short column filled with C18 silica (2 g) and eluted with acetonitrile−water mixture (first with 2:3, then 3:2 mixtures; TFA 0.1%). The fractions with the product were combined and lyophilized. The dark-red solid was dissolved in a mixture DCM−TFA 1:1 (1 mL) and stirred for 1 h at rt. The volatile components were evaporated under vacuum, the residue was redissolved in methanol (0.1 mL), and the solution was poured into ether (15 mL). The solid was collected, washed with ether, and dried under vacuum, yielding final compound **1** as TFA salt (8 mg, 60% over 2 steps). ^{1}H NMR (600 MHz, methanol-d_4) δ 7.77 (d, J = 8.9 Hz, 1H), 7.64 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 9.7 Hz, 1H), 7.45 (d, J = 8.4 Hz, 2H), 7.41−7.32 (m, 4H), 7.29 (d, J = 6.8 Hz, 2H), 7.13 (dd, J = 8.9, 2.6 Hz, 1H), 7.09 (d, J = 2.6 Hz, 1H), 6.84 (dd, J = 9.7, 2.0 Hz, 1H), 6.31 (d, J = 2.0 Hz, 1H), 5.22 (s, 2H), 4.46 (dd, J = 8.8, 5.4 Hz, 1H), 4.39 (dd, J = 9.3, 6.3 Hz, 1H), 4.36 (dd, J = 8.6, 4.4 Hz, 1H), 3.54−3.47 (m, 1H), 3.24 (t, J = 7.0 Hz, 2H), 3.16 (dd, J = 13.1, 6.1 Hz, 1H), 3.14−3.09 (m, 1H), 2.64 (dt, J = 9.6, 7.2 Hz, 1H), 2.05−1.88 (m, 3H), 1.88−1.75 (m, 3H), 1.74−1.66 (m, 1H), 1.55−1.46 (m, 1H). ^{13}C NMR (151 MHz, methanol-d_4) δ 186.99, 172.90, 170.71, 167.51, 163.39, 161.61, 157.24, 150.61, 145.77, 144.91, 138.15, 135.14, 134.03, 133.17, 131.95, 131.48, 129.24, 128.72, 128.60, 128.20, 127.72, 119.97, 114.66, 105.35, 100.83, 70.27, 60.21, 53.89, 52.88, 40.63, 36.97, 29.42, 28.71, 25.09, 23.95. ESI-HRMS (MH^+) calcd for $C_{39}H_{43}N_8O_6$ 719.3306, found 719.3308. RP-HPLC analysis, 99.5% at 214 nm; t_R = 22.52 min (conditions see in General Methods).

*Bzls-D-Arg-Gly-Arg-PAB-Resorufin·2TFA (**2**).* The dipeptide **9** (20 mg, 0.031 mmol) and TBTU (12.8 mg, 0.04 mmol) were dissolved in dry DMF (1 mL), and DIEA (10.3 mg, 14 μL, 0.08 mmol) was added, followed by stirring for 15 min at rt. Resorufin conjugate **5** (21 mg, 0.03 mmol) was dissolved in DMF (0.1 mL). DIEA (7.7 mg, 10.4 μL, 0.06 mmol) was added, and the resulted mixture was added to the activated dipeptide. The reaction was stirred overnight at rt. DMF was evaporated under vacuum, and the residue was purified by C18 silica using a gradient 20−60% acetonitrile in water with 0.1% TFA. The fractions were lyophilized, and Pbf-deprotection was carried out with a mixture TFA−DCM 1:1 (1 mL) for 3 h at rt. The solvents were evaporated under vacuum, and the residue was purified by HPLC using a gradient 5−40% acetonitrile in water with 0.1% TFA. The desired fractions were combined and lyophilized, obtaining **2** (3.1 mg, 10% over 2 steps) as a TFA salt. ^{1}H NMR (600 MHz, methanol-d_4) δ 7.79 (d, J = 8.9 Hz, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 9.8 Hz, 1H), 7.47−7.39 (m, 4H), 7.35 (dd, J = 4.8, 1.9 Hz, 3H), 7.14 (dd, J = 8.9, 2.6 Hz, 1H), 7.10 (d, J = 2.7 Hz, 1H), 6.85 (dd, J = 9.8, 2.1 Hz, 1H), 6.32 (d, J = 2.1 Hz, 1H), 5.23 (s, 2H), 4.48−4.43 (m, 1H), 4.43−4.35 (m, 2H), 4.00−3.88 (m, 2H), 3.84 (t, J = 6.7 Hz, 1H), 3.18 (t, J = 7.1 Hz, 2H), 3.15 (t, J = 7.0 Hz, 2H), 2.01−1.92 (m, 1H), 1.86−1.57 (m, 7H). ^{13}C NMR (151 MHz, methanol-d_4) δ 187.00, 173.71, 170.75, 170.22, 163.37, 161.25, 157.19, 150.63, 145.79, 144.94, 138.04, 135.15, 133.18, 132.03, 131.48, 130.75, 129.46, 128.61, 128.21, 128.11, 120.23, 114.69, 105.35, 100.84, 70.26, 58.48, 56.64, 53.85, 42.32, 40.58, 40.44, 29.59, 28.61, 24.98, 24.68. ESI-HRMS (MH^+) calcd for $C_{40}H_{48}N_{11}O_8S^+$ 842.3403, found 842.3394. RP-HPLC analysis, 97.9% at 214 nm; t_R = 22.17 min (conditions, see in General Methods).

*Boc-Arg(Boc)$_2$-PAB-OH (**3**).* To a solution of Boc-Arg(Boc)$_2$-OH (100 mg, 0.21 mmol) and TBTU (80 mg, 0.25 mmol) in DMF (5 mL), DIEA (64.5 mg, 87 μL, 0.5 mmol) was added and the resulting solution was stirred at rt for 15 min. PABA (30 mg, 0.25 mmol) was added as solid, and the reaction mixture was stirred at rt overnight. DMF was evaporated under vacuum, and the residue was taken up in ethyl acetate (10 mL). The organic phase was washed with water twice, dried over $MgSO_4$, and the solvent evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using 5% methanol in DCM as an eluent, yielding **3** as a foam (113 mg; 93%). ^{1}H NMR (600 MHz, acetonitrile-d_3) δ 7.49 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 4.51 (d, J = 5.7 Hz, 2H), 4.21−4.11 (m, 1H), 3.94−3.85 (m, 1H), 3.85−3.78 (m, 1H), 3.26 (t, J = 5.8 Hz, 1H), 1.80−1.72 (m, 1H), 1.69−1.60 (m, 3H), 1.47 (s, 9H), 1.41 (s, 9H), 1.40 (s, 9H). ^{13}C NMR (151 MHz, acetonitrile-d_3) δ 164.08, 162.95, 161.37, 155.54, 138.39, 137.82, 127.82, 120.40, 84.43, 78.82, 63.91, 44.54, 38.43, 36.17, 30.88, 28.15, 28.00, 27.71, 25.40. ESI-HRMS (MNa^+) calcd for $C_{28}H_{45}N_5NaO_8$ 602.3166, found 602.3176. According to ^{1}H NMR spectra, 95% pure.

*Boc-Arg(Boc)$_2$-PAB-Cl (**4**).* To a solution of **3** (160 mg, 0.27 mmol) in dry DMSO (4 mL) was added cyanuric chloride (24.4 mg, 0.13 mmol) in 3−4 portions under stirring at rt. The resulting mixture was stirred for 1 h at rt. The DMSO solution was poured into a $NaHCO_3$ solution (20 mL) and extracted with ethyl acetate (20 mL). The extract was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The residue was purified by column chromatography using 10% ethyl acetate in DCM as eluent, yielding **4** as white foam (47 mg, 30%). ^{1}H NMR (200 MHz, chloroform-d) δ 7.47 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 4.76−4.63 (m, 1H), 4.58 (s, 2H), 4.21−3.84 (m, 2H), 2.24−1.64 (m, 4H), 1.55 (s, 9H), 1.49 (s, 9H), 1.47 (s, 9H). ESI-HRMS (MNa^+) calcd for $C_{28}H_{44}ClN_5NaO_7$ 620.2827, found 620.2839. According to ^{1}H NMR spectra, 99% pure.

*Arg-PAB-Resorufin·2TFA (**5**).* Resorufin (32 mg, 0.15 mmol) and dry K_2CO_3 (41.4 mg, 0.3 mmol) were first kept under vacuum for 2 h. Dry DMF (1 mL) was added under inert atmosphere and the mixture stirred for 30 min. The solvent was evaporated under vacuum, and a new portion of DMF (3 mL) was added. Benzylic chloride **4** (45 mg, 0.075 mmol) in DMF (1 mL) was added dropwise, and the resulting mixture was stirred overnight. DMF was evaporated under vacuum, water was added to the residue, and the product was extracted with DCM. The extracts were washed with brine, dried over $MgSO_4$, and the solvent evaporated under reduced pressure. The residue was purified by column chromatography using 5% methanol in DCM as eluent, yielding Boc-protected **5** as an orange solid (55 mg, 95%). ^{1}H NMR (600 MHz, chloroform-d) δ 9.44 (s, 1H), 9.28 (s, 1H), 9.08 (s,

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1H), 7.69 (d, J = 8.8 Hz, 1H), 7.53 (d, J = 8.0 Hz, 2H), 7.41 (d, J = 9.8 Hz, 1H), 7.38 (d, J = 8.1 Hz, 2H), 6.98 (dd, J = 8.9, 2.6 Hz, 1H), 6.85 (d, J = 2.6 Hz, 1H), 6.83 (dd, J = 9.8, 2.0 Hz, 1H), 6.31 (d, J = 2.0 Hz, 1H), 6.05−5.89 (m, 1H), 5.13 (s, 2H), 4.59−4.41 (m, 1H), 4.12−3.95 (m, 1H), 3.79−3.63 (m, 1H), 1.93−1.79 (m, 2H), 1.79−1.71 (m, 1H), 1.70−1.59 (m, 1H), 1.51 (s, 9H), 1.46 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (151 MHz, chloroform-d) δ 186.35, 163.08, 162.62, 161.11, 154.83, 149.82, 145.66, 145.59, 137.95, 134.72, 134.26, 131.60, 131.44, 128.48, 128.17, 121.36, 114.35, 106.76, 101.12, 84.33, 79.98, 79.61, 70.56, 53.99, 43.96, 29.05, 28.45, 28.03, 24.60. ESI-HRMS (MH^+) calcd for $C_{40}H_{51}N_6O_{10}$ 775.3667, found 775.3684. Boc-groups were cleaved using a 1:1 TFA/DCM mixture (3 mL) at rt for 3 h. The solvents were evaporated under reduced pressure, and the resulting solid was dissolved in a minimal amount of methanol (0.5 mL). The methanolic solution was poured in ether (10 mL), and the resorufin conjugate was collected as an orange solid as TFA salt. It was washed with ether several times and dried under vacuum, yielding 44 mg (88%) of pure product **5**. ^{1}H NMR (600 MHz, methanol-d_4) δ 7.79 (d, J = 8.9 Hz, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 9.8 Hz, 1H), 7.49 (d, J = 8.4 Hz, 2H), 7.14 (dd, J = 8.9, 2.6 Hz, 1H), 7.11 (d, J = 2.6 Hz, 1H), 6.85 (dd, J = 9.8, 2.1 Hz, 1H), 6.33 (d, J = 2.1 Hz, 1H), 5.24 (s, 2H), 4.04 (t, J = 6.5 Hz, 1H), 3.25 (t, J = 7.0 Hz, 2H), 2.10−1.88 (m, 2H), 1.83−1.66 (m, 2H). ^{13}C NMR (151 MHz, methanol-d_4) δ 187.01, 166.79, 163.34, 157.28, 150.63, 145.79, 144.98, 137.66, 135.16, 133.20, 132.51, 131.49, 128.63, 128.33, 128.33, 119.83, 114.63, 105.36, 100.84, 70.17, 53.33, 48.17, 48.03, 40.40, 28.48, 24.07. ESI-HRMS (MH^+) calcd for $C_{25}H_{27}N_6O_4$ 475.2094, found 475.2082. According to ^{1}H NMR spectra and HPLC, 99% pure.

*Boc-D-Phe-Pro-OMe (**6**).* To a solution of Boc-D-Phe-OH (250 mg, 0.95 mmol) and TBTU (369 mg, 1.15 mmol) in DMF (10 mL) was added DIEA (296 mg, 400 μL, 2.3 mmol), and the solution was stirred for 15 min at rt. H-Pro-OMe·HCl (190 mg, 1.15 mmol) was dissolved in DMF (2 mL), mixed with DIEA (148 mg, 200 μL, 1.15 mmol), and added to the reaction mixture. After stirring overnight at rt, the solvent was evaporated and to the residue was added water and ethyl acetate. The extracts were dried over $MgSO_4$, the solvent was evaporated, and the residue was chromatographed on silica with 10% ethyl acetate in DCM as eluent. The dipeptide **6** (260 mg, 73%) was obtained as white foam. ^{1}H NMR (600 MHz, chloroform-d) δ 7.32−7.13 (m, 5H), 5.37 (d, J = 8.6 Hz, 1H), 4.62 (td, J = 9.1, 5.4 Hz, 1H), 4.28 (dd, J = 8.4, 3.9 Hz, 1H), 3.71 (s, 3H), 3.57−3.47 (m, 1H), 3.05 (dd, J = 12.9, 5.5 Hz, 1H), 2.91 (dd, J = 12.8, 9.5 Hz, 1H), 2.68−2.56 (m, 1H), 1.95−1.88 (m, 1H), 1.88−1.78 (m, 2H), 1.53−1.46 (m, 1H), 1.43 (s, 9H).

*Boc-D-Phe-Pro-OH (**7**).* To the solution of methyl ester **6** (260 mg, 0.7 mmol) in THF (2 mL) was added water (10 mL), and the emulsion was cooled to 0 °C. A solution of NaOH (0.5 N, 20 mL) was added dropwise, and the reaction mixture was stirred for 3 h at 0 °C. The base was neutralized with acetic acid, and the product was extracted with ethyl acetate. The extracts were dried over $MgSO_4$, the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography on silica using 5% methanol in DCM with 1% acetic acid. The product **7** (200 mg, 79%) was obtained as white crystals. ^{1}H NMR (600 MHz, chloroform-d) δ 7.32−7.15 (m, 5H), 5.35 (d, J = 8.5 Hz, 1H), 4.64 (td, J = 9.3, 5.6 Hz, 1H), 4.36 (d, J = 5.9 Hz, 1H), 3.62−3.50 (m, 1H), 3.07 (dd, J = 12.8, 5.5 Hz, 1H), 2.94 (dd, J = 12.8, 10.0 Hz, 1H), 2.53 (td, J = 9.4, 6.5 Hz, 1H), 2.32−2.19 (m, 1H), 1.88−1.76 (m, 1H), 1.65−1.51 (m, 2H), 1.43 (s, 9H).

*Bzls-D-Arg(Pbf)-OH (**8**).* To a suspension of H-D-Arg(Pbf)-OH (400 mg, 0.94 mmol) in water (0.95 mL) was added dropwise 1 M solution of NaOH (0.95 mL) at 0 °C. Phenylmethanesulfonyl chloride (200 mg, 1.05 mmol) was added portionwise to the reaction mixture at 0 °C, followed by triethylamine (111 mg, 153 μL, 1.1 mmol) and acetone (2 mL). Stirring was continued at 0 °C for 1 h then at rt overnight. The solution was acidified with 10% $KHSO_4$ and extracted 5 times with ethyl acetate. The extracts were washed with brine and concentrated under reduced pressure. The residue was purified by column chromatography using 7.5% methanol in DCM with 1% of acetic acid, yielding 150 mg (27%) of **8** as white powder. ^{1}H NMR (600 MHz, chloroform-d) δ 7.35−7.26 (m, 5H), 4.20 (q, J = 13.8 Hz, 2H), 3.30−3.27 (m, 1H), 3.12−3.01 (m, 2H), 2.88 (s, 2H), 2.48 (s, 3H), 2.41 (s, 3H), 2.01 (s, 3H), 1.74−1.63 (m, 1H), 1.60−1.43 (m, 3H), 1.38 (s, 6H). ESI-HRMS (M^-) calcd for $C_{26}H_{35}N_4O_7S_2^-$ 579.1953, found 579.1940. According to ^{1}H NMR spectra, 95% pure.

*Bzls-D-Arg(Pbf)-Gly-OH (**9**).* Carboxylic acid **8** (150 mg, 0.26 mmol) and NHS (38.6 mg, 0.33 mmol) were dissolved in 1,2-dimethoxyethane (3 mL), and DCC (68 mg, 0.33 mmol) was added as solid at 0 °C. The resulting solution was stirred for 3 h at 0 °C, followed by 1 h at room temperature. The urea was filtered off and solvent was evaporated under vacuum, obtaining the succinimide Bzls-Arg(Pbf)-OSuc as a foam which was used in the next step without purification. The solution of succinimide in dioxane (10 mL) was added to a solution of glycine (60 mg, 0.8 mmol) and $NaHCO_3$ (67.2 mg, 0.8 mmol) in water (6 mL) at 10 °C. The suspension was stirred vigorously for 1 h at 5−10 °C and then left overnight in the fridge. Most of the dioxane was evaporated under vacuum, and the resulting water solution was filtered, the filtrate was washed with ethyl acetate, and the water phase was collected and then acidified with acetic acid to pH 4. The water phase was again extracted with ethyl acetate, and the extracts were washed with brine then dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by SiO_2 column chromatography using 8% methanol in CH_2Cl_2 with 1% acetic acid, yielding the dipeptide **9** (31 mg, 19%) as a white foam. ^{1}H NMR (600 MHz, methanol-d_4) δ 7.44−7.37 (m, 2H), 7.37−7.27 (m, 3H), 4.40−4.24 (m, 2H), 3.99−3.82 (m, 3H), 3.28−3.07 (m, 2H), 2.99 (s, 2H), 2.58 (s, 3H), 2.52 (s, 3H), 2.08 (s, 3H), 1.77−1.67 (m, 1H), 1.66−1.52 (m, 3H), 1.44 (s, 6H). ^{13}C NMR (151 MHz, methanol-d_4) δ 173.13, 171.53, 158.50, 156.79, 138.05, 132.89, 132.16, 130.74, 129.63, 128.11, 128.01, 124.68, 117.07, 86.29, 58.84, 56.37, 53.43, 42.54, 40.55, 30.19, 27.31, 18.22, 17.04, 11.11. ESI-HRMS (M^-) calcd for $C_{28}H_{38}N_5O_8S_2^-$ 636.2167, found 636.2186. According to ^{1}H NMR spectra, 99% pure.

Enzyme Kinetic Studies. Michaelis−Menten kinetics were measured for the substrate **1a** using UV−vis and for the substrate **1** using both fluorescence and UV−vis. Thrombin solution (200 μL, 1 nM) which also contained Tris buffer (50 mM, pH 8.3) and NaCl (130 mM) was placed in a cuvette. A 20× solution of substrate (10 μL) in water was added to the enzyme (substrate final concentration 1−20 μM), and the enzymatic reaction was monitored over time. For substrate **1a** the absorption increase at 405 nm (ε = 9600 M^{-1} cm^{-1}) was measured and for **1**, the absorption increase at 570 nm (ε = 53000 M^{-1} cm^{-1}) or emission increase at 583 nm. The reaction rate ν was calculated for each substrate concentration [s]. The data were fit to the following equation: $\nu = \nu_{max} \times [s]/(K_M + [s])$.

Specificity of Substrate 1 for Thrombin. The activity of a series of enzymes was assayed in the presence of substrate **1**. The following enzymes were used: myoglobin horse heart (17 kDa), cytochrome C horse heart (12.4 kDa), trypsin (23.3 kDa), BSA (66.5 kDa), factor Xa (43 kDa), and human thrombin (36 kDa). A 96-well microtiter plate was charged with the enzymes solutions (350 μL, 100 pM), also containing Tris buffer (50 mM, pH 8.3) and NaCl (130 mM) and the emission increase at 583 nm started to be measured immediately after substrate **1** addition (5 μM). The initial reaction rates were determined.

Thrombin Detection Assay. A series of thrombin solutions with concentrations in low range (0.5−10 pM) and high range (5−100 pM) were prepared and loaded onto a 96-well microtiter plate. The thrombin solution contained also Tris buffer (50 mM, pH 8.3), NaCl (130 mM), and BSA (0.1 mg/mL). The emission increase measurement started immediately after substrate (5 μM) addition. For the calibration curve, the initial reaction rates were plotted versus thrombin concentration. Each measurement was carried out in triplicate.

Dabigatran Quantification in Human Plasma. Human plasma was spiked with dabigatran at different concentrations (30−300 ng/mL). The thrombin solution (100 pM) with Tris buffer (50 mM, pH 8.3), NaCl (130 mM), urea (500 mM), aprotinin (150 mU/mL), polybrene (100 ng/mL), and BSA (0.1 mg/mL) was loaded onto a 96-well microtiter plate (350 μL per well). Plasma spiked with dabigatran (14 μL) was added to thrombin solution and incubated for 10 min. The substrate **1** (5 μM) was added, and the emission increase at 583

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nm was monitored. The reciprocal of the initial reaction rate ν was calculated and plotted against dabigatran concentration [I] to obtain the calibration curve. All measurements were carried out in triplicate.

Dabigatran Quantification in Whole Blood. Fresh blood was collected from donor finger (DA) using standard sterile lancets and capillaries coated with EDTA. Each sample (20 μL) was spiked with dabigatran (2 μL) to obtain the final inhibitor concentration ranging from 100 to 400 ng/mL. The blood with a known dabigatran concentration (20 μL) was added to the thrombin solution (250 pM, 2 mL) in a fluorescence plastic cuvette, agitated, and incubated for 5 min at rt. The thrombin solution also contained Tris buffer (50 mM, pH 8.3), NaCl (130 mM), urea (500 mM), aprotinin (40 mU/mL), polybrene (100 ng/mL), and BSA (0.1 mg/mL). The substrate **1** (10 μM) was added at the end, and the resorufin emission was measured using the portable fluorescence device Aquafluor from Turner Designs (filter: excitation 530–550 nm; emission ≥570 nm). The initial rates were determined and plotted against the dabigatran concentration to obtain the calibration curve.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00652.

Proton (^{1}H) and carbon (^{13}C) NMR spectra of key compounds **5**, **9**, **1**, and **2** (PDF)

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49 6221 548438. Fax: +49 6221 54 8599. E-mail: kraemer@aci.uni-heidelberg.de.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

PABA, *p*-aminobenzyl alcohol; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TLC, thin layer chromatography; DMF, *N*,*N*-dimethylformamide; DIEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; DCM, dichloromethane; Bzls, benzylsulfonyl; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; DME, 1,2-dimethoxyethane; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; BSA, bovine serum albumin; AMC, 7-amino-4-methylcoumarine

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