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Fluorescently Labeled Amino Acids as Building Blocks for Bioactive Molecules

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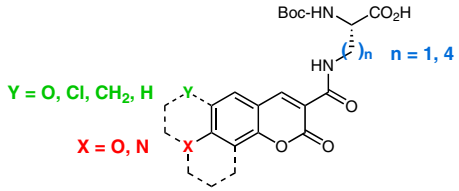

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Abstract A series of twelve fluorescently labeled amino acids were designed by assembling different coumarin, fluorescein, or nitrobenzofurazan fluorophores with N-protected lysine or 2-aminopropionic acid. The synthesized amino acids were evaluated with regard to their spectroscopic properties. The easy introduction of the amino acids into peptides and peptidomimetics was exemplarily shown for one coumarin-labeled amino acid.

Key words amino acids, coumarins, fluorescence, guanidines, peptidomimetics

Fluorescence labeling of biologically active peptides is of vital importance in clinical and biomedical research. There is a broad field of application of such compounds, for example, as peptidic enzyme substrates, environmental indicators, and chemical probes to study biological structures and functions. Fluorescently labeled peptides are useful for visualizing intracellular processes, or investigating protein folding, localization of proteins, and interactions between peptides and biological membranes.[1–5] Imaging by fluorescence provides many advantages, such as high sensitivity and selectivity, as well as a safe and ready detection. The probes and fluorescent substrates can be provided by facile syntheses with opportunities for manifold structural variations. Their photophysical properties can be adjusted by the selection of an appropriate fluorophore. Several fluorescent labels are valued for their high chemical and biological stability.[1] To achieve adequate detection of the peptide fluorescence and to maintain its native structure, the peptide should contain either natural fluorescent amino acids, namely tryptophan or tyrosine, or non-natural fluorescent analogues. Various types of fluorophores have been employed. Besides fluorescein and nitrobenzofurazan derivatives, coumarins represent a widely used class of fluorescent dyes and are distinguished by their small molecular size, low bleaching, and large Stokes shifts.[3–7] Whereas the unsubstituted coumarin does not possess fluorescent properties, introduction of an electron-donating group at the 7-position provokes fluorescence caused by an intramolecular charge transfer, arising from an electron transfer from the donor in the 7-position to the α,β-unsaturated lactone system. A carbonyl group at the 3-position enhances the fluorescence outcome.

The introduction of an additional electron-donating substituent at the 6-position further contributes to the charge transfer.[8] Methoxy groups have been commonly applied as electron-donating groups at the 7- and 6-positions,[8,9] and 7-amino substituents produce a typical red shift to longer wavelengths of absorption and emission, a favorable feature with regard to background fluorescence.[4,5,7,10,11]

In this study, we synthesized a series of fluorescently labeled amino acids and evaluated their potential as fluorescent building blocks by examining their spectroscopic properties. In addition to a fluorescein and a nitrobenzofurazan-labeled amino acid, ten coumarin-labeled derivatives were prepared covering the blue to green region of the visible light spectrum. Exemplarily, the applicability of one labeled amino acid was shown by incorporation into a peptidomimetic bis-benzguanidine and demonstration of the biological activity.

To prepare coumarin building blocks, we followed a literature procedure,[9] involving a Knoevenagel reaction of *ortho*-hydroxy aldehydes **1**–**5** (Figure 1) with Meldrum's acid and catalytic amounts of piperidinium acetate to form the coumarin-3-carboxylic acids **8**–**12** (Scheme 1). Whereas aldehydes **1**, **2**, **3**, and **4** were commercially available, **5** and **6** were obtained by multi-step syntheses involving a Vilsmeier formylation as depicted in the Supporting Information (Schemes S1 and S2). The coumarin acids **8**–**12**

were coupled either to the N-protected lysine **15** or to 3-amino-2-(*tert*-butoxycarbonylamino)propanoic acid (**14**) using NHS and EDC.

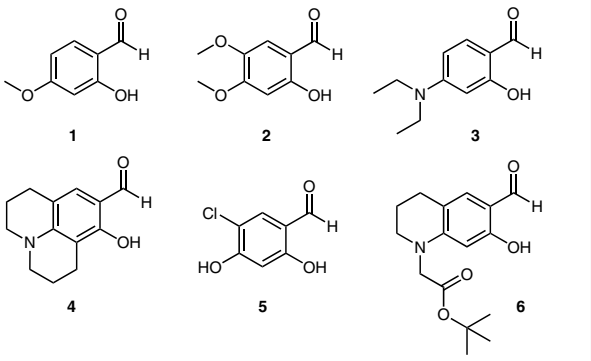
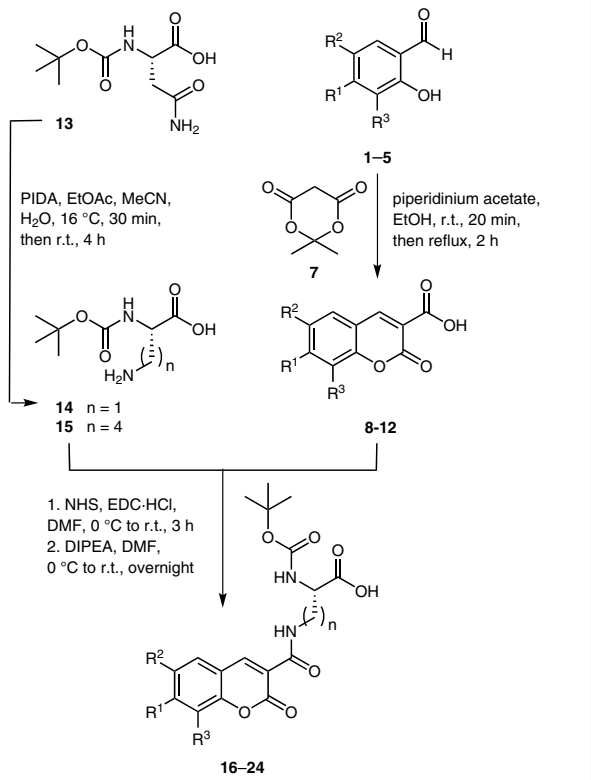


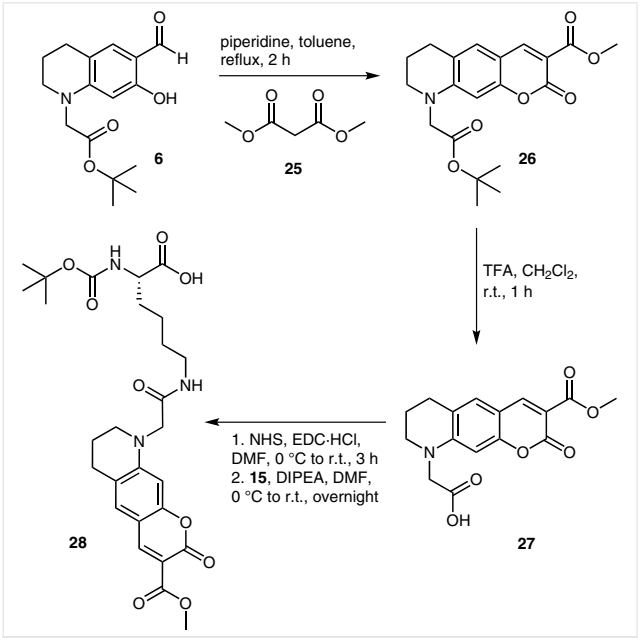
Figure 1 Salicylaldehydes for coumarin syntheses

To access the required compound **14**, Boc-asparagine (**13**) was subjected to a Hofmann rearrangement of the terminal carboxamide moiety utilizing PIDA as the oxidant.[12] This route provided the nine coumarin-labeled amino acids **16**–**24** as final compounds (Scheme 1 and Figure 2). The



Scheme 1 Synthesis of the fluorescently labeled amino acids **16**–**24**. PIDA: Phenyliodine(III) diacetate; NHS: *N*-hydroxysuccinimide; EDC: *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; DIPEA: diisopropylethylamine.

synthesis of the labeled amino acid **22** using NHS and *N*,*N*′-dicyclohexylcarbodiimide (DCC) as coupling reagents has already been described.[13] However, the authors used racemic lysine, whereas our compounds exhibit the natural L-configuration. The ethyl esters of **21** and **23** and their spectroscopic properties have been reported by Murase et al.[4] Compound **28** (Scheme 2) was prepared in a different manner by performing the Knoevenagel condensation of the salicylaldehyde **6** with dimethyl malonate (**25**),[7] yielding coumarin **26** with two orthogonally protected carboxyl groups. The *tert*-butyl ester was cleaved under acidic conditions, followed by the NHS and EDC-mediated amide coupling with N^{α}-Boc-L-lysine (**15**) to synthesize the labeled amino acid **28**. Furthermore, two new fluorescent amino acids, **29** and **30** (Figure 2), were assembled from N^{α}-Boc-L-lysine (**15**) and the established fluorophores fluorescein and nitrobenzofurazan, respectively; similar labeled amino acids have been described.[14] Synthetic details for **29** and **30** are given in the Supporting Information (Schemes S3 and S4). The final fluorescently labeled amino acids **16**–**24** and **28**–**30** can easily be introduced into bioactive molecules, as they exhibit the free carboxyl group. In a subsequent step, the amino functionality can be readily deprotected to allow for a further extension of the structure.



Scheme 2 Synthesis of the fluorescently labeled amino acid **28**

The fluorescently labeled amino acids **16**–**24** and **28**–**30** were analyzed with regard to their spectroscopic properties. Absorption and emission spectra were recorded in buffer pH 8; maxima are outlined in Table 1, and representative spectra covering all different fluorophores incorporated are depicted in Figure 3. A comparison of compounds **16**/**17** with **18**/**19** revealed that the introduction of the

second methoxy group in 6-position led to a bathochromic shift, caused by an enhanced push-pull system that contributes to the charge transfer.[8] Furthermore, **18** and **19** exhibited the largest Stokes shift (79 nm) of all final compounds. The amino acids bearing 7-aminocoumarins **20**–**23**, **28** possessed a typical red shift to longer wavelengths of absorption and emission, an advantageous attribute to facilitate fluorescence measurements and to overcome background fluorescence. A further well established fluorophore, 7-diethylaminocoumarin-3-carboxylic acid,[4,10,15] was used for the preparation of **20** and **21**. However, its fluorescence is clearly quenched in an aqueous environment.[4,10] By rigidification of the amino group into a ring structure as in **22** and **23** containing coumarin 343, as well as in **28** with a tricyclic coumarin moiety, fluorescence returned in the aqueous medium and an additional bathochromic shift occurred. This expected effect of rigidified 7-aminocoumarins relies on a maximal interaction of the nitrogen lone pair with the aromatic system.[4,7,16] These amino acids possessed the highest fluorescence maxima of the coumarin-labeled derivatives of this series.

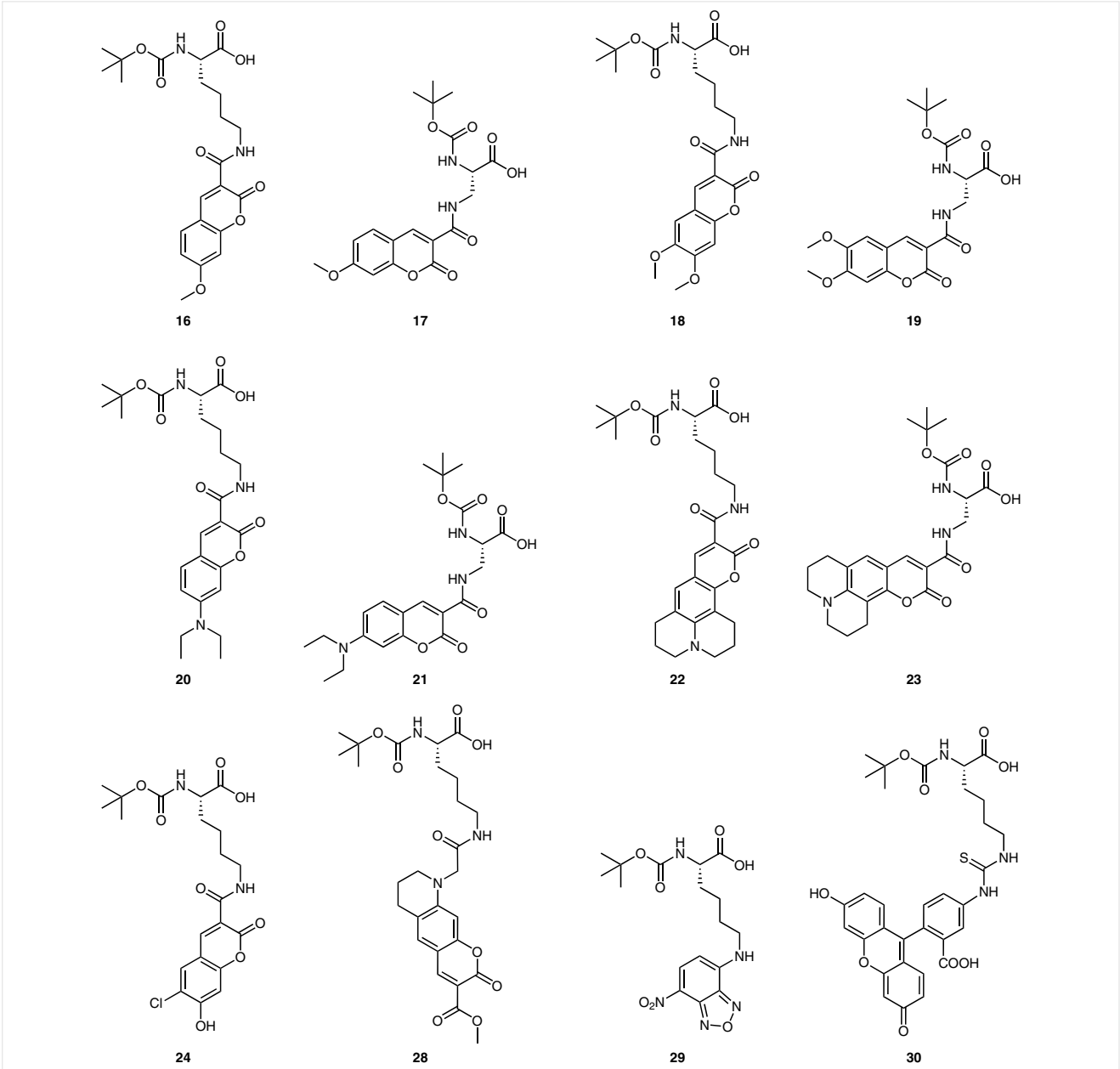


Figure 2 Fluorescently labeled amino acids **16**–**24**, **28**–**30**

Table 1 Absorption (UV λ_{max}) and Fluorescence Emission (F λ_{max}) Maxima of Compounds **16**–**24**, **28**–**30**[a]

Compound	UV λ_{max} (nm)	F λ_{max} (nm)	Stokes shift (nm)
16	351	404	53
17	350	406	56
18	369	448	79
19	371	450	79
20	428	472	44
21	429	478	49
22	444	492	48
23	447	492	45
24	404	448	44
28	423	490	67
29	481	552	71
30	493	516	23

[a] Recorded in buffer, pH 8, at concentrations of 10 µM for the absorption spectra and 1 µM for the emission spectra.

The fluorescein derivative **30** showed a maximum of 516 nm, 24 nm more shifted to longer wavelengths than that of the coumarin-labeled compounds **22**/**23**, but it also possessed the smallest Stokes shift. The 7-nitrobenzofurazan fluorophore present in compound **29** provided an emission maximum of 552 nm, which was the most red-shifted within this series. However, this fluorophore has the drawback that its fluorescence is quenched in aqueous media; for comparison, see the non-normalized spectra in the Supporting Information (Figure S1).

To demonstrate the applicability of such coumarin-labeled amino acids, compound **19** was chosen for insertion into a dibasic molecule of a chemotype known to exhibit inhibitory properties against several serine proteases of the trypsin family. Benzamidine and benzguanidine moieties represent prominent arginine mimetics. They have frequently been used to address the S1 and S3/S4 binding site of trypsin-like serine proteases, such as matriptase, matriptase-2, thrombin, factor Xa, and trypsin.[17–19] Moreover, such compounds possess antiprotozoal and antifungal activities. Their mode of action has been attributed to the insertion into the minor groove of adenine/thymine-rich sites of double-strand DNA.[20] In addition, it has been shown that certain bis-benzamidines exhibit DNA-dependent fluorescent properties.[21]

The synthesis of such a compound comprising two benzguanidine substructures and a central coumarin-labeled amino acid is shown in Scheme 3. Using HATU as coupling reagent and DIPEA as base, the amino acid **19** and 4-nitrobenzylamine (**31**), the first amide bond was formed. After trifluoroacetic acid-promoted removal of the Boc-protecting group of **32**, the resulting free amine was reacted with 4-nitrobenzoic acid (**33**) reapplying the same coupling protocol. The following simultaneous conversion of both nitro groups into primary amine moieties occurred after treatment with $SnCl_2$ acting as the reductive agent. The bis-aniline **35** was converted in a $HgCl_2$-catalyzed reaction with *N*,*N*′-di-Boc-*S*-methylisothiourea (**36**)[22] and triethylamine to produce an intermediate with two protected guanidine groups. After the subsequent removal of the Boc-protecting groups with trifluoroacetic acid, the desired bis-benzguanidine **37** was obtained and purified by means of preparative HPLC. The hydrochloride salt was obtained by adding few drops of HCl to a solution of the guanidinium trifluoroacetate in water, followed by lyophilization.

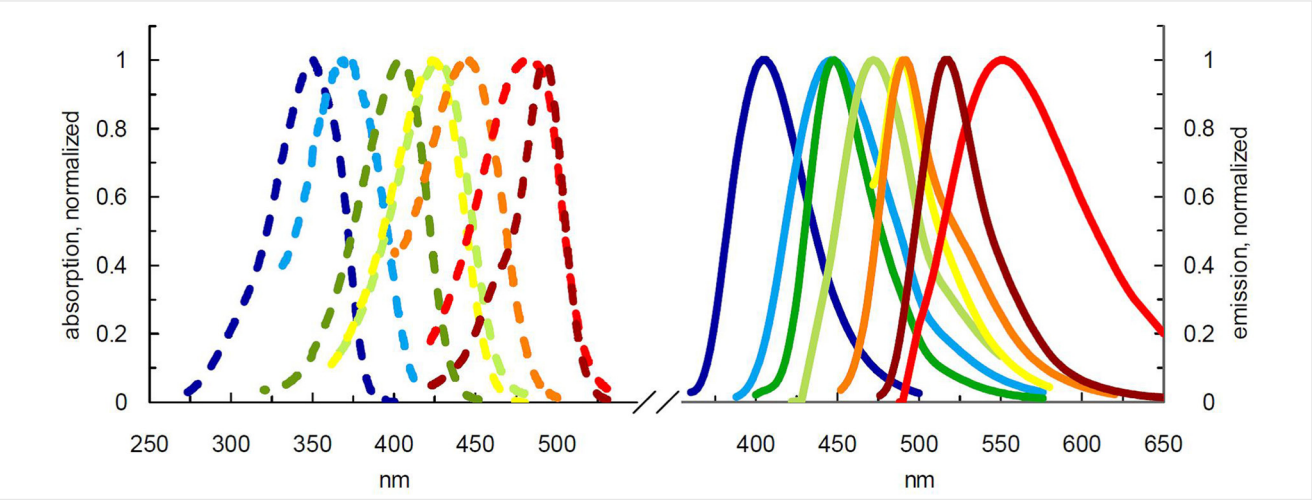
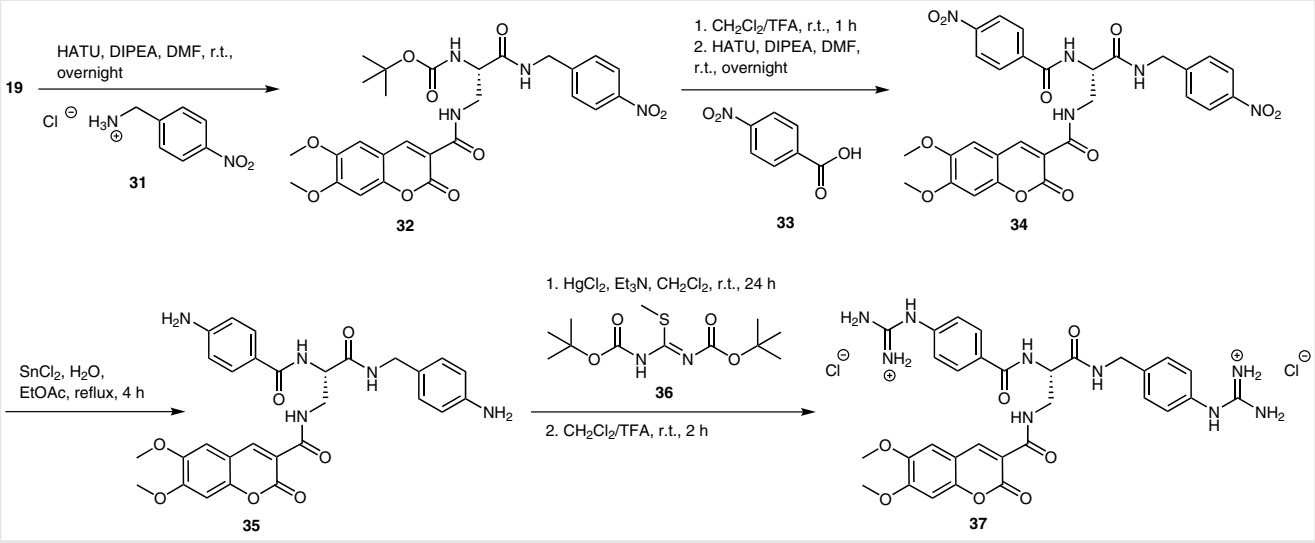


Figure 3 Normalized absorption (10 µM, 1% DMSO, dashed lines) and emission spectra (1 µM, 1% DMSO, continuous lines) in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl). Dark blue: **16**; light blue: **18**; dark green: **24**; yellow: **28**; light green: **20**; orange: **22**; red: **29**; brown: **30**.



Scheme 3 Synthesis of the bis-benzguanidine **37**. HATU: *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; TFA: trifluoroacetic acid.

Compound **37** was evaluated as an inhibitor of the serine proteases matriptase-2, thrombin, factor Xa, and trypsin and with respect to its binding to DNA. The results of duplicate measurements are depicted in Table 2. The enzymes were assayed as described,[18,19] using fluorogenic peptide substrates. The progress curves were analyzed and IC_{50} values were obtained from plots of the rates versus inhibitor concentrations. K_i values were calculated with the Cheng–Prusoff equation. Whereas **37** did not inhibit factor Xa, it exhibited a moderate inhibition of thrombin, and K_i values of less than 10 µM were obtained for matriptase-2 and trypsin. DNA binding of **37** was determined by anisotropy titration, using short hairpin oligonucleotides, as described.[21] Compound **37** did not bind to a guanine/cytosine-rich oligonucleotide, but showed a concentration dependent, albeit weak affinity (K_D > 20 µM) to a short hairpin oligonucleotide featuring the adenine/thymine-rich binding site AAATTT.

Table 2 Kinetic Parameters of Inhibition (K_i) of Compound **37** towards Four Serine Proteases and Dissociation Constant (K_D) for the DNA Binding of **37**

Assay	K_i or K_D value
human matriptase-2	K_i = 6.3 ± 0.8 µM
human thrombin	K_i = 28 ± 2 µM
bovine factor Xa	K_i > 40 µM
bovine trypsin	K_i = 8.4 ± 0.4 µM
DNA binding	K_D > 20 µM

In conclusion, a small library of fluorescently labeled amino acids were synthesized. The labels comprise different coumarin moieties, as well as fluorescein and nitrobenzofurazan, connected to either a C-1 or C-4 linker via an amide bond. In aqueous medium, the products show fluorescence in the blue to green region of the visible light spectrum. A 6,7-dimethoxycoumarin-containing amino acid was exemplarily introduced into a peptidomimetic compound, whose bioactivity was evaluated. Accordingly, the labeled amino acids reported herein are expected to be useful building blocks for the assembly of various biologically active, fluorescent compounds.

Solvents and reagents were obtained from Acros (Geel, Belgium), Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), Bachem (Bubendorf, Switzerland), Carbolution (Saarbrücken, Germany), and Fluorochem (Hadfield, UK). TLC was carried out on aluminum sheets, coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany). Compounds were visualized under UV light (254 nm). Preparative column chromatography was performed using Merck silica gel 60, 0.060–0.200 mm. Preparative HPLC was performed on a Eurospher 100 column with reversed phase silica gel C-18, and a spectrophotometer Wellchrome K-2600 (Knauer, Berlin, Germany) was applied.

Mass spectra were recorded on an API 2000 mass spectrometer (electron spray ion source, Applied Biosystems, Darmstadt, Germany) coupled with an Agilent 1100 HPLC system using a Phenomenex Luna HPLC C18 column (50 × 2.00 mm, particle size 3 µm). The purity of the tested compounds was determined by HPLC-UV obtained on an LC-MS instrument (Applied Biosystems API 2000 LC-MS/MS, HPLC Agilent 1100). HRMS was recorded on a microTOF-Q mass spectrometer (Bruker, Köln, Germany) with ESI-source coupled with a HPLC Dionex Ultimate 3000 (Thermo Scientific, Braunschweig, Germany) using a EC50/2 Nucleodur C18 Gracity 3 µm column (Macherey-Nagel, Düren, Germany). Elemental analyses were performed with a Vario

EL apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer. DMSO-d_6 was used as the solvent as indicated below. NMR spectra were recorded at 30 °C. Chemical shifts are given in parts per million (ppm) referring to the signal center using the solvent peaks for reference: DMSO-d_6 2.49/39.7 ppm. Melting points were determined on a Büchi 510 oil bath apparatus and are uncorrected.

Coumarin Acids of 8–12; General Prodecure

The respective aldehyde **1**–**5** (1.0 equiv) was dissolved in absolute EtOH (7–60 mL). Isopropylidene malonate (1.0 equiv) and piperidinium acetate (0.02 equiv) were added to the solution. The mixture was stirred for 20 min at r.t. and refluxed for 2 h. The mixture was allowed to cool down to r.t. and kept in an ice bath for 30 min. The product was collected by filtration and washed with EtOH (50 mL).

7-Methoxy-2-oxo-2*H*-chromene-3-carboxylic Acid (8)

From 2-hydroxy-4-methoxybenzaldehyde (**1**; 2.28 g, 15 mmol), 2,2-dimethyl-1,3-dioxane-4,6-dione (**7**; 2.16 g, 15 mmol), and piperidinium acetate (0.044 g, 0.3 mmol) in anhydrous EtOH (60 mL); yield: 2.38 g (72%, 10.81 mmol); colorless solid; mp 196–198 °C (Lit.[9] mp 192–194 °C).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 3.88 (s, 3 H, OCH_3), 6.99 (dd, ^4J = 2.6 Hz, ^3J = 8.6 Hz, 1 H, 6-H), 7.02 (d, ^4J = 2.6 Hz, 1 H, 8-H), 7.81 (d, ^3J = 8.6 Hz, 1 H, 5-H), 8.70 (s, 1 H, 4-H), 12.93 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 56.4, 100.4, 111.8, 113.4, 114.0, 131.7, 149.1, 157.0, 157.4, 164.2, 164.8.

LC-MS (ESI): 100% purity; m/z = 238.23 ($[M + NH_4]^+$), 219.03 ($[M – H]^-$).

Anal. Calcd for $C_{11}H_8O_5$: C, 60.00; H, 3.66; N, 0.00. Found: C, 59.80; H, 3.62; N, 0.25.

7,6-Dimethoxy-2-oxo-2*H*-chromene-3-carboxylic Acid (9)

From 2-hydroxy-4,5-dimethoxybenzaldehyde (**2**; 2.73 g, 15 mmol), **7** (2.16 g, 15 mmol), and piperidinium acetate (0.044 g, 0.3 mmol) in anhydrous EtOH (60 mL); yield: 2.63 g (70%, 10.51 mmol); light yellow solid; mp 265–269 °C (Lit.[23] mp 266 °C).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 3.80 (s, 3 H, OCH_3), 3.89 (s, 3 H, OCH_3), 7.10 (s, 1 H, 8-H), 7.43 (s, 1 H, 5-H), 8.67 (s, 1 H, 4-H), 12.87 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 56.1, 56.6, 99.8, 110.2, 110.7, 113.8, 146.4, 149.2, 151.6, 155.2, 157.9, 164.3.

LC-MS (ESI): 100% purity; m/z = 249.11 ($[M – H]^-$).

Anal. Calcd for $C_{12}H_{10}O_6$: C, 57.60; H, 4.03; N, 0.00. Found: C, 57.36; H, 4.08; N, 0.00.

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic Acid (10)

From 4-(diethylamino)-2-hydroxybenzaldehyde (**3**; 1.93 g, 10 mmol), **7** (1.44 g, 10 mmol), and piperidinium acetate (0.029 g, 0.2 mmol) in anhydrous EtOH (40 mL); yield: 1.65 g (63%, 6.32 mmol); yellow solid; mp 220–223 °C (Lit.[10] mp 222 °C).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.13 (t, ^3J = 7.1 Hz, 6 H, CH_2CH_3), 3.47 (q, ^3J = 7.1 Hz, 4 H, CH_2CH_3), 6.55 (t, ^4J = 2.3 Hz, 1 H, 8-H), 6.78 (dd, ^4J = 2.3 Hz, ^3J = 8.9 Hz, 1 H, 6-H), 7.62 (d, ^3J = 8.9 Hz, 1 H, 5-H), 8.57 (s, 1 H, 4-H), 12.47 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 12.5, 44.5, 96.1, 107.3, 107.5, 110.2, 132.0, 149.6, 153.1, 158.0, 159.7, 164.6.

LC-MS (ESI): 98% purity; m/z = 262.1 ($[M + H]^+$).

Anal. Calcd for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.03; H, 5.75; N, 5.72.

2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano-[6,7,8-*ij*]quinolizine-10-carboxylic Acid (11)

From 8-hydroxyjulolidine-9-carboxaldehyde (**4**; 2.17 g, 10 mmol), **7** (1.44 g, 10 mmol), and piperidinium acetate (0.029 g, 0.2 mmol) in anhydrous EtOH (10 mL); yield: 0.69 g (24%, 2.42 mmol); orange solid; mp 248–251 °C (Lit.[24] mp 253 °C).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.84–1.90 [m, 4 H, $N(CH_2CH_2)_2$], 2.69–2.73 {m, 4 H, $N[(CH_2)_2CH_2]_2$}, 3.33–3.36 [m, 4 H, $N(CH_2)_2$], 7.23 (s, 1 H, 5-H), 8.44 (s, 1 H, 4-H), 12.38 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 19.7, 19.7, 20.6, 26.9, 49.3, 49.8, 104.9, 105.2, 107.4, 119.7, 127.6, 148.9, 149.3, 152.8, 160.7, 164.8.

LC-MS (ESI): 99% purity; m/z = 286.0 ($[M + H]^+$).

Anal. Calcd for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91. Found: C, 67.69; H, 5.74; N, 5.11.

6-Chloro-7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic Acid (12)

From 5-chloro-2,4-dihydroxybenzaldehyde (**5**; 0.30 g, 1.73 mmol), **7** (0.25 g, 1.73 mmol), and piperidinium acetate (0.005 g, 0.035 mmol) in anhydrous EtOH (7 mL), yield: 0.12 g (29%, 0.50 mmol); yellow solid; mp >250 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 6.88 (s, 1 H, 8-H), 7.97 (s, 1 H, 5-H), 8.63 (s, 1 H, 4-H), 11.99 (br s, 1 H, OH), 12.81 (br s, 1 H, OH).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 103.0, 111.4, 114.2, 117.8, 130.9, 148.4, 155.3, 157.0, 158.9, 164.1.

LC-MS (ESI): 97% purity; m/z = 241.1 ($[M + H]^+$).

Anal. Calcd for $C_{10}H_5ClO_5$: C, 49.92; H, 2.09; N, 0.00. Found: C, 49.83; H, 2.14; N, 0.30.

(*S*)-3-Amino-2-(*tert*-butoxycarbonylamino)propanoic Acid (14)

N-Boc-L-asparagine (**13**; 3.48 g, 15 mmol) and PIDA (5.81 g, 18 mmol) were dissolved in a mixture of EtOAc, MeCN, and H_2O (2:2:1, 62.5 mL) and stirred at 16 °C for 30 min. Afterwards, the reaction mixture was allowed to warm up to r.t. and stirred for 4 h whereupon a precipitate was formed, which was collected by filtration and dried; yield: 2.02 g (66%, 9.89 mmol); colorless solid; mp 211–213 °C (Lit.[25] mp 216 °C).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.40 [s, 9 H, $C(CH_3)_3$], 2.76 (dd, ^3J = 9.2 Hz, ^2J = 11.8 Hz, 1 H, CH_2), 3.06 (dd, ^3J = 5.1 Hz, ^2J = 11.8 Hz, 1 H, CH_2), 3.65–3.69 (m, 1 H, COCH), 6.08 (s, 1 H, OCONH). Protons of CO_2H and the NH_2 group were not detected.

^{13}C NMR (125 MHz, DMSO-d_6): δ = 28.3, 40.8, 51.1, 78.2, 155.2, 171.2.

LC-MS (ESI): 100% purity; m/z = 205.19 ($[M + H]^+$), 203.09 ($[M – H]^-$).

HRMS (ESI$^+$): m/z calcd for $C_8H_{16}N_2O_4$ ($[M + H]^+$): 205.1183; found: 205.1177.

Fluorescently Labeled Amino Acids 16–24; General Procedure

The appropriate coumarin-3-carboxylic acid **8**–**12** (1.0 equiv) and *N*-hydroxysuccinimide (1.5 equiv) were dissolved in DMF (15–60 mL) and the solution was cooled to 0 °C. EDC·HCl (1.5 equiv) was added and the mixture was stirred for 3 h. During this time, r.t. was reached and the mixture was diluted with EtOAc (50–300 mL) and washed with aq 10% citric acid (50–150 mL), aq sat. $NaHCO_3$ (50–150 mL), and brine (50–150 mL). The organic phase was dried (Na_2SO_4), filtered, and evaporated. The NHS-ester was dissolved in DMF (25–50 mL) and cooled to 0 °C. DIPEA (2.4 equiv) and N^{α}-Boc-L-Lys-OH (**15**) or com-

pound **14** (1.2 equiv) were added and the solution was stirred at r.t. overnight. The mixture was diluted with EtOAc (50–300 mL) and washed with aq 10% citric acid (3 × 50–150 mL) and brine (50–150 mL), dried (Na_2SO_4), filtered, and evaporated.

2-(*tert*-Butoxycarbonylamino)-6-(7-methoxy-2-oxo-2*H*-chromene-3-carboxamido)hexanoic Acid (16)

From **8** (1.10 g, 5 mmol), activated with *N*-hydroxysuccinimide (0.86 g, 7.5 mmol) and EDC·HCl (1.44 g, 7.5 mmol) in DMF (15 mL); and subsequent coupling with **15** (1.48 g, 6 mmol) in the presence of DIPEA (1.55 g, 2.04 mL, 12 mmol) in DMF (25 mL). The product was crystallized from EtOAc; yield: 0.89 g (40%, 1.98 mmol); colorless solid; mp 104–105 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.35 [s, 11 H, $C(CH_3)_3$, $CH_2CH_2CH_2CH_2NH$], 1.45–1.54 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.55–1.62 (m, 1 H, $CHCH_2CH_2CH_2NH$), 1.63–1.71 (m, 1 H, $CHCH_2CH_2CH_2NH$), 3.29–3.31 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.89 (s, 3 H, OCH_3), 4.02 (q, ^3J = 7.3 Hz, 1 H, COCH), 6.99 (d, ^3J = 7.3 Hz, 1 H, OCONH), 7.03 (dd, ^4J = 2.4 Hz, ^3J = 8.7 Hz, 1 H, 6-H), 7.09 (d, ^4J = 2.4 Hz, 1 H, 8-H), 7.88 (d, ^3J = 8.7 Hz, 1 H, 5-H), 8.61 (t, ^3J = 5.8 Hz, 1 H, CONH), 8.79 (s, 1 H, 4-H), 12.36 (s, 1 H, CO_2H).

^{13}C NMR (500 MHz, DMSO-d_6): δ = 20.9, 23.2, 28.3, 30.6, 39.0, 53.5, 56.4, 78.1, 100.4, 112.3, 113.7, 115.1, 131.6, 147.8, 155.7, 156.3, 161.0, 161.5, 164.5, 174.3.

LC-MS (ESI): 99% purity; *m*/*z* = 449.28 ([M + H]$^+$), 447.15 [M – H]$^-$.

Anal. Calcd for $C_{22}H_{28}N_2O_8$: C, 58.92; H, 6.29; N, 6.25. Found: C, 58.46; H, 6.45; N, 5.72.

2-(*tert*-Butoxycarbonylamino)-3-(7-methoxy-2-oxo-2*H*-chromene-3-carboxamido)propanoic Acid (17)

From **8** (0.88 g, 4 mmol), activated using *N*-hydroxysuccinimide (0.86 g, 7.5 mmol) and EDC·HCl (1.14 g, 6 mmol) in DMF (15 mL); and subsequent coupling with **14** (0.98 g, 4.8 mmol) in the presence of DIPEA (1.24 g, 1.63 mL, 9.6 mmol) in DMF (30 mL). The product was crystallized from EtOAc; yield: 0.58 g (36%, 1.43 mmol); colorless solid; mp 190–191 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.36 [s, 9 H, $C(CH_3)_3$], 3.35–3.40 (m, 1 H, CH_2), 3.80–3.85 (m, 1 H, CH_2), 3.89 (s, 3 H, OCH_3), 4.10–4.14 (m, 1 H, COCH), 7.37 (dd, ^4J = 2.5 Hz, ^3J = 8.8 Hz, 1 H, 6-H), 7.10 (d, ^4J = 2.5 Hz, 1 H, 8-H), 7.20 (d, ^3J = 7.9 Hz, 1 H, OCONH), 7.90 (d, ^3J = 8.8 Hz, 1 H, 5-H), 8.82–8.84 (m, 2 H, CONH, 4-H), 12.72 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 28.3, 53.5, 56.4, 78.4, 100.4, 112.2, 113.8, 114.5, 131.8, 148.3, 155.6, 156.4, 160.9, 161.9, 164.7, 172.3. The CH_2 and DMSO signals overlapped.

LC-MS (ESI): 99% purity; *m*/*z* = 407.32 ([M + H]$^+$), 405.26 ([M – H]$^-$).

Anal. Calcd for $C_{19}H_{22}N_2O_8$: C, 56.15; H, 5.46; N, 6.89. Found: C, 56.15; H, 5.24; N, 6.82.

2-(*tert*-Butoxycarbonylamino)-6-(6,7-methoxy-2-oxo-2*H*-chromene-3-carboxamido)hexanoic Acid (18)

From **9** (1.25 g, 5 mmol), activated using *N*-hydroxysuccinimide (0.86 g, 7.5 mmol) and EDC·HCl (1.44 g, 7.5 mmol) in DMF (60 mL); and subsequent coupling with **15** (1.48 g, 6 mmol) in the presence of DIPEA (1.55 g, 2.04 mL, 12 mmol) in DMF (25 mL); yield: 0.52 g (22%, 1.09 mmol); yellow solid; mp 178–182 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.36 [s, 11 H, $C(CH_3)_3$, $CH_2CH_2CH_2CH_2NH$], 1.47–1.54 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.55–1.62 (m, 1 H, $CHCH_2CH_2CH_2NH$), 1.63–1.71 (m, 1 H, $CHCH_2CH_2CH_2NH$), 3.27–3.31 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.81 (s, 3 H, OCH_3), 3.82–3.87 (m, 1 H, COCH), 3.90 (s, 3 H, OCH_3), 6.99 (d, ^3J = 7.9 Hz, 1 H, OCONH), 7.17 (s, 1 H, 8-H), 7.50 (s, 1 H, 5-H), 8.67 (t, ^3J = 5.7 Hz, 1 H, CONH), 8.78 (s, 1 H, 4-H), 12.37 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 23.3, 28.3, 28.8, 30.6, 39.0, 53.5, 56.2, 56.6, 78.1, 99.1, 110.1, 111.3, 114.9, 146.7, 147.8, 150.8, 154.2, 155.7, 161.2, 161.5, 174.3.

LC-MS (ESI): 97% purity; *m*/*z* = 479.28 ([M + H]$^+$), 477.18 ([M – H]$^-$).

Anal. Calcd for $C_{23}H_{30}N_2O_9$: C, 57.73; H, 6.32; N, 5.85. Found: C, 56.67; H, 6.37; N, 6.01.

2-(*tert*-Butoxycarbonylamino)-3-(6,7-dimethoxy-2-oxo-2*H*-chromene-3-carboxamido)propanoic Acid (19)

From **9** (1.00 g, 4 mmol), activated using *N*-hydroxysuccinimide (0.69 g, 6 mmol) and EDC·HCl (1.14 g, 6 mmol) in DMF (15 mL); and subsequent coupling with **14** (0.98 g, 4.8 mmol) in the presence of DIPEA (1.24 g, 1.63 mL, 9.6 mmol) in DMF (30 mL). The product was crystallized from EtOAc; yield: 0.78 g (45%, 1.78 mmol); yellow solid; mp 199–200 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.36 [s, 9 H, $C(CH_3)_3$], 3.34–3.40 (m, 2 H, CH_2), 3.81 (s, 3 H, OCH_3), 3.90 (s, 3 H, OCH_3), 4.09–4.13 (m, 1 H, COCH), 7.17 (s, 1 H, OCONH), 7.18 (s, 1 H, 8-H), 7.53 (s, 1 H, 5-H), 8.82 (s, 1 H, 4-H), 8.89 (t, ^3J = 5.7 Hz, 1 H, CONH), 12.73 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 28.3, 53.5, 56.2, 56.6, 78.4, 99.8, 110.2, 111.2, 114.4, 146.7, 148.3, 150.9, 155.1, 155.6, 161.1, 161.9, 172.3. The CH_2 and DMSO signals were overlapped.

LC-MS (ESI): 99% purity; *m*/*z* = 437.37 ([M + H]$^+$), 435.38 [M – H]$^-$.

HRMS (ESI$^+$): *m*/*z* calcd for $C_{20}H_{24}N_2O_9$ ([M + H]$^+$): 437.1555; found: 437.1556.

(*S*)-2-(*tert*-Butoxycarbonylamino)-6-[7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamido]hexanoic Acid (20)

From **10** (0.131 g, 0.5 mmol), activated using *N*-hydroxysuccinimide (0.086 g, 0.75 mmol) and EDC·HCl (0.14 g, 0.75 mmol) in DMF (10 mL); and subsequent coupling with **15** (0.18 g, 0.6 mmol) in the presence of DIPEA (0.16 g, 0.20 mL, 1.2 mmol) in DMF (20 mL); yield: 0.20 g (82%, 0.41 mmol); yellow solid; mp 80–82 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.13 (t, ^3J = 7.0 Hz, 6 H, CH_2CH_3), 1.36 [s, 11 H, $C(CH_3)_3$, $CH_2CH_2CH_2CH_2NH$], 1.44–1.53 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.54–1.61 (m, 1 H, $CHCH_2CH_2CH_2NH$), 1.62–1.71 (m, 1 H, $CHCH_2CH_2CH_2NH$), 3.25–3.27 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.47 (q, ^3J = 7.0 Hz, 4 H, CH_2CH_3), 3.82–3.86 (m, 1H, COCH), 6.59 (d, ^4J = 2.3 Hz, 1 H, 8-H), 6.79 (dd, ^4J = 2.3 Hz, ^3J = 9.1 Hz, 1 H, 6-H), 6.99 (d, ^3J = 7.6 Hz, 1 H, OCONH), 7.66 (d, ^3J = 9.1 Hz, 1 H, 5-H), 8.61 (t, ^3J = 6.0 Hz, 1 H, CONH), 8.63 (s, 1 H, 4-H), 12.35 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 12.5, 23.3, 28.3, 28.3, 30.6, 38.8, 44.5, 53.5, 78.1, 96.0, 107.8, 109.7, 110.3, 131.7, 147.7, 152.5, 155.7, 157.3, 161.9, 162.2, 174.3.

LC-MS (ESI): 97% purity; *m*/*z* = 490.4 ([M + H]$^+$).

HRMS (ESI$^+$): *m*/*z* calcd for $C_{25}H_{35}N_3O_7$ ([M + H]$^+$): 490.2548; found: 490.2542.

(*S*)-2-(*tert*-Butoxycarbonylamino)-3-[7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamido]propanoic Acid (21)

From **10** (1.05 g, 4 mmol), activated using *N*-hydroxysuccinimide (0.69 g, 6 mmol) and EDC·HCl (1.14 g, 6 mmol) in DMF (20 mL); and subsequent coupling with **14** (0.98 g, 4.8 mmol) in the presence of DIPEA (1.24 g, 1.63 mL, 9.6 mmol) in DMF (30 mL); yield: 1.31 g (73%, 2.93 mmol); bright yellow solid; mp 207–210 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.13 (t, ^3J = 7.0 Hz, 6 H, CH_2CH_3), 1.35 [s, 9 H, $C(CH_3)_3$], 3.26–3.37 (m, 1 H, CH_2), 3.47 (q, ^3J = 7.0 Hz, 4 H, CH_2CH_3), 3.78–3.85 (m, 1 H, CH_2), 4.08–4.12 (m, 1 H, COCH), 6.59 (s, 1 H, 8-H), 6.79 (dd, ^4J = 2.4 Hz, ^3J = 9.3 Hz, 1 H, 6-H), 7.19 (d, ^3J = 7.9 Hz, 1 H, OCONH), 7.67 (d, ^3J = 9.3 Hz, 1 H, 5-H), 8.65 (s, 1 H, 4-H), 8.82 (t, ^3J = 5.7 Hz, 1 H, CONH), 12.69 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 12.4, 28.3, 44.5, 53.7, 78.4, 96.0, 107.8, 109.1, 110.3, 131.8, 148.1, 152.7, 155.6, 157.4, 161.7, 162.7, 172.3. The CH_2 and DMSO signals overlapped.

LC-MS (ESI): 99% purity; *m*/*z* = 448.0 ([M + H]$^+$).

Anal. Calcd for $C_{22}H_{29}N_3O_7$: C, 59.05; H, 6.53; N, 9.39. Found: C, 58.98; H, 6.74; N, 9.28.

(*S*)-2-(*tert*-Butoxycarbonylamino)-6-(2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano[6,7,8-*ij*]quinolizine-10-carboxamido)hexanoic Acid (22)

From **11** (0.54 g, 1.88 mmol), activated using *N*-hydroxysuccinimide (0.33 g, 2.82 mmol) and EDC·HCl (0.54 g, 2.82 mmol) in DMF (30 mL); and subsequent coupling with **15** (0.56 g, 2.26 mmol) in the presence of DIPEA (0.77 g, 1.01 mL, 4.51 mmol) in DMF (30 mL). The product was chromatographed over silica gel using CH_2Cl_2–MeOH–AcOH (29:1:0.03) as eluent; yield: 0.59 g (61%, 1.16 mmol); orange resin.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.30–1.34 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.35 [s, 9 H, $C(CH_3)_3$], 1.43–1.51 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.53–1.60 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 1.62–1.69 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 1.84–1.89 [m, 4 H, $N(CH_2CH_2)_2$], 2.69–2.73 {m, 4 H, $N[(CH_2)_2CH_2]_2$}, 3.24–3.28 (q, ^3J = 5.5 Hz, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.30–3.32 [m, 4 H, $N(CH_2)_2$], 3.80–3.84 (m, 1 H, COCH), 6.94 (d, ^3J = 6.6 Hz, 1 H, OCONH), 7.22 (s, 1 H, 5-H), 8.48 (s, 1 H, 4-H), 8.63 (t, ^3J = 4.8 Hz, 1 H, CONH), 12.10 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 19.8, 21.2, 23.3, 27.0, 28.4, 29.0, 30.7, 38.8, 49.2, 49.7, 53.6, 78.0, 104.8, 107.5, 108.3, 119.6, 127.2, 147.6, 148.1, 152.2, 155.7, 162.1, 162.5, 172.1.

LC-MS (ESI): 93% purity; *m*/*z* = 514.4 ([M + H]$^+$).

HRMS (ESI$^+$): *m*/*z* calcd for $C_{27}H_{35}N_3O_7$ ([M + H]$^+$): 514.2548; found: 514.2548.

(*S*)-2-(*tert*-Butoxycarbonylamino)-3-(2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano[6,7,8-*ij*]quinolizine-10-carboxamido)propanoic Acid (23)

From **11** (0.66 g, 2.31 mmol), activated using *N*-hydroxysuccinimide (0.40 g, 3.47 mmol) and EDC·HCl (0.66 g, 3.47 mmol) in DMF (15 mL); and subsequent coupling with **14** (0.56 mg, 2.77 mmol) in the presence of DIPEA (0.72 g, 0.94 mL, 5.54 mmol) in DMF (30 mL); yield: 0.66 g (61%, 1.40 mmol); orange oil.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.35 [s, 9 H, $C(CH_3)_3$], 1.83–1.89 [m, 4 H, $N(CH_2CH_2)_2$], 2.67–2.73 {m, 4 H, $N[(CH_2)CH_2]_2$}, 3.29–3.36 [m, 5 H, $N(CH_2)_2$, CH_2], 3.77–3.82 (m, 1 H, CH_2), 4.06 (q, ^3J = 7.0 Hz, 1 H, COCH), 7.13 (d, ^3J = 7.6 Hz, 1 H, OCONH), 7.22 (s, 1 H, 5-H), 8.49 (s, 1 H, 4-H), 8.83 (t, ^3J = 5.7 Hz, 1 H, CONH). CO_2H was not detected.

^{13}C NMR (125 MHz, DMSO-d_6): δ = 19.7, 20.7, 26.9, 28.3, 49.2, 49.7, 53.9, 78.4, 104.8, 107.5, 107.7, 119.6, 127.3, 147.8, 148.3, 152.3, 155.1, 161.8, 163.0, 172.4. The CH_2 and DMSO signals overlapped.

LC-MS (ESI): 97% purity; *m*/*z* = 472.2 ([M + H]$^+$).

HRMS (ESI$^+$): *m*/*z* calcd for $C_{24}H_{29}N_3O_7$ ([M + H]$^+$): 472.2078; found: 472.2073.

(*S*)-2-(*tert*-Butoxycarbonylamino)-6-(6-chloro-7-hydroxy-2-oxo-2*H*-chromene-3-carboxamido)hexanoic Acid (24)

From **12** (0.13 g, 0.53 mmol), activated using *N*-hydroxysuccinimide (0.10 g, 0.80 mmol) and EDC·HCl (0.15 g, 0.80 mmol) in DMF (50 mL); and subsequent coupling with **15** (0.16 g, 0.64 mmol) in the presence of DIPEA (0.16 g, 0.22 mL, 1.27 mmol) in DMF (25 mL); yield: 0.19 g (76%, 0.41 mmol); yellow solid; mp 125–128 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.32–1.38 [s, 11 H, $C(CH_3)_3$, $CH_2CH_2CH_2CH_2NH$], 1.44–1.54 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.54–1.60 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 1.62–1.70 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 3.30 (q, ^3J = 5.6 Hz, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.81–3.85 (m, 1 H, COCH), 6.95 (s, 1 H, 8-H), 7.00 (d, ^3J = 6.6 Hz, 1 H, OCONH), 8.05 (s, 1 H, 5-H), 8.58 (t, ^3J = 4.8 Hz, 1 H, CONH), 8.74 (s, 1 H, 4-H), 11.83 (br s, 1 H, OH), 12.36 (br s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 23.3, 28.4, 28.1, 30.6, 39.0, 53.6, 78.1, 103.0, 111.9, 115.4, 118.2, 130.9, 147.1, 154.5, 155.8, 158.6, 160.7, 161.3, 174.3.

LC-MS (ESI): 97% purity; *m*/*z* = 469.1 ([M + H]$^+$).

HRMS (ESI$^+$): *m*/*z* calcd for $C_{21}H_{25}ClN_2O_8$ ([M + H]$^+$): 469.1372; found: 469.1360.

Methyl 9-(2-*tert*-Butoxy-2-oxoethyl)-2-oxo-(6,7,8,9-tetrahydro-2*H*-pyrano[3,2-*g*]quinoline-3-carboxylate (26)

tert-Butyl 2-(6-formyl-7-hydroxy-3,4-dihydroquinolin-1(2*H*)-yl)acetate (**6**; 0.39 g, 1.33 mmol) was dissolved in toluene (10 mL). Dimethyl malonate (**25**; 0.19 g, 0.17 mL, 1.46 mmol) and piperidine (0.13 mL, 1.33 mmol) were added and the mixture was refluxed for 2 h. After evaporation in vacuo, the residue was purified by column chromatography using petroleum ether–EtOAc (3:1 to 1:1); yield: 0.44 g (88%, 1.18 mmol); green solid; mp 143–146 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.41 [s, 9 H, $C(CH_3)_3$], 1.87 (quint, ^3J = 4.9 Hz, 2 H, NCH_2CH_2), 2.72 [t, ^3J = 4.9 Hz, 2 H, $N(CH_2)_2CH_2$], 3.40 (t, ^3J = 4.9 Hz, 2 H, NCH_2), 3.75 (s, 3 H, CH_3), 4.20 (s, 2 H, CH_2CO), 6.63 (s, 1 H, 8-H), 7.36 (s, 1 H, 5-H), 8.49 (s, 1 H, 4-H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 20.9, 26.8, 27.9, 50.1, 51.9, 53.2, 81.5, 95.6, 107.7, 108.0, 120.7, 129.5, 149.3, 151.5, 156.8, 157.1, 164.0, 168.4.

LC-MS (ESI): 99% purity; *m*/*z* = 374.1 ([M + H]$^+$), 764.3 ([2 M + NH_4]$^+$).

Anal. Calcd for $C_{20}H_{23}NO_6$: C, 64.33; H, 6.21; N, 3.75. Found: C, 64.43; H, 6.34; N, 3.76.

(*S*)-2-(*tert*-Butoxycarbonylamino)-6-{2-[3-(methoxycarbonyl)-2-oxo-7,8-dihydro-2*H*-pyrano[3,2-*g*]quinolin-9(6*H*)-yl]acetamido}hexanoic Acid (28)

Compound **26** (0.69 g, 1.85 mmol) was dissolved in a mixture of CH_2Cl_2–TFA (1:1, 20 mL) and stirred for 1 h at r.t. Afterwards, the mixture was evaporated to obtain **27**, which was used in the next step without further purification. Amide coupling was performed by applying the aforementioned protocol. Compound **27** (0.59 g, 1.85 mmol) was activated with *N*-hydroxysuccinimide (0.32 g, 2.78 mmol) and EDC·HCl (0.53 g, 2.78 mmol) in DMF (50 mL) and coupled with N^{α}-Boc-L-Lys-OH (**15**; 0.55 g, 2.22 mmol) in the presence of DIPEA (0.57 g, 0.76 mL, 4.44 mmol) in DMF (50 mL) to afford compound **28**; yield: 0.53 g (53%, 0.97 mmol); orange solid; mp 194–197 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.32–1.38 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.36 [s, 9 H, $C(CH_3)_3$], 1.38–1.43 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.49–1.58 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 1.59–1.67 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 1.88 (quint, ^3J = 5.7 Hz, 2 H, NCH_2CH_2), 2.70–2.73 [m, 2 H, $N(CH_2)_2CH_2$], 3.02–3.10 (m, 2 H,

$CH_2CH_2CH_2CH_2NH$), 3.44 (t, ^3J = 5.7 Hz, 2 H, NCH_2), 3.74 (s, 3 H, CH_3), 3.79–3.85 (m, 1 H, COCH), 4.02 (s, 2 H, CH_2), 6.29 (s, 1 H, 8-H), 6.96 (d, ^3J = 8.7 Hz, 1 H, OCONH), 7.33 (s, 1 H, 5-H), 8.07 (t, ^3J = 5.5 Hz, 1 H, CH_2CONH), 8.47 (s, 1 H, 4-H), 12.36 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 20.8, 23.2, 26.8, 28.3, 28.8, 30.5, 38.5, 50.6, 51.9, 54.2, 53.2, 78.1, 95.4, 107.5, 107.5, 120.8, 129.3, 149.3, 151.8, 155.7, 156.9, 157.1, 164.1, 167.7, 174.3.

LC-MS (ESI): 94% purity; m/z = 546.3 ([M + H]$^+$).

HRMS (ESI$^+$): m/z calcd for $C_{27}H_{35}N_3O_9$ ([M + H]$^+$): 546.2446; found: 546.2441.

(*S*)-2-(*tert*-Butoxycarbonylamino)-6-(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-ylamino)hexanoic Acid (29)

$NaHCO_3$ (1.19 g, 14.13 mmol), Nα-Boc-L-Lys-OH (**15**; 0.70 g, 2.83 mmol), and 4-chloro-7-nitrobenzofurazan (0.49 g, 2.43 mmol) were dissolved in a mixture of H_2O and MeCN (1:1; 20 mL). The solution was stirred at 75 °C for 1 h. The solvents were removed in vacuo, and the remaining crude product was dissolved in CH_2Cl_2 (100 mL) and washed with aq 10% $KHSO_4$ (2 × 100 mL) and H_2O (100 mL), dried (Na_2SO_4), filtered, and evaporated; yield: 0.83 g (83%, 2.03 mmol); brown solid; mp 99–102 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.34 [s, 9 H, $C(CH_3)_3$], 1.37–1.46 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 1.55–1.72 (m, 4 H, $CH_2CH_2CH_2CH_2NH$), 3.44–3.45 (m, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.82–3.86 (m, 1 H, COCH), 6.39 (d, ^3J = 8.9 Hz, 1 H_{arom}), 7.04 (d, ^3J = 7.9 Hz, 1 H, OCONH), 8.49 (d, ^3J = 8.9 Hz, 1 H_{arom}), 9.54 (t, ^3J = 5.2 Hz, 1 H, NH), 12.42 (s, 1 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 23.3, 27.3, 28.4, 30.6, 43.3, 53.5, 78.2, 99.3, 120.7, 138.2, 144.4, 144.7, 145.4, 155.8, 174.4.

LC-MS (ESI): 95% purity; m/z = 410.4 ([M + H]$^+$).

HRMS (ESI$^+$): m/z calcd for $C_{17}H_{23}N_5O_7$ ([M + H]$^+$): 410.1670; found: 410.1672.

(*S*)-5-{3-[5-(*tert*-Butoxycarbonylamino)-5-carboxypentyl]thioureido}-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic Acid (30)

Fluorescein isothiocyanate (0.39 g, 1.0 mmol) was added to a solution of Nα-Boc-L-Lys-OH (**15**; 0.25 g, 1.0 mmol) and Et_3N (0.15 g, 0.21 mL, 1.5 mmol) in EtOH (15 mL) and the mixture was stirred at 60 °C for 6 h. Afterwards, the mixture was evaporated, and the residue was purified by column chromatography using EtOAc to EtOAc–MeOH–AcOH (9:1:0.01) as eluent; yield: 0.33 g (52%, 0.52 mmol); red solid; mp 110–112 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.36 [s, 11 H, $C(CH_3)_3$, $CH_2CH_2CH_2CH_2NH$], 1.52–1.63 (m, 3 H, $CH_2CH_2CH_2CH_2NH$), 1.65–1.73 (m, 1 H, $CH_2CH_2CH_2CH_2NH$), 3.48 (br s, 2 H, $CH_2CH_2CH_2CH_2NH$), 3.84–3.89 (m, 1 H, COCH), 6.54–6.57 (m, 2 H_{arom}), 6.59–6.60 (m, 2 H_{arom}), 6.66 (s, 1 H_{arom}), 6.67 (s, 1 H_{arom}), 7.01 (d, ^3J = 8.2 Hz, 1 H_{arom}), 7.16 (d, ^3J = 8.5 Hz, 1 H_{arom}), 7.73 (d, ^3J = 6.6 Hz, 1 H, OCONH), 8.07 (s, 1H, NH or OH), 8.21 (s, 1 H_{arom}), 9.83 (s, 1 H, NH or OH), 10.07 (s, 1 H, NH or OH), 12.07 (s, 2 H, CO_2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 21.2, 23.3, 28.4, 30.7, 43.8, 53.6, 78.1, 83.1, 102.4, 109.9, 112.7, 116.5, 124.1, 126.7, 129.1, 141.6, 147.2, 152.0, 155.8, 159.6, 168.7, 172.1, 174.3, 180.5.

LC-MS (ESI): 99% purity; m/z = 636.44 ([M + H]$^+$), 634.39 ([M – H]$^-$).

HRMS (ESI$^+$): m/z calcd for $C_{32}H_{33}N_3O_9S$ ([M + H]$^+$): 636.2010; found: 636.2008.

(*S*)-*tert*-Butyl 3-(6,7-Dimethoxy-2-oxo-2*H*-chromene-3-carboxamido)-1-(4-nitrobenzylamino)-1-oxopropan-2-ylcarbamate (32)

Compound **19** (0.80 g, 1.83 mmol) was activated using HATU (0.70 g, 1.83 mmol) and DIPEA (0.47 g, 0.62 mL, 3.66 mmol) in anhydrous DMF (20 mL) for 15 min at r.t. Afterwards, 4-nitrobenzylamine hydrochloride (**31**; 0.35 g, 1.83 mmol) was added and the mixture was stirred for 16 h overnight. Solvents were then removed in vacuo. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with aq 10% citric acid (100 mL), aq 10% $NaHCO_3$ (100 mL), H_2O (100 mL) and brine (100 mL), dried (Na_2SO_4), filtered, and the solvent was removed under reduced pressure. The product was crystallized from EtOAc; yield: 0.93 g (89%, 1.64 mmol); yellow solid; mp 222 °C (dec.).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.57 [s, 9 H, $C(CH_3)_3$], 3.40–3.46 (m, 1 H, CH_2), 3.73–3.80 (m, 1 H, CH_2), 3.82 (s, 3 H, OCH_3), 3.90 (s, 3 H, OCH_3), 4.17–4.21 (q, ^3J = 8.2 Hz, 1 H, COCH), 4.35–4.44 (m, 2 H, $CHCONHCH_2$), 7.18 (s, 1 H, 8-H), 7.19 (d, ^3J = 7.9 Hz, 1 H, OCONH), 7.51 (d, ^3J = 8.7 Hz, 1 H_{arom}), 7.53 (s, 1 H, 5-H), 8.10 (d, ^3J = 8.7 Hz, 2 H_{arom}), 8.69 (t, ^3J = 5.5 Hz, 1 H, CONH), 8.81 (s, 1 H, 4-H), 8.86 (t, ^3J = 5.5 Hz, 1 H, CONH).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 28.3, 40.7, 41.9, 54.5, 56.2, 56.7, 78.6, 99.8, 110.2, 111.2, 114.4, 123.3, 128.3, 146.5, 146.7, 147.7, 148.2, 150.9, 155.1, 155.6, 161.1, 162.0, 170.7.

LC-MS (ESI): 97% purity; m/z = 571.5 ([M + H]$^+$).

HRMS (ESI$^+$): m/z calcd for $C_{27}H_{30}N_4O_{10}$ ([M + H]$^+$): 571.2035; found: 571.2032.

(*S*)-*N*-[2-(4-Guanidinobenzamido)-3-(4-guanidinobenzylamino)-3-oxopropyl]-6,7-dimethoxy-2-oxo-2*H*-chromene-3-carboxamide Dihydrochloride (37)

The N-protecting group was cleaved by dissolving **32** (0.93 g, 1.64 mmol) in a solution of CH_2Cl_2 and TFA (1:1, 20 mL) and stirring the mixture for 1 h at r.t. The corresponding ammonium trifluoroacetate was obtained after removing the solvent in vacuo. 4-Nitrobenzoic acid (**33**; 0.27 g, 1.64 mmol) was dissolved in anhydrous DMF (20 mL) and activated for 15 min at r.t. using HATU (0.62 g, 1.64 mmol) and DIPEA (0.64 g, 0.84 mL, 4.92 mmol). Afterwards, the ammonium trifluoroacetate (0.959 g, 1.64 mmol) was added and the mixture was stirred overnight at r.t. The solvent was removed and the residue was suspended in EtOAc (100 mL). The insoluble material, compound **34**, was collected by filtration and directly subjected to the next reaction step. To a stirred suspension **34** (0.73 g, 1.18 mmol) in EtOAc (100 mL) were added $SnCl_2{\cdot}2\ H_2O$ (2.66 g, 11.8 mmol) and H_2O (0.85 g, 47.20 mmol). The solution was stirred under reflux for 4 h. The solvent was removed in vacuo and the residue was suspended in aq 2 M NaOH (200 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic extracts were washed with H_2O (150 mL) and brine (150 mL), dried (Na_2SO_4), and evaporated. A mixture of the obtained bis-aniline derivative **35** (98 mg, 0.18 mmol), *N*,*N*′-di-Boc-*S*-methylisothiourea (**36**; 94 mg, 0.32 mmol), $HgCl_2$ (93 mg, 0.34 mmol), Et_3N (55 mg, 0.07 mL, 0.54 mmol), and CH_2Cl_2 (20 mL) was stirred at r.t. for 24 h. The solution was passed through a pad of Celite and the pad was washed with CH_2Cl_2 (50 mL) and MeOH (50 mL). The solvents were removed in vacuo. The resulting Boc-protected bis-guanidine was purified by column chromatography using EtOAc–MeOH–Et_3N (9:1:0.01) as eluent. The material (148 mg, 0.14 mmol) was dissolved in CH_2Cl_2 (5 mL) and a 50% solution of TFA in CH_2Cl_2 (10 mL) was added. After stirring at r.t. for 2 h, the solvent was evaporated. The resulting oil was purified by preparative HPLC. The HPLC eluent was MeOH–H_2O (30:70) for 8 min. To the product, few drops of aq 1 M HCl were added to form

the hydrochloride salt. The final product **37** was obtained after lyophilization; yield: 23 mg (2% over five steps, 0.033 mmol); yellow solid; mp 173–176 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 3.60–3.67 (m, 1 H, CH_2), 3.81 (s, 3 H, OCH_3), 3.89–3.98 (m, 1 H, CH_2), 3.90 (s, 3 H, OCH_3), 4.27–4.35 (m, 2 H, CHCONHCH_2), 4.66–4.71 (m, 1 H, COCH), 7.14 (d, ^3J = 8.6 Hz, 2 H_{arom}), 7.17 (s, 1 H, 8-H), 7.32 (d, ^3J = 8.6 Hz, 4 H_{arom}), 7.46 (s, 4 H, $NH_{2guanidine}$), 7.54 (s, 1 H, 5-H), 7.70 (s, 4 H, $NH_{2guanidine}$), 7.96 (d, ^3J = 8.6 Hz, 2 H_{arom}), 8.70 (t, ^3J = 6.2 Hz, 1 H, CONH), 8.79 (d, ^3J = 7.6 Hz, 1 H, CONH), 8.84 (s, 1 H, 4-H), 8.99 (t, ^3J = 6.0 Hz, 1 H, CONH), 9.97 (s, 1 H, $NH_{guanidine}$), 10.35 (s, 1 H, $NH_{guanidine}$).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 41.8, 45.5, 54.0, 56.2, 56.7, 99.8, 110.2, 111.2, 114.3, 123.0, 124.3, 128.4, 129.2, 131.2, 133.9, 137.8, 138.6, 146.7, 148.3, 150.9, 155.1, 155.9, 156.2, 161.1, 162.2, 166.0, 170.0.

LC-MS (ESI): 98% purity; m/z = 644.5 ([M + H]$^+$).

HRMS (ESI$^+$): m/z calcd for $C_{31}H_{33}N_9O_7$ ([M + AcOH]$^+$): 351.6357; found: 351.6369.

Biochemical Investigations

Kinetic parameters of inhibition of the serine proteases human matriptase-2, human thrombin, bovine factor Xa, and bovine trypsin were determined in duplicate measurements by using literature protocols.[18,19] Assays were performed in the presence of five different concentrations of inhibitor **37** at pH 8, either at 25 °C or 37 °C. Reactions were followed over 15 min.

Affinity to DNA of **37** was measured by anisotropy titration, following a literature protocol.[21] The short hairpin oligonucleotide (GG-C*AAATTT*CAGTTTTTCTG*AAATTT*GCC) was used containing the hairpin loop TTTTT and the adenine/thymine-rich binding site *AAATTT*. The experiments were performed in duplicate at pH 7.5 and 25 °C.

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Supporting Information

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