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Synthesis of peptide-adenine conjugates as a new tool for the protease activity monitoring

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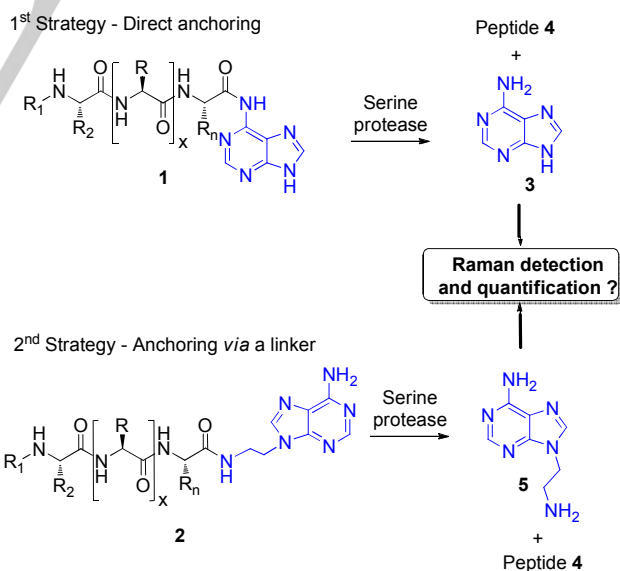
Abstract: We took advantage of the powerful adenine SERS (Surface Enhanced Raman Spectroscopy) probe to design peptide-adenine conjugates as candidates for their use as serine protease substrates. Whereas the direct introduction of the peptide sequence on the adenine exocyclic N6 amine gave an imidazopurinone derivative, the introduction of an aminoethyl linker between the adenine group and the peptide chain led to the expected candidate probes. These potential substrates were then evaluated for the monitoring of the hydrolytic activity of trypsin, used as a model of protease, by HPLC and by SERS. We demonstrated that Boc-VPR-adenine conjugate was a substrate of trypsin and constitute a good starting point to design optimized substrates to monitor the protease activity by SERS.

Introduction

Serine proteases are major actors in cell signalling and tissue remodelling. Their deregulation is implicated in many diseases including cancer, neurodegeneration, and inflammation.^[1–3] Thus, many efforts have been made to develop serine protease inhibitors.^[4–7] In such a context, fluorescence spectrometry is a lead technique to identify and characterize inhibitors. Fluorescence assays are based on the use of peptide substrates bearing fluorogenic groups that generate highly fluorescent entities during hydrolytic digestion. However, during screening for enzyme inhibition, quenching and/or autofluorescence could hamper the proper selection and evaluation of inhibitors. To circumvent this issue, other methods, generally based on chromatographic techniques (electrophoresis or liquid chromatography) must be used. Alternate approaches, such as surface enhanced Raman spectroscopy or surface-enhanced

Raman scattering (SERS) have been also considered. SERS is a surface-sensitive technique involving signal enhancement with molecular probe adsorption on rough metal or nanostructured surfaces, allowing the detection of single molecules.^[8] The use of SERS in biomedical applications, in particular in enzymology, is advantageous, since it is ideal for dilute media and SERS circumvents fluorescence background.^[8] Another striking element is that it provides vibrational fingerprints of adsorbed molecules even in mixtures.^[9] Based on SERS principles, several hydrolytic enzymes including lipases, esterases as well as proteases were successfully investigated.^[10–14]

On the other hand, adenine constitutes a very powerful SERS probe, as little as 10^{-13} mol of adenylyl residue can be detected when brought in contact with the silver colloidal particles used for SERS.^[15–17] Consequently, we proposed to design, synthesize and evaluate adenylyl containing peptides as potential substrates to monitor protease activity. First, the synthesis of peptide probes was optimized. In a second step, these modified peptides were evaluated by SERS for their ability to be recognized and cleaved by trypsin as a serine protease model. In parallel, a selected probe was evaluated using HPLC and compared to a known substrate incorporating an amino-methylcoumarin (AMC) group.^[18]



Scheme 1. Design of the peptide-adenine conjugates as potential SERS probes.

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Results and Discussion

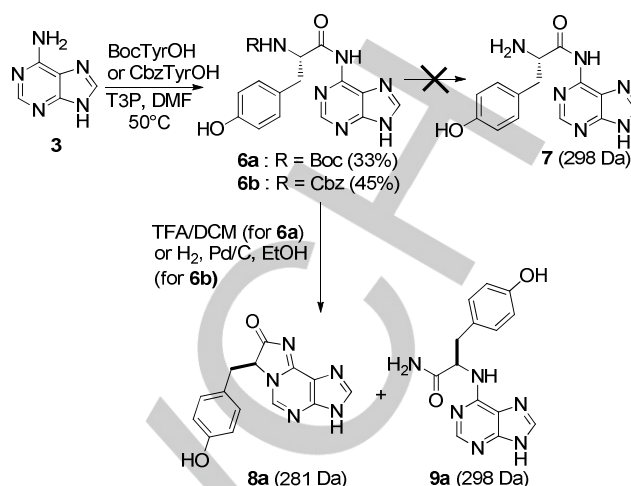
Based on previously reported AMC-containing substrates,^[7,13,18] two peptide sequences were selected to design the targeted probes: Succinyl-LLVY was chosen as a substrate for chymotrypsin-like proteases and Boc-VPR for trypsin-like enzymes. Two strategies were envisioned to anchor the adenine moiety (Scheme 1).

The first strategy consisted of the direct linking of the peptide sequence on the adenine exocyclic N6 amine (compound **1**). With such a sequence, the native adenine **3** will be released during enzymatic hydrolysis. The second strategy consisted of the anchoring of the peptide onto the adenine N1 via a linker (compound **2**). With this framework, N1-aminoethyladenine moiety **5** will be released during proteolysis.

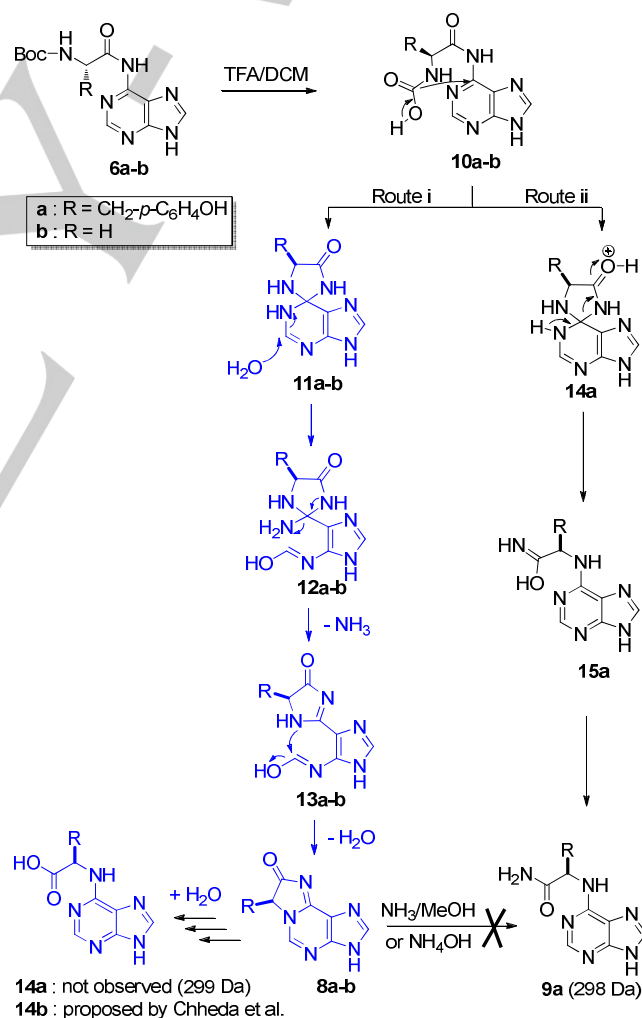
Direct anchoring approach

Considering the first strategy (Scheme 2), adenine **3** was coupled to Boc-Tyr-OH, using carbonyldiimidazole (CDI) as activating agent and dry dimethylformamide (DMF) as the solvent. N6-acyladenine derivative **6a** was isolated in 22% yield. After testing several additional coupling reactants (EDCI/HOBt, HBTU, T3P), we found that polyphosphonic anhydride (T3P) in DMF at 50°C for one night improved slightly the yield of compound **6a** (33% yield). Then, the Boc group was removed, using a 1:1 trifluoroacetic acid/dichloromethane (TFA/DCM) mixture. The mass spectrometry (MS) analysis of the resulting crude mixture showed the formation of a main compound, which possesses a molecular weight of 281 Da instead of 298 Da for the expected Boc-protected adenine peptide conjugate **7**. It was identified as the tricyclic derivative **8a** (Scheme 2). Such a reactivity has already been observed by Chheda et al. with N6-glycyladenine **6b**,^[19,20] which is converted into the tricyclic derivative **8b** in water after the loss of ammonia (scheme 3, route i). These authors reported also that the tricyclic derivative **8b** could further evolved to N-(6-purinyl)glycine **14b** after ring opening by a molecule of water. According to our experimental conditions, the formation of a minority compound **9a** (~15%) was also detected in the crude with compound **8a** (Scheme 2 and ESI, Figures S1 and S2). Its molecular weight of 298 Da don't match the acid **14a**. Based on its fragmentation pattern (ESI, Figures S3 and S4), compound **9a** was identified as the amide derivative (Schemes 2 and 3). As compound **9a** was not obtained after treatment of compound **8a** with aqueous or methanolic solution of ammonia (Scheme 3), the formation of **9a** could not result from the aminolysis of the lactame ring of **8a**. A possible mechanism to explain the formation of **9a** is proposed Scheme 3, route ii.

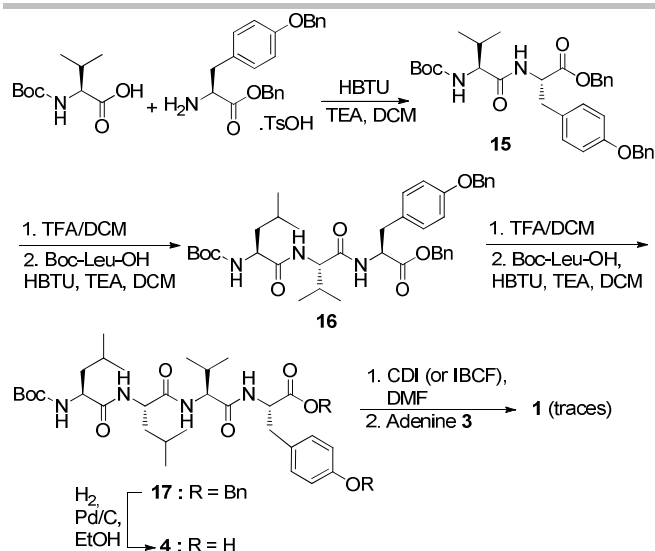
As the synthesis of the peptide-adenine conjugate **1** was not successful from N-acyladenine **6**, we then envisioned its synthesis by direct coupling adenine **3** and the protected tetrapeptide Boc-LLVY-OH **4**, which was prepared in solution, using a classical Boc/Bn strategy (Scheme 4). Coupling of peptide **4** with adenine **3** in DMF was studied, after activation of **4** by CDI or isobutylchloroformate (IBCF). However, even after 48h, only traces of conjugate **1** were detected by LC/MS analysis. It is worth noting that this strategy could also yield to tyrosine epimerization by oxazolone formation. Taken together, these limitations lead us to envision the second strategy.



Scheme 2. Attempts to prepare the peptide-adenine conjugate using the direct anchoring approach (First strategy).



Scheme 3. Putative mechanisms for the formation of **8** and **9**. In blue, intermediates and structures proposed by Chheda et al.^[19,20]

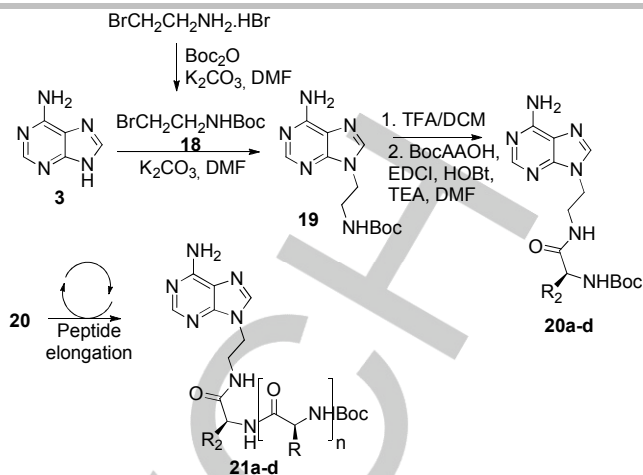


Scheme 4. Synthesis of peptide Boc-LLVY-OH **4** and direct coupling between adenine **3** and **4**.

Anchoring via a linker

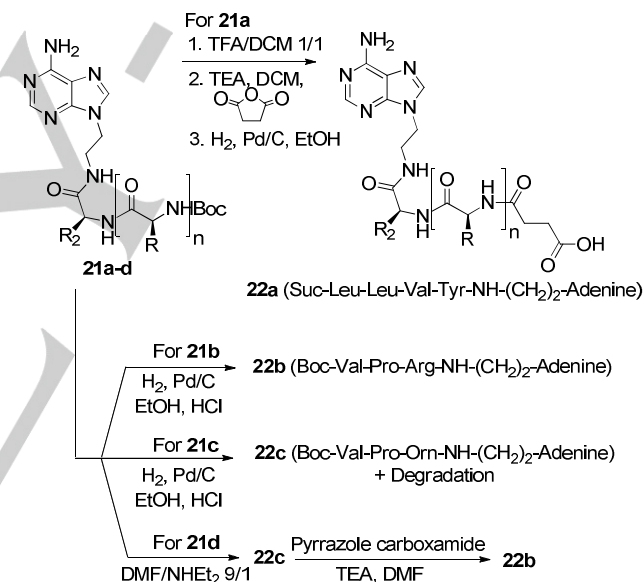
Firstly, N-Boc protected amino-ethyladenine **19** was prepared. For that purpose, *tert*-butyl 2-bromo-ethylcarbamate **18** was synthesized by protection of commercial 2-bromo-ethylamine hydrobromide with *tert*-butylcarbonate, according to a slightly modification of the procedure described by Narayanaswamy et al.^[21] Compound **18** was then used to alkylate adenine, using potassium carbonate in DMF (Scheme 5). Compound **19** was isolated in 46% yield after precipitation. The Boc group was then removed in acidic conditions. The resulting deprotected compound was then coupled with Boc-Tyr(Bn)-OH in DMF, using EDCI/HOBt as coupling reagents to offer compound **20a** in 20% yield. Chain elongation was then carried out using a standard Boc strategy for peptide synthesis, in the same conditions as previously, to lead protected compound **21a**. The Boc group of **21a** was removed by treatment with a TFA/DCM mixture (Scheme 6). The terminal amine was then condensed with succinic anhydride. Finally, the benzyl group was removed by hydrogenolysis yielding Suc-LLVY-adenine **22a**, which was purified by preparative HPLC.

To access the second substrate which is based on a Boc-VPR sequence, compound **19** was deprotected as previously and the deprotected derivative was coupled with Boc-Arg(NO₂)-OH. The resulting compound **20b** as well as all the other intermediates during the peptide chain elongation revealed to be highly soluble in water. Therefore, each coupling intermediate needed to be purified by preparative HPLC, leading to a very poor final yield in compound **21b** (2% overall yield, starting from compound **19**). Nitro group was finally removed quantitatively by hydrogenolysis, using Pd/C in ethanol to offer compound **22b** (Scheme 6). To improve the global yield, we envisioned to generate the guanidine function of the arginine in the last step of the synthesis. To achieve this goal, an ornithine derivative with a Cbz-protected lateral chain was prepared (compound **20c**, Scheme 5).



21a : Boc-Leu-Leu-Val-Tyr(Bn)-NH-(CH₂)₂-Adenine
21b : Boc-Val-Pro-Arg(NO₂)-NH-(CH₂)₂-Adenine
21c : Boc-Val-Pro-Orn(Cbz)-NH-(CH₂)₂-Adenine
21d : Boc-Val-Pro-Orn(Fmoc)-NH-(CH₂)₂-Adenine

Scheme 5. Synthesis of the protected peptide-adenine conjugates **21a-d**.



Scheme 6. Synthesis of the peptide-adenine conjugates.

Because of the higher lipophilicity of all the intermediates, by-products could be easily removed by simple washing with sodium hydrogenocarbonate solution. Compound **21c** could be recovered in 44% overall yield from **19**. Cbz group of **21c** was then removed by hydrogenolysis, using Pd/C, with one equivalent of hydrochloric acid. Surprisingly, this deprotection step revealed to be slow and led finally to compound **22c** and several other unidentified derivatives, whatever the solvent tested (EtOH, THF, AcOEt). Thus, we attempted to protect the ornithine lateral chain with a Fmoc group. Fmoc-ornithine derivative was prepared by protection of the commercially available Boc-Orn-OH with Fmoc-O-succinimide in a mixture of THF and water. Boc-Orn(Fmoc)OH derivative was obtained in 81% yield. After acidic deprotection of **19**, the resulting product was then coupled with Boc-Orn(Fmoc)OH, to offer compound **20d** in 75% yield. Peptide chain elongation was then realized as previously to obtain **21d** in 46% yield. Fmoc group removal was

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realized with diethylamine in DMF. The deprotected derivative **22c** was used in the next step without purification. Ornithine was converted into arginine, using pyrazole-carboxamide hydrochloride, as the guanidinylation agent, in DMF. Compound **22b** was finally obtained after purification by preparative HPLC in 12% overall yield, from compound **19**.

Monitoring of the trypsin activity by SERS

The ability of compounds **22a** and **22b** to be recognized and cleaved by trypsin was then evaluated by SERS, using the Creighton silver colloid.^[22] Experiments were performed in triplicates. Several silver colloid batches were synthesized using the classical Creighton' method^[22] and analyzed as described in Soualmia et al.^[13] Compound **22a** revealed to be poorly soluble in the aqueous medium at micromolar concentrations used for the spectra registration. Thus, we focused our study on compound **22b**. The SERS spectrum of the peptide conjugate Boc-VPR-Adenine **22b** was compared to that of compound **5**, which illustrates the spectral fingerprint of the released amino-ethyladenine moiety after the proteolysis.

Very low Raman signal was observed for **22b** (Figure 1A, $t = 0$ min), whereas spectrum of compound **5** showed several more intense Raman bands (spectrum identical to that obtained after $t = 40$ min on Figure 1A). These bands (Table 1) could be attributed to the adenine moiety, even if their positions shifted slightly from canonical adenyl bands.^[15,16] The overall fingerprint confirmed a privilege-concerted (cooperative) C_6-NH_2/N_7 binding available for adenyl group with the intensity of the bands which increased proportionally to the concentration of compound **5** (data not shown). Time-course of the tryptic hydrolysis of **22b** was then followed using a sampling method, with a substrate concentration adjusted at 100 μM in order to have an optimum SERS detection during the investigated time scale (Figure 2A). Successive samples were collected over time from the reaction medium and subjected to SERS measurement. Thus, registered SERS spectra illustrated the composition of reaction media at a given stage. The time course of compound **5** release is shown in Figure 1A. At $t = 0$ min, the spectrum intensity is low. During digestion process, the contribution of compound **5** in the spectra increased over the time. Pattern spectra revealed the apparition of adenyl characteristic bands (noted by asterisks in Figure 1A). In addition, intensity of typical bands of the released adenyl group (see above) increased over the time. Figure 1B presents the increase of the intensity of the characteristic band at 1384 cm^{-1} as a function of time, these latter correlated to the increase of compound **5** concentration. However, the evaluation of kinetics parameters was not achieved due probably to a weaker affinity of compound **5** compared to free adenine or improper adsorption orientation onto silver colloid nanoparticles.

Table 1. Main SERS observed bands and assignments of adenyl moieties.

Wavenumber (cm^{-1})	Assignments
2928, 1014, 953	DMSO
685-785	C_6NH_2 vibrational modes
980-1045	C_6NH_2 vibrational modes
1380-1423	$N_7[Ag]_n$ interaction
1482	$N_7[Ag]_n$ interaction

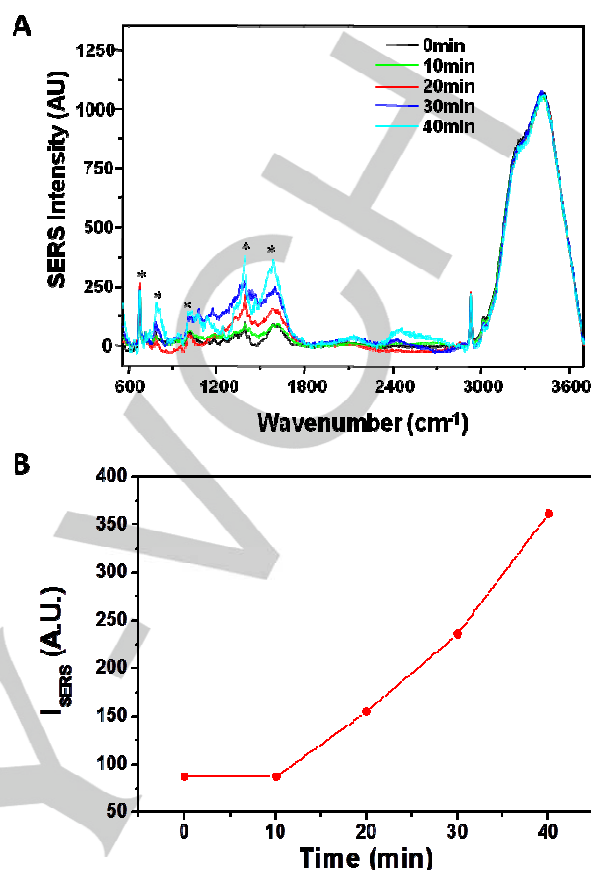


Figure 1. A: Time-course evolution of SERS spectrum of 100 μM **22b** after incubation with 200 nM of trypsin in Tris buffer 10 mM at pH 7.5. The final percentage of DMSO was 2% for all samples. The spectra were normalized to the water band at 3500 cm^{-1} . Asterisks showed the adenyl characteristic bands. B: Progression of SERS intensity 1384 cm^{-1} adenyl band over the time.

Monitoring of the trypsin activity by HPLC

The proteolytic cleavage of VPR-Adenine **22b** by trypsin was then studied using a HPLC technique. Firstly, 75 μM of **22b** was incubated at 37°C with trypsin in a TRIS-HCl buffer (100 mM, pH 8.0). The time-course of the reaction was followed by measuring the apparition of Boc-VPR-OH at $\lambda = 214$ nm (Figure 2A and ESI Figures S7 and S8). Rapidly, a new product was detected and **22b** was totally hydrolyzed after around 1 hour of incubation. LC-MS analysis (ESI+) of the final hydrolysis medium showed that Boc-VPR-OH ($R_T = 1.11$ min, $[M+H]^+ = 471.2$ Da) and compound **5** (injection peak) were formed, demonstrating that **22b** was a substrate of trypsin. The rates of hydrolysis of **22b** were then determined using 5 different concentrations of **22b** (37 to 150 μM). The rate data for **22b** were plotted as double reciprocal plots (Lineweaver-Burk plots of $1/\text{rate}$ vs $1/[\text{substrate}]$) as shown in Figure 2B. From these plots, the values of K_m (the Michaelis constant) and V_{max} (the maximal velocity of the reaction) were determined. The catalytic constant was calculated from $k_{cat} = V_{max}/E_0$, where E_0 is the enzyme concentration (200 nM) and finally the ratio k_{cat}/K_m was obtained. These data are presented in Table 2. These data were compared to those obtained with the classical Boc-VPR-AMC substrate, using the same experimental procedure (see ESI,

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Figure S9). Based on the second-order rate constant (k_{cat}/K_m), the value for compound **22b** is about 6 times smaller than that for Boc-VPR-AMC. The K_m value for **22b** is also smaller than that for the AMC derivative. If K_m reflects the binding affinity of these two substrates to trypsin, **22b** is a weaker substrate relatively to Boc-VPR-AMC for trypsin, as already observed using SERS.

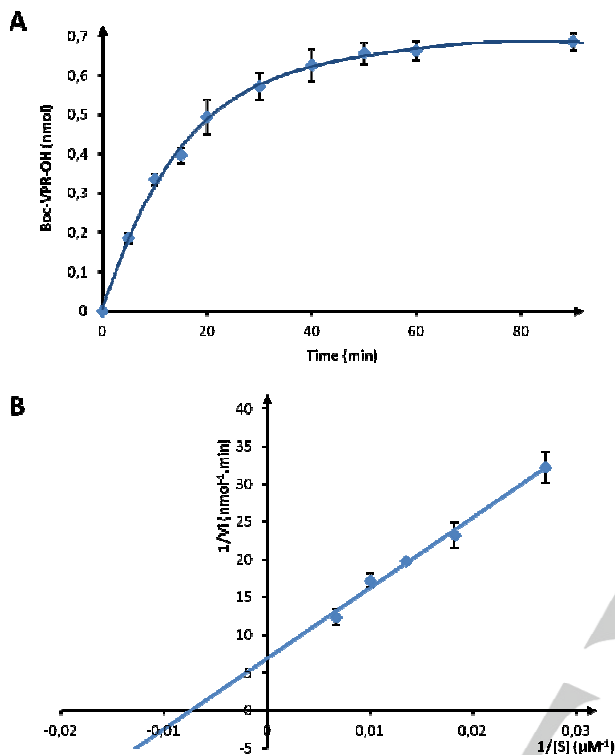


Figure 2 **A.** Time course of tryptic hydrolysis of **22b** (75 μM) by HPLC ($\lambda = 214$ nm). The amount of Boc-VPR-OH in nmol refers to the amount contained in 10 μL of the reaction mixture applied to the column at each time (average of 4 independent reactions). **B.** Double reciprocal (Lineweaver-Burk) plot of kinetic data for the action of trypsin (200 nM) on **22b**. Each point represents the average of triplicate measurements.

Table 2. Kinetic data.

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{min}^{-1}$)
22b	135.3 ± 2.7	72.6 ± 1.8	0.53 ± 0.02
Boc-VPR-AMC	11.1 ± 0.3	34.1 ± 0.6	3.08 ± 0.13

Conclusions

We investigated the synthesis of adenine-peptide conjugates as alternative to the AMC-serine proteases substrates. Whereas the direct anchoring of the peptide chain onto the adenine exocyclic N6 amine led only to the tricyclic compound **8**, the introduction of an aminoethyl linker between the adenine group and the peptide chain led to the candidate probes. Then, we bring experimental evidence that compound **22b** may be recognized although not very efficiently and could be cleaved by

trypsin, its cleavage product was detected by SERS. Kinetic parameters were then determined using an HPLC technique and compared to those obtained with Boc-VPR-AMC. Compound **22b** revealed to be a weaker substrate for trypsin than the AMC derivative (K_m of 135.3 ± 2.7 μM compared to 11.1 ± 0.3 μM). Ongoing studies especially on the linker chemistry may allow to produce optimized substrates for SERS detection since adenine constitutes one of the best SERS probe that could also be expanded to other proteases.

Experimental Section

All reagents and solvents were from AlfaAesar and Acros and were used without further purification. The following abbreviations were used: DCM, dichloromethane; DMF, N,N'-dimethylformamide; HPLC, High Performance Liquid Chromatography; LC/MS, Tandem Liquid Chromatography/ Mass Spectroscopy; TFA, trifluoroacetic acid; THF, Tetrahydrofuran. Other abbreviations used were those recommended by the IUPAC-IUB Commission.^[23] Chemical and enzymatic reactions were monitored by HPLC using an analytical Chromolith Speed Rod RP-C18 185 Pm column (50 x 4.6 mm, 5 mm), at a flow rate of 3.0 mL/min, and gradients of 100/0 to 0/100 eluents A/B for 5 min (eluent A= H₂O/0.1% TFA and B= CH₃CN/0.1% TFA). Detection was performed at 214 nm using a photodiode array detector. Retention times (R_T) are reported in min. ¹H and ¹³C NMR spectra were recorded at room temperature in deuterated solvents. Chemical shifts (δ) are expressed in parts per million (ppm), relative to the resonance of CDCl₃ = 7.26 ppm for ¹H (77.16 ppm for ¹³C), CD₃OD = 3.31 ppm for ¹H (49.00 ppm for ¹³C) or DMSO d_6 = 2.50 ppm for ¹H (39.52 ppm for ¹³C). The following abbreviations were used: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad). LC-MS spectra (ESI+) were performed on a Waters Alliance 2695, coupled with Waters MS ZQ and UV 2489 detectors. Detection was performed at $\lambda = 214$ nm, sample cone 20 V. LC-MS-MS (ESI+) were performed on a Waters Alliance 2695, coupled with Waters MS Quattro micro and UV 2488 detectors. Detection was performed at 214 nm. Samples were analyzed using an analytical Chromolith Speed Rod RP-C18 185 Pm column (25 x 4.6 mm, 2.5 mm); solvent A, H₂O/HCOOH 0.1%; solvent B, CH₃CN/HCOOH 0.1%; gradient, 0% solvent B to 100% solvent B in solvent A in 2.5 min; flow rate, 3.0 mL/min. RP-preparative HPLC purification was performed on a Waters HPLC 4000 instrument, equipped with a UV detector 486 and a C18 reversed-phase column. Standard conditions were eluent system A (water/0.1% TFA), system B (acetonitrile/0.1% TFA). The crude sample was dissolved in dimethylsulfoxide (~100 mg in 3 mL). A flow rate of 50 mL/min and a gradient of (0–35)% B over 30 min were used, detection $\lambda = 214$ nm.

General procedure for N6-acylation of adenine

To a stirred solution of Boc-Tyr-OH or Cbz-Tyr-OH (1.48 mmol) in 5 mL of DMF, 1036 μL of polyphosphonic anhydride solution at 50% in DMF (1.62 mmol, 1.1 eq) and then 400 mg of adenine (2.96 mmol, 2 eq) were added. The suspension was stirred at 50°C for one night. After evaporation under vacuum, 50 mL of ethyl acetate were added and then washed with water (25 mL) and then by an aqueous saturated NaHCO₃ solution (2 x 25 mL). The organic layer was dried over Na₂SO₄, filtered, and the solvent was concentrated under vacuum to offer compound **6** as a white powder.

N6-(N-Boc-tyrosyl)adenine **6a** : White solid (196 mg, yield 33%); ¹H NMR (DMSO d_6 , 300 MHz): δ ppm 1.81 (s, 9H), 3.47 (dd, 1H, $J = 14.3$ Hz, 8.4 Hz), 3.68 (dd, 1H, $J = 14.3$ Hz, 5.5 Hz), 5.20 (m, 1H), 7.20 (d, 2H, $J = 8.5$ Hz), 7.63 (d, 2H, $J = 8.5$ Hz), 9.05 (s, 1H), 9.12 (s, 1H); HPLC, $R_T = 1.09$ min; MS (ESI+): m/z calc for C₁₉H₂₃N₆O₄ [M+H]⁺ 399.2, found 399.3

N6-(N-Cbz-tyrosyl)adenine **6b** : White solid (290 mg, yield 45%); ¹H NMR (DMSO-*d*₆, 300 MHz): δ ppm 2.74 (d, 1H, *J* = 13.0 Hz), 3.03 (d, 1H, *J* = 13.0 Hz), 4.65 (bs, 1H), 4.97 (s, 2H), 6.66 (d, 2H, *J* = 7.0 Hz), 7.10-7.30 (m, 10H), 8.44 (s, 1H), 8.66 (s, 1H), 9.25 (bs, 1H); ¹³C NMR (DMSO-*d*₆, 100 MHz): δ ppm 36.2, 57.0, 65.4, 114.9, 121.0, 126.8, 127.5, 127.6, 127.8, 128.3, 130.3, 136.8, 145.8, 151.2, 152.4, 155.9, 156.0, 172.7; HPLC, *R*_T = 1.19 min; MS (ESI+): *m/z* calc for C₂₂H₂₁N₆O₄ [M+H]⁺ 433.2, found 433.0

Synthesis of compound 8a+9a

To compound **6a** (0.13 mmol) were added 10 mL of a mixture of TFA/DCM 50/50 v/v. The solution was stirred at room temperature for 1 hour. The solution was concentrated to dryness and co-evaporated several times with acetonitrile to completely remove TFA. The residue was dissolved in DCM (10 mL) and washed with a saturated NaHCO₃ solution (2 x 10 mL) to offer compound **8a** and compound **9a** as a mixture. Compound **8a** : HPLC, *T*_R = 0.70 min; MS (ESI+): *m/z* calc for C₁₄H₁₂N₅O₂ [M+H]⁺ 282.1, found 282.3; compound **9a** : HPLC, *R*_T = 0.66 min; MS (ESI+): *m/z* calc for C₁₄H₁₅N₆O₂ [M+H]⁺ 299.1, found 299.3

Synthesis of compound 4

- *Peptide coupling conditions*: Boc-Tyr(Bn)OH.Ts or peptide **15** or **16** (0.75 mmol) was dissolved in DCM (10 mL) and basified with triethylamine. HBTU (313 mg, 1.1 eq), Boc-Val-OH or Boc-Leu-OH (1.1 eq) were added. The solution was stirred at room temperature for 2 h and then evaporated to dryness. The residue was dissolved in 25 mL of ethyl acetate and washed with a saturated NaHCO₃ solution (2 x 25 mL) and then with brine (1 x 25 mL). The organic layer was dried over Na₂SO₄, filtered, and the solvent was concentrated under vacuum.

Boc deprotection: removal of the Boc protecting group was carried out using a solution of TFA/DCM 1/1 v/v at room temperature for 1h. The solution was then evaporated to dryness under vacuum and the residue was used in the next step without purification.

- *Benzyl deprotection*: purified benzyl-protected derivative **17** was dissolved in ethanol (20 mL). Two drops of 37% hydrochloric acid, followed by 10% activated palladium on carbon were added. Hydrogen gas was bubbled into the solution at 25°C until completion of the reaction (HPLC monitoring). Then, the solution was filtered on a pad of celite. Deprotected derivative **4** was obtained after evaporation to dryness under vacuum, as a white powder (22% yield). HPLC, *R*_T = 1.57 min; MS (ESI+) *m/z*: calcd for C₃₁H₅₁N₄O₈ [M+H]⁺ 607.4, found 607.3.

Synthesis of tert-butyl 2-bromoethylcarbamate 18

To a stirred solution of 2-bromo-ethylamine hydrobromide (4 g, 20 mmol) in 40 mL of chloroform at 0°C, di-*tert*-butyl dicarbonate (4.29 g, 20 mmol, 1 eq) and then triethylamine (4.5 mL, 32 mmol, 1.7 eq) were added. The reaction was allowed to stir at room temperature for 24 h. Aminomethyl PS resin was then added (2 g, loading of 1.1 mmol.g⁻¹, 0.1 eq) and the suspension was gently stirred for 1 h. After filtration, the solution was washed with brine (2 x 40 mL), dried over Na₂SO₄. The residue was purified by chromatography on silica, eluted with nHex/AcOEt 4/1 v/v to offer a colourless liquid (4.33 g, 99% yield). Spectral characteristics were in agreement with the published data.^[24]

Synthesis of 9-(2-N-Boc-aminoethyl)-9H-purin-6-ylamine 19

A suspension of adenine **3** (2 g, 14.8 mmol), *tert*-butyl 2-bromoethylcarbamate **18** (3.33 g, 14.8 mmol, 1 eq), potassium carbonate (4.1 g, 29.7 mmol, 2 eq) in 60 mL of DMF was stirred at room temperature for 3 days. The suspension was filtered and evaporated to dryness under reduced pressure. The residue was triturated with water (5 mL), filtered and dry at 40°C under vacuum to offer compound **19** as a

white powder (1.91 g, 46% yield). Spectral characteristics were in agreement with the published data.^[24]

Synthesis of Boc-Orn(Fmoc)-OH

Boc-Orn-OH (500 mg, 2.15 mmol) was dissolved in 16 mL of a mixture of THF/H₂O 1/1 v/v. Sodium bicarbonate (360 mg, 4.28 mmol, 2eq) was then added, followed by Fmoc-OSu (730 mg, 2.15 mmol, 1 eq). The reaction was stirred at room temperature for 2 hours. THF was removed under vacuum and the aqueous layer was washed with Et₂O (2 x 20 mL). The aqueous layer was then acidified by 1N HCl and then extracted by EtOAc (2 x 20 mL). The organic layers were dried over Na₂SO₄, filtered and the solvent was concentrated under vacuum to offer compound as a white powder (795 mg, 81% yield). ¹H NMR (CDCl₃, 300 MHz): δ ppm 1.23 (bs, 1H), 1.42 (s, 9H), 1.58-1.84 (m, 4H), 3.00-3.19 (m, 2H), 4.16-4.22 (m, 4H), 5.22 (bs, 1H), 6.13 (bs, 1H), 7.27 (t, 2H, *J* = 6.7 Hz), 7.36 (t, 2H, *J* = 6.7 Hz), 7.55 (d, 2H, *J* = 6.7 Hz), 7.72 (d, 2H, *J* = 6.7 Hz); ¹³C NMR (CDCl₃, 75 MHz): δ ppm 25.5, 28.4, 29.8, 40.5, 47.3, 53.1, 66.8, 80.4, 120.1, 125.2, 127.2, 127.8, 141.4, 144.0, 155.9, 156.9, 176.0; HPLC, *R*_T = 1.85 min; MS (ESI+): *m/z* calcd for C₂₅H₃₁N₂O₆ [M+H]⁺ 455.2, found 477.2 [M+Na]⁺, 355.2 (M+H-Boc)⁺.

General procedure for the synthesis of compounds 20

A solution of compound **19** (200 mg, 0.72 mmol, 1 eq) in 10 mL of DCM/TFA 1/1 v/v was stirred at room temperature for 1 h. After evaporation to dryness under reduce pressure, the residue was dissolved in DMF (5 mL), basified with triethylamine. 1-Hydroxybenzotriazole monohydrate (121 mg, 0.79 mmol, 1.1 eq), EDCI (151 mg, 0.79 mmol, 1.1 eq) and Boc-Tyr(Bn)-OH or Boc-Arg(NO₂)-OH or Boc-Orn(Cbz)-OH or Boc-Orn(Fmoc)-OH (0.72 mmol, 1 eq) were added. The solution was stirred at room temperature for 2 h and then evaporated to dryness. For compound **20b**, the crude was purified by preparative HPLC. For compounds **20a**, **20c** and **20d**, the residue was dissolved in 25 mL of ethyl acetate and washed with a saturated NaHCO₃ solution (2 x 25 mL) and then with brine (1 x 25 mL). The organic layer was dried over Na₂SO₄, filtered, and the solvent was concentrated under vacuum to offer compound **20c** and **20d**.

Compound **20a** : white powder (153 mg, 40 % yield). ¹H NMR (CDCl₃, 300 MHz): δ ppm 1.38 (s, 9H), 2.90 (m, 2H), 3.48-3.65 (m, 2H), 4.27 (m, 1H), 4.97 (s, 2H), 5.18 (bs, 1H), 6.75 (bs, 1H), 6.81 (d, 2H, *J* = 8.6 Hz), 7.03 (d, 2H, *J* = 8.6 Hz), 7.25-7.38 (m, 5H), 7.64 (s, 1H), 8.06 (s, 1H); HPLC, *R*_T = 1.29 min; MS (ESI+): *m/z* calcd for C₂₅H₃₅N₅O₅ [M+H]⁺ 527.2, found 527.1.

Compound **20b** : white powder (165 mg, 48 % yield). ¹H NMR (DMSO-*d*₆, 300 MHz): δ ppm 1.36 (s, 9H), 3.09 (m, 4H), 3.49-3.76 (m, 5H), 4.26 (t, 2H, *J* = 5.7 Hz), 6.86 (d, 1H, *J* = 8.5 Hz), 7.92 (bs, 2H), 8.05 (t, 1H, *J* = 5.6 Hz), 8.28 (s, 1H), 8.36 (s, 1H), 8.62 (bs, 2H); ¹³C NMR (DMSO-*d*₆, 100 MHz): δ ppm 28.2, 28.9, 31.3, 38.4, 43.2, 51.9, 54.0, 78.1, 118.2, 143.1, 147.2, 149.0, 152.0, 155.3, 159.2, 172.4; HPLC, *R*_T = 0.82 min; MS (ESI+): *m/z* calcd for C₁₈H₃₀N₁₁O₅ [M+H]⁺ 480.2, found 480.1.

Compound **20c** : white powder (352 mg, 93 % yield). ¹H NMR (DMSO-*d*₆, 400 MHz): δ ppm 1.30-1.48 (m, 12H), 2.93 (m, 2H), 3.47 (m, 2H), 3.77 (m, 2H), 4.19 (t, 2H, *J* = 5.5 Hz), 5.00 (s, 2H), 6.80 (d, 1H, *J* = 7.9 Hz), 7.15 (bs, 2H), 7.19 (t, 1H, *J* = 5.9 Hz), 7.29-7.35 (m, 5H), 8.01 (s, 1H), 8.04 (t, 1H, *J* = 6.1 Hz), 8.13 (s, 1H); ¹³C NMR (DMSO-*d*₆, 75 MHz): δ ppm 26.5, 28.7, 29.6, 39.0, 40.7, 43.1, 54.6, 65.6, 78.5, 119.2, 128.2, 128.8, 137.7, 141.4, 144.0, 150.1, 152.8, 155.8, 156.4, 156.5, 172.9; HPLC, *R*_T = 1.29 min; MS (ESI+): *m/z* calcd for C₂₅H₃₅N₈O₅ [M+H]⁺ 527.2, found 527.1.

Compound **20d** : white powder (302 mg, 75 % yield). ¹H NMR (DMSO-*d*₆, 300 MHz): δ ppm 1.31 (m, 2H), 1.36 (s, 9H), 1.40-1.57 (m, 2H), 2.91 (m, 2H), 3.50 (m, 2H), 3.72 (m, 1H), 4.26 (m, 5H), 6.83 (d, 1H,

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$J = 7.7$ Hz), 7.27 (t, 1H, $J = 5.6$ Hz), 7.32 (t, 2H, $J = 7.4$ Hz), 7.41 (t, 2H, $J = 7.4$ Hz), 7.67 (d, 2H, $J = 7.4$ Hz), 7.88 (d, 2H, $J = 7.4$ Hz), 8.01 (t, 1H, $J = 5.4$ Hz), 8.35 (s, 1H), 8.44 (s, 1H), 8.97 (bs, 2H); ^{13}C NMR (DMSO- d_6 , 75 MHz): δ ppm 26.1, 28.2, 29.0, 38.4, 39.9, 43.4, 46.8, 54.2, 65.2, 78.0, 118.0, 120.1, 125.1, 127.0, 127.6, 140.7, 143.8, 143.9, 145.7, 148.9, 150.9, 155.4, 156.1, 172.6; HPLC, $R_T = 1.54$ min; MS (ESI+): m/z calcd for $\text{C}_{32}\text{H}_{39}\text{N}_8\text{O}_5$ 615.3 [M+H] $^+$, found 615.2.

Synthesis of compounds 22

- *Boc deprotection*: removal of the Boc protecting group was carried out using a solution of TFA/DCM 1/1 v/v at room temperature for 1h. The solution was then evaporated to dryness under vacuo and the residue was used in the next step without purification.

- *Peptide coupling conditions*: after deprotection, the residue was dissolved in DMF (5 mL), basified with triethylamine. 1-Hydroxybenzotriazole monohydrate (1.1 eq), EDCI (1.1 eq) and Boc-AA-OH (1 eq) were added. The solution was stirred at room temperature for 2 h and then evaporated to dryness. The residue was dissolved in 25 mL of ethyl acetate and washed with a saturated NaHCO_3 solution (2 x 25 mL) and then with brine (1 x 25 mL). The organic layer was dried over Na_2SO_4 , filtered, and the solvent was concentrated under vacuum.

- *Cbz deprotection*: the Cbz-protected derivative was dissolved in ethanol (20 mL) and 10% activated palladium on carbon was added. Hydrogen gas was bubbled into the solution at 25°C until completion of the reaction (HPLC monitoring). Then, the solution was filtered on a pad of celite. Deprotected derivative was obtained after evaporation to dryness under vacuum.

- *Fmoc deprotection*: removal of the Fmoc protecting group was carried out using a solution of diethylamine in DMF 1/9 v/v at room temperature for 1h. The solution was then evaporated to dryness under vacuum and the residue was used in the next step without purification.

- *Introduction of the succinic residue*: after Boc deprotection of compound **21a** in acidic conditions, the crude was dissolved in 10 mL of DCM. Then, were added triethylamine until alkaline pH (~9) and then 1.1 eq of succinic anhydride. The solution was stirred at room temperature until completion (HPLC monitoring). The solution was evaporated to dryness under vacuum. The crude was dissolved in DMSO and then purified by preparative HPLC.

- *Guanidinium formation*: to a solution of crude compound **22c** in 10 mL of DMF were added triethylamine until alkaline pH (~9) and then 3 eq. of pyrrolole-1-carboxamide. The solution was stirred at room temperature until completion (HPLC monitoring). The solution was evaporated to dryness under vacuum and the residue was washed with EtOAc and then with Et_2O . The crude was dissolved in DMSO (~2 mL) and then purified by preparative HPLC.

Compound **22a** (Suc-Leu-Leu-Val-Tyr- $\text{CH}_2\text{-CH}_2$ -Adenine). HPLC, $R_T = 1.54$ min; MS (ESI+) m/z : calcd for $\text{C}_{37}\text{H}_{55}\text{N}_{10}\text{O}_8$ [M+H] $^+$ 767.0, found 767.3

Compound **22b** (Boc-Val-Pro-Arg- $\text{CH}_2\text{-CH}_2$ -Adenine). HPLC, $R_T = 1.03$ min; MS (ESI+) m/z : calcd for $\text{C}_{28}\text{H}_{47}\text{N}_{12}\text{O}_5$ [M+H] $^+$ 631.4, found 631.3

SERS evaluation

The Raman spectra were recorded on a Senterra Bruker Optics instrument (Ettlingen, Germany) using a 532nm diode laser source and a 1200 lines/mm grating (resolution < 4 cm^{-1}). The Rayleigh filtering is achieved using Notch Filters. The laser power was generally 20 mW, the acquisition time was 20s (up to 10 accumulations). The spectra of the various samples were recorded in at least three independent

experiments. Several silver colloid batches were synthesised using the classical Creighton' method^[22] and analyzed as described in Soualmia et al.^[13] For optimal reproducibility of SERS experiments, the samples were inserted in glass tubes using the following order: 80 μL of colloid, 10 μL of salt and 10 μL of tested aqueous solution. The SERS spectra of these latter were immediately recorded.

Monitoring of the trypsin activity by HPLC

The reaction mixture (1.2 mL) contained 100 mM TRIS-HCl and 10 mM CaCl_2 , pH 8.0, 37°C, with **22b** or Boc-VPR-AMC varying from 37 to 150 μM . Reactions were initiated by the addition of trypsin (final concentration 0.2 μM). Aliquots of 100 μL were periodically removed from the reaction mixture and trypsin was inactivated by the addition of 2 μL of 2 N HCl to each aliquot. 10 μL of the mixture was injected and the quantity of Boc-VPR-OH released at various times was quantified by HPLC ($\lambda = 214$ nm).

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Nicolas Masurier,* Feryel Soualmia, Pierre Sanchez, Valérie Lefort, Mia Roué, Ludovic T. Maillard, Gilles Subra, Aline Percot and Chahrazade El Amri*

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Synthesis of peptide-adenine conjugates as a new tool for the protease activity monitoring

We report an efficient way to synthesize challenging peptide-adenine conjugates, as alternative to the aminomethylcoumarine (AMC)-serine proteases substrates.

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