SUPPLEMENTARY INFORMATION

Rational design of NT-PSMA heterobivalent probes for prostate cancer theranostics

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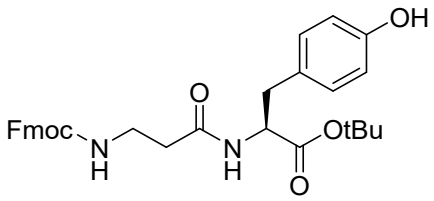
1. Chemistry

Solvents and reagents used both for the synthesis in solution and in solid phase (SPPS) were purchased from Merck. All *N*-Fmoc-protected amino acids were obtained from Fluorochem, whereas DOTA(tBu)$_3$-OH was obtained from TCI. A VWR Microplate Shaker was employed for SPPS. The appropriate syringes of different volumes for SPPS equipped with a polypropylene filter were purchased from Merck. **JMV 7489** was synthesized using a preliminary activated 2-Chloro-Trytil Chloride (2-CTC) resin with a loading of Fmoc-Glu(tBu) equal to 0.77 mmol/g, whereas the Fmoc-Glu(OMe)-βAla-βAla-Lys(Boc)-Lys(Boc)-Pro-OH fragment (**7**) was synthesized using the same resin with a loading of Fmoc-Pro equal to 0.68 mmol/g. The intermediates obtained from synthesis in batch were purified by column chromatography using silica gel (Merck 60, 230-400 mesh). Silica gel 60 F254 plates (Merck) were used for TLC. The target final compound was purified using a Gilson PLC 2250 preparative apparatus equipped with a C18 Deltapak column (100 mm x 40 mm, 15 μM, 100 Å), with a flow rate equal to 15 mL/min. A mixture of water- and ACN-containing 0.1% TFA was employed as the eluent, starting from 100:0 % to 0:100% of water and ACN, respectively, over 20 minutes. All the HPLC analysis were performed using a C18 Chromolith Flash 25 x 4.6 mm column, operating with a flow rate of 3 mL/min. Running HPLC was performed in gradient using water- and ACN-containing 0.1% of TFA as the solvents: starting from 100% of water (solvent A), the % of ACN (solvent B) was increased to 100% over 3 min or 10 min. Characterization of all compounds was performed by LC-MS, which consists of a Water Alliance 2629 HPLC coupled with a ZQ spectrometer fitted with an electrospray source operating in positive ionization mode (ESI$^+$). All data regarding the LC-MS system were already reported.[1] High-resolution mass spectra (HRMS) were performed by "Laboratoire de Mesures Physiques" of University of Montpellier (FR) as we previously reported.[1]

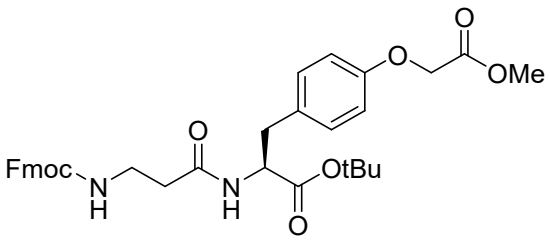
Synthesis of PMSA linker 5 in solution

Synthesis of Fmoc-βAla-Tyr-O*t*Bu 3



In a round bottom flask, Fmoc-βAla-OH (1.3 eq.) was dissolved in DMF (10mL/mmol) and HATU (1.3 eq.) and DIPEA (3 eq) were added. The resulting solution was maintained in stirring for 30 minutes at room temperature (rt). After this time, H-Tyr-O*t*Bu (1 eq.) was added and the solution was kept in vigorously stirring over night (on) at rt. Subsequently, DMF was removed *in vacuo* and the resulting residue was dissolved in EtOAc, washed with 1 M HCl (x 2), $NaHCO_3$ saturated solution (x 2), and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The desired product **3** was purified using a mixture of hexane/EtOAc 3:2 as the eluent. Yield = 72%. Consistency = pale orange powder. R_f = 0.36 in hexane/EtOAc 3:2. Retention Time (RT) in LC-MS system = 1.76 min. Exact mass determined for $C_{31}H_{34}N_2O_6$ = 530.2417 [M]; found = 531.3 $[M + H]^+$ and 553.2 $[M + Na]^+$. Fragment: 475.2 [M – *t*Bu].

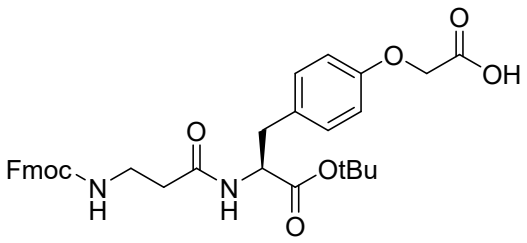
Synthesis of Fmoc-βAla-Tyr(O-CH_2-COOMe)-O*t*Bu 4



In a round bottom flask, intermediate **3** (1 eq.) was dissolved in ACN (5 mL/mmol) and K_2CO_3 (3 eq.) was added portion-wise at rt. The resulting suspension was maintained in vigorously stirring for 1h. After this time, methyl bromoacetate (1.2 eq.) was added dropwise over 10 min at 0°C, and the reaction was maintained in stirring on at rt. After this time, water was added and ACN was removed *in vacuo*. The organic phase was extracted with EtOAc (x 3), washed with brine (x 3), dried over Na_2SO_4, and concentrated *in vacuo*. The desired product was purified using a mixture of hexane/EtOAc 1:1 as the eluent. Yield = 53%. Consistency: pale orange powder. R_f = 0.28 in hexane/EtOAc 1:1. RT in LC-MS

system = 1.89 min. Exact mass determined for $C_{34}H_{38}N_2O_8$ = 602.2628 [M]; found = 603.3 $[M + H]^+$ and 625.3 $[M + Na]^+$. Fragment: 547.2 [M – *t*Bu].

Synthesis of Fmoc-βAla-Tyr(O-CH_2-COOH)-O*t*Bu (5)



In a round bottom flask, intermediate **4** (1 eq.) was dissolved in a mixture of *i*PrOH/H_2O 7:3, in which $CaCl_2$ (0.8 M) was dissolved at rt. A mild heating not over 40° C was provided until the total solubilization of $CaCl_2$. NaOH (1.2 eq.) was added over 10 min and the reaction was maintained in stirring at rt for 7 h. After this time, no presence of starting material was detected in TLC monitoring (hexane/EtOAc 2:3) and HPLC. Subsequently, the reaction was neutralized with 1 M AcOH, and solvents were removed *in vacuo*. The resulting solid residue was dissolved in MeOH and cold water was added dropwise, in order to precipitate the desired product, which was filtered and washed with cold water. The desired product **5** was used for the next step without further purification. Yield = 82%. Consistency: opalescent solid. R_f = 0.00 in hexane/EtOAc 1:1. RT in LC-MS system = 3.64 min. Exact mass determined for $C_{33}H_{36}N_2O_8$ = 588.2472 [M]; found = 589.3 $[M + H]^+$ and 612.5 $[M + Na]^+$. Fragment: 533.4 [M – *t*Bu].

Synthesis of NT arm (12)

Fmoc-Ile-Leu-O*t*Bu (6). The desired intermediate was synthesized following the procedure above-described for compound **3**. Fmoc-Ile-OH and H-Leu-O*t*Bu were used as the acid and amine, respectively. The title compound was purified by column chromatography using a mixture of hexane/EtOAc (7:3) as the eluent. Yield = 68%. Consistency = pale brown solid. RT in LC-MS system = 2.36 min. Exact mass determined for $C_{31}H_{42}N_2O_5$= 522.3094 [M]; found: 523.3 $(M + H)^+$; found: 467.2 (M – *t*Bu), 245.2 (M – *t*Bu – Fmoc).

H-Ile-Leu-O*t*Bu (7). In a round bottom flask, the intermediate **6** (1 eq.) was dissolved in DMF (8 mL/mmol) and piperidine (2 mL/mmol) was added dropwise at rt. The reaction was maintained in

stirring for 1h. After this time, all volatiles were removed *in vacuo* and the obtained amine was used for the next step without purification. Yield = not determined. Consistency = pale yellow oil. RT in LC-MS system = 1.26 min. Exact mass determined for $C_{16}H_{32}N_2O_3$= 300.4369 [M]; found: 301.2 $(M + H)^+$; fragment: 245.0 (M – *t*Bu).

Fmoc-Tyr(O*t*Bu)-Ile-Leu-O*t*Bu (8). The Intermediate **8** was obtained following the procedure above described for compound **3** using Fmoc-Tyr(O*t*Bu)-OH and H-Ile-Leu-O*t*Bu **7** as the acid and amine, respectively. The desired product was purified by column chromatography using a mixture of hexane/EtOAc (3:2) as the eluent. Yield = 75%. Consistency = pale orange solid. RT in LC-MS system = 2.60 min. Exact mass determined for $C_{44}H_{59}N_3O_7$ = 741.4353 [M]; found: 742.4 $(M + H)^+$, 764.4 $(M + Na)^+$; fragments: 686.4 (M – *t*Bu), 555.3 (M – Leu-O*t*Bu).

H-Tyr(O*t*Bu)-Ile-Leu-O*t*Bu (9). Intermediate **9** was obtained following the procedure above described for compound **7** starting from *N*-Fmoc-protected analogue **8**. The resulting residue was used for the next step without purification. Consistency = pale yellow oil. RT in LC-MS system = 3.10 min. Exact mass determined for $C_{29}H_{49}N_3O_5$ = 519.3672 [M]; found: 520.7 $(M + H)^+$, 542.7 $(M + Na)^+$; fragment: 464.7 (M - *t*Bu).

Fmoc-Glu(OMe)-βAla-βAla-Lys(Boc)-Lys(Boc)-Pro-OH (10).

The *N*-Fmoc-pentapeptide **10** was synthesized by SPPS starting from 1360 mg of Fmoc-Pro-2-CTC resin with a loading of 0.68 mmol/g (Scheme S1). A 20 mL syringe equipped with a teflon filter was used. The resin was swollen in a mixture of DCM/DMF (10 mL, 4:1) for 20 min. After this time, the solvents were sucked off by filtration *in vacuo*.

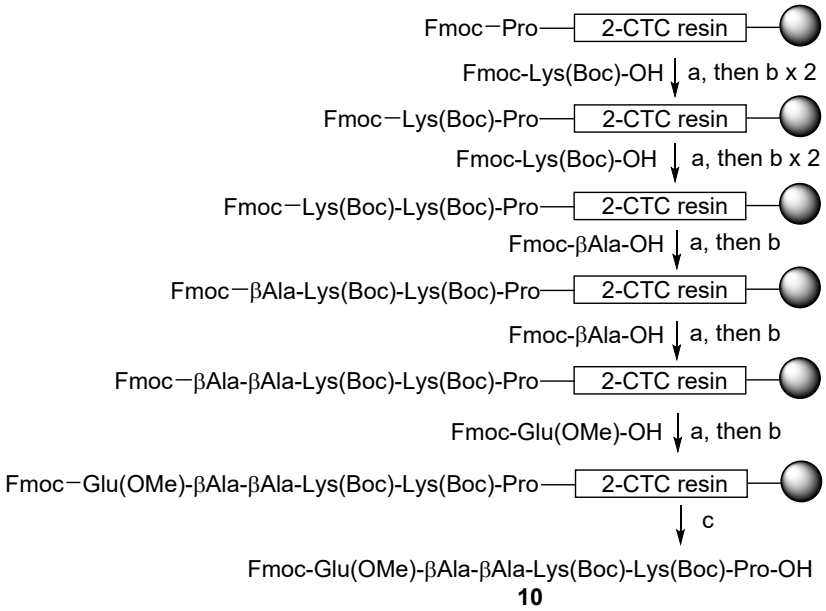
Reagents and conditions “a”: the Fmoc-deprotection was carried-out using a mixture of 20% piperidine in DMF (10 mL, 5 min x 3). The resin was subsequently washed with DCM (10 mL x 2).

Reagents and conditions “b”: DMF (10 mL), the appropriate Fmoc-amino acid (4 eq.), HATU (4 eq.), and DIPEA (5 eq.) were added to the syringe, which was shaken for 1h.

The incorporation of both Fmoc-Lys(Boc)-OH residues was performed in duplicate.

Reagents and conditions “c”: the peptide was cleaved from the resin using a mixture of DCM/TFE/AcOH 8:1:1 (12 mL) in shaking for 12h.

Then, the volatiles were removed *in vacuo* and the intermediate **10** was used for the next step without purification. Consistency: colorless oil. RT in LC-MS system = 3.61 min. Exact mass determined for $C_{54}H_{78}N_8O_{15}$ = 1078.5587 [M]; found: 1080.0 $(M + H)^+$, 1102.0 $(M + Na)^+$; fragment: 979.9 (M – Boc),



a: 20% piperidine in DMF (5 min x 3)
b: Fmoc-AA-OH (4 eq.), HATU (4 eq.), DIPEA (5 eq.) in DMF (1 h)
c: DCM/TFE/AcOH 8:1:1, 12h

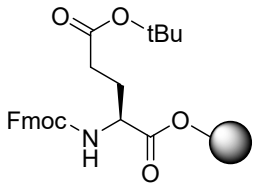
Scheme S1. Synthesis of intermediate 10.

Fmoc-Glu(OMe)-βAla-βAla-Lys(Boc)-Lys(Boc)-Pro-Tyr(O*t*Bu)-Ile-Leu-O*t*Bu (11). The intermediate **11** was obtained following the procedure above described for compound **6**. In this reaction, Fmoc-Glu(OMe)-βAla-Lys(Boc)-Lys(Boc)-Pro-OH **10** and H-Tyr(O*t*Bu)-Ile-Leu-O*t*Bu **9** were employed as the acid and amine, respectively. The title compound was purified using a mixture of DCM/MeOH in gradient from 1% to 10% of MeOH. Yield = 34%. Consistency = pale orange solid. RT in LC-MS system = 4.84 min. Exact mass determined for $C_{83}H_{125}N_{11}O_{19}$ = 1579.9153 [M]; found: 791.7 $[M/2 + 2H]^{2+}$.

Fmoc-Glu-βAla-βAla-Lys(Boc)-Lys(Boc)-Pro-Tyr(O*t*Bu)-Ile-Leu-O*t*Bu (12). he intermediate **11** was obtained following the procedure above described for compound **5**. In this reaction, Fmoc-Glu(OMe)-βAla-βAla-Lys(Boc)-Lys(Boc)-Pro-Tyr(O*t*Bu)-Ile-Leu-O*t*Bu (**11**) was used as the ester. Yield = 61%. Consistency = pale orange solid. RT in LC-MS system = 4.68 min. Exact mass determined for $C_{82}H_{123}N_{11}O_{19}$ = 1565.8997 [M]; found: 784.5 $[M/2 + 2H]^{2+}$.

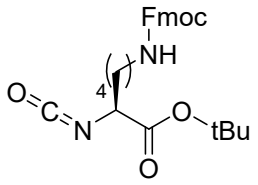
Synthesis of PSMA binding region (14)

Loading of Fmoc-Glu(OMe)-OH on 2-CTC.



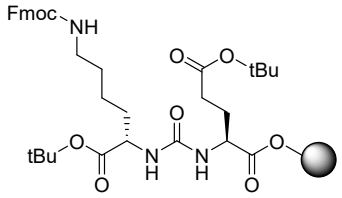
The 2-CTC resin (1.6 mmol/g) was dried on, then moved on a SPPS syringe, and treated with $SOCl_2$ (2 eq/resin loading) in DCM (3 mL/100 mg resin) for 2h at rt. Subsequently, the resin was washed with DCM (10 mL x 5) and dried *in vacuo* for 4h. The resin was then swollen with DCM (1 mL/100 mg resin) and Fmoc-Glu(OMe)-OH (1 eq.) dissolved in DCM (1 mL/100 mg resin) was added. DIPEA (1 eq.) was rapidly added and the syringe was shacked for 5 minutes. A further 1.5 eq. of DIPEA was then added, and the shaking was maintained on. Subsequently, MeOH (0.1 mL/100 mg resin) was added and the syringe was kept in shacking for further 30 min. The resin was dried and washed with DCM (10 mL x 3), DMF (10 mL x 2), and MeOH (10 mL x 2), and dried *in vacuo*. The loading was spectrophotometrically determined.

***tert*-Butyl (*S*)-6-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-isocyanatohexanoate (13)**



In a round bottom flask, H-Lys(Fmoc)-O*t*Bu (1 eq.) was dissolved in DCM (5 mL/mmol) and TEA (4 eq.) and triphosgene (0.6 eq.) were added in this order at 0°C. The reaction was maintained in stirring for 6h at rt. After this time, a few mL of water were added, the organic phase was separated, washed with brine (x 2), and dried over $MgSO_4$. The obtained intermediate **13** was used for the next step without further purification.

O*t*Bu-Lys(Fmoc)-urea- Glu(O*t*Bu)-2-CTC resin (14)



14

In a syringe for SPPS (20 mL), Fmoc-Glu(OMe)-2-CTC resin (0.77 mmol/g) was added. The resin was swollen in a mixture of DCM/DMF (10 mL, 4:1) for 20 min. The Fmoc protecting group was removed as above reported for intermediate **10** (procedure "a"). The resin was then suspended in DCM (10 mL/mmol) and compound **13** (4 eq.) was added. The reaction was kept in shacking for 1h. After this time, the resin was filtered, washed with DCM (10 mL x 3), and dried. A few beads of the resin were cleaved using a mixture of DCM/TFE/acetic acid (8:1:1) and LC-MS analysis was performed. Exact mass determined for $C_{35}H_{47}N_3O_9$: 653.33; found 654.4 [M + H], 676.5 [M + Na]; fragments: 597.4 [M – *t*Bu], 541.3 [M – *t*Bu – *t*Bu], 320.3 [M- *t*Bu – *t*Bu- Fmoc]. The new resin loading was found to be: 0.37 mmol/g (spectrophotometrically determined).

Synthesis of the target compound JMV 7489

Starting from intermediate **14** (200 mg, 0.37 mmol/g), the target compound **JMV 7489** was synthesized by SPPS in a syringe of 10 mL (Scheme S2). The resin was swollen in a mixture of DCM/DMF (5 mL, 4:1) for 20 min. After this time, the solvents were sucked off by filtration *in vacuo*.

Reagents and conditions "a": the Fmoc-deprotection was carried-out using a mixture of 20% piperidine in DMF (5 mL, 5 min x 3). The resin was washed with DCM (10 mL x 2).

Reagents and conditions "b": DMF (5 mL), the appropriate Fmoc-amino acid (4 eq.), HATU (4 eq.), and DIPEA (5 eq.) were added to the syringe, which was shaken for 1h.

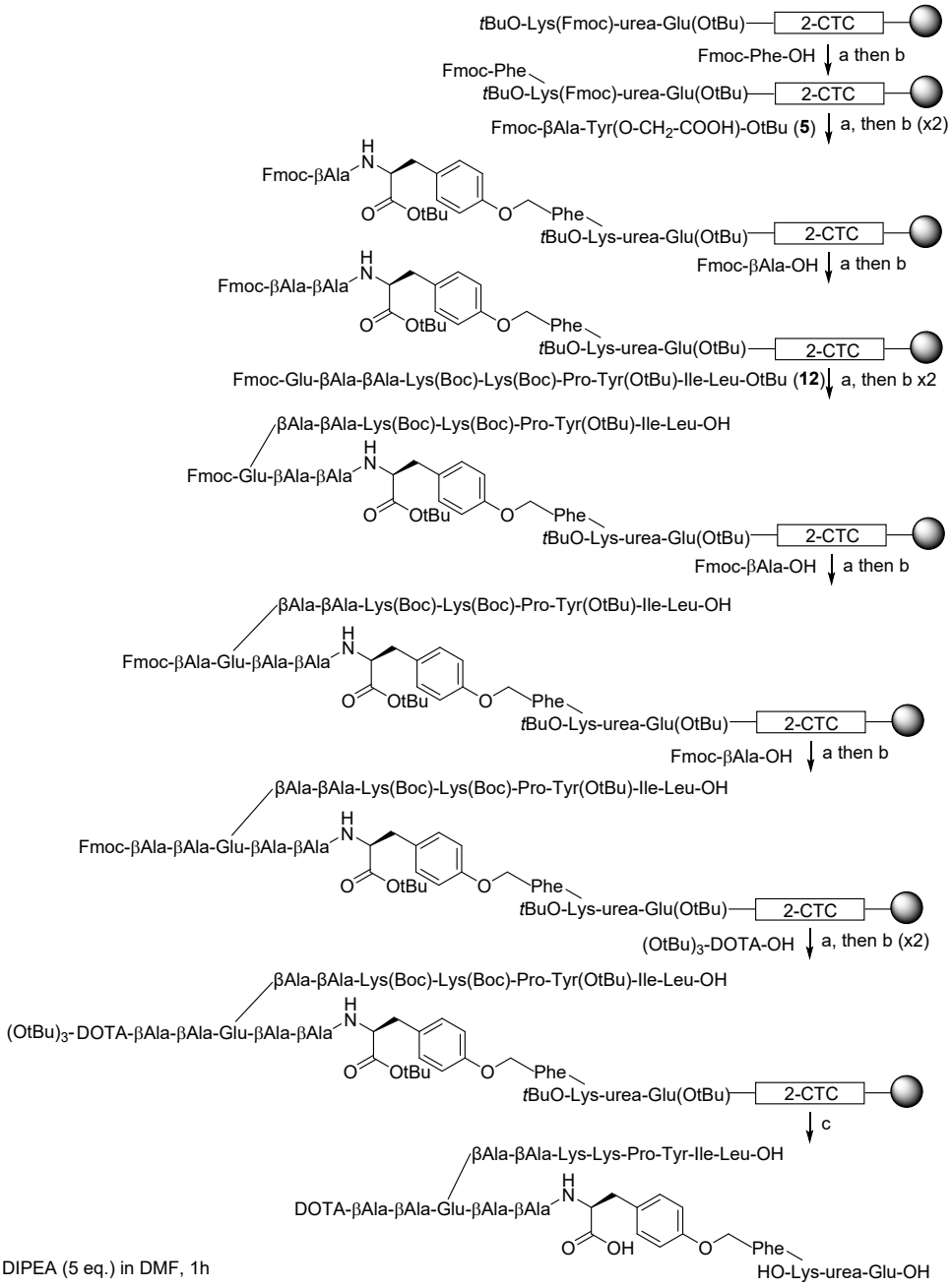
The incorporation of intermediates **5**, **12**, and DOTA-(O*t*Bu)$_3$-OH was performed in duplicate.

Reagents and conditions "c": the peptide was cleaved from the resin using a mixture of TFA/TIS/H_2O (95/2.5/2.5, 6 mL) in shaking for 7h.

Then, the volatiles were removed *in vacuo* and the desired compound was purified by preparative HPLC.

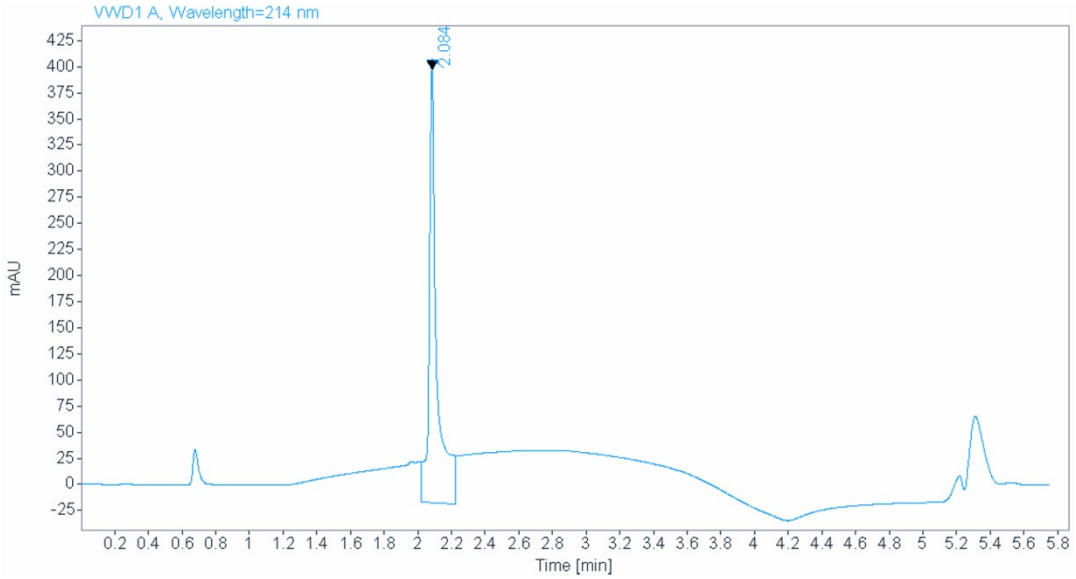
Consistency: white solid. Yield: 4%. RT in HPLC system = 2.08 over 3 min; 3.66 over 10 min. RT in LC-MS system = 1.98 min. Exact mass determined for $C_{109}H_{166}N_{24}O_{35}$: 2371.1947 [M]; found: 1187.3

[M/2], 791.8 [M/3], 594.2 [M/4]. HR-MS = determined: 1187.1067 [M/2], 791.7404 [M/3], and 594.0573 [M/4]; found: 1187.1072 [M/2], 791.7415 [M/3], and 594.0580 [M/4].



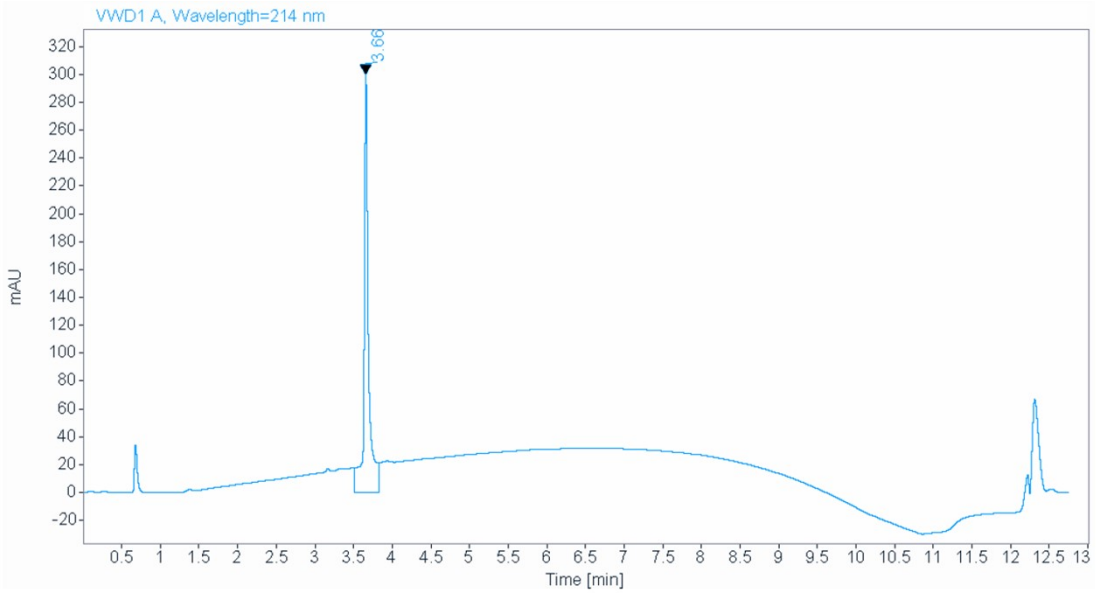
a: 20% piperidine in DMF, 5 min x 3
b: Fmoc-AA-OH (4 eq.), HATU (4 eq.), DIPEA (5 eq.) in DMF, 1h
c: TFA/TIS/H_2O (95/2.5/2.5), 7h

Scheme S2. Synthesis of the target hybrid probe JMV 7489.



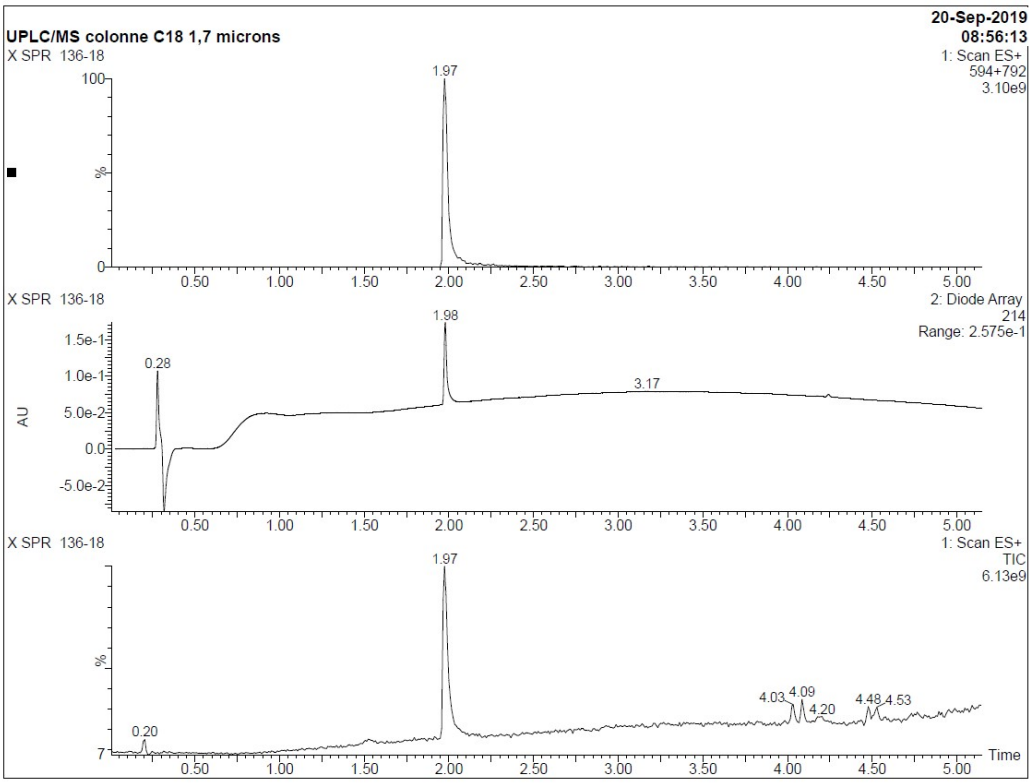
Signal:	VWD1 A, Wavelength=214 nm		
RT [min]	Area	Height	Peak Area Percent
2.084	1305.15088	414.41245	100.00

Chart S1. HPLC profile of JMV 7489 over 3 min.



Signal:	VWD1 A, Wavelength=214 nm		
RT [min]	Area	Height	Peak Area Percent
3.660	1177.22974	299.69958	100.00

Chart S2. HPLC profile of JMV 7489 over 10 min.



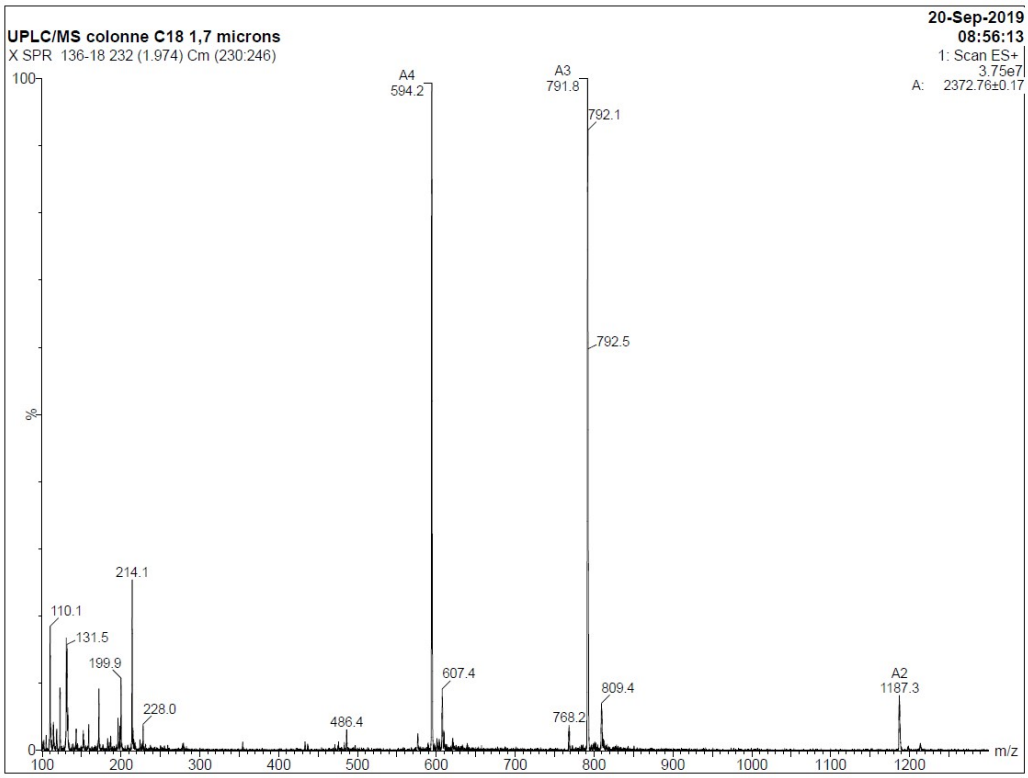


Chart S3. LC-MS profile of JMV 7489.

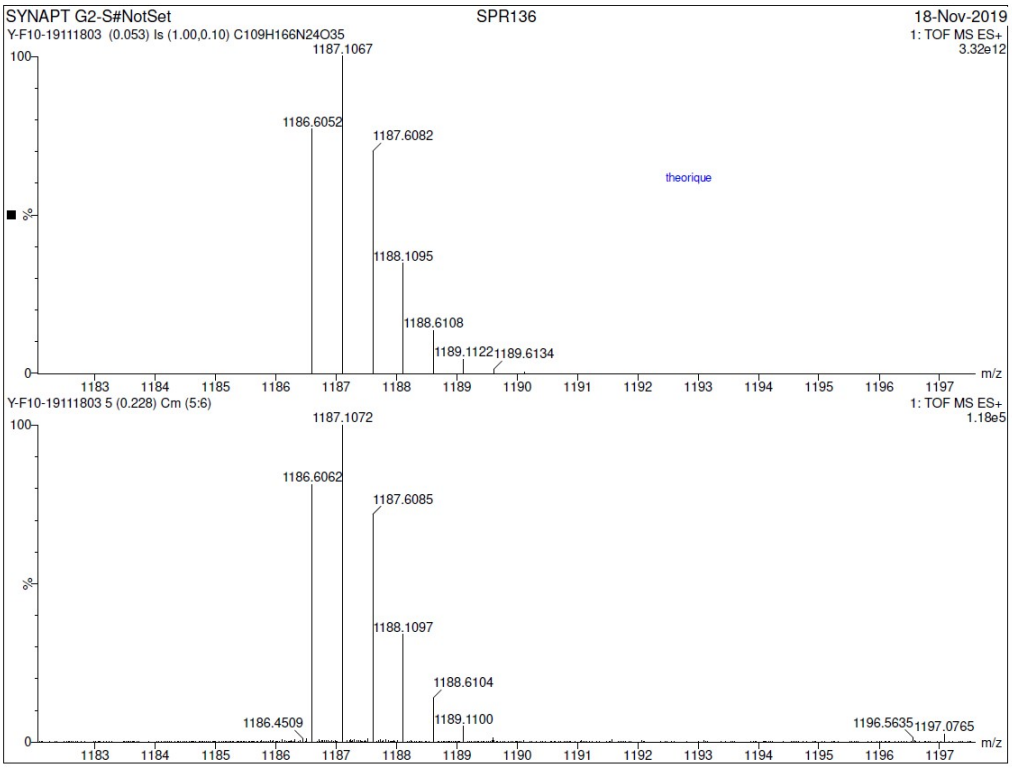


Chart S4. HRMS profile of JMV 7489 with (M+2H)/2 (M = 2371.1947, (M+2H)/2 = 1187.1067).

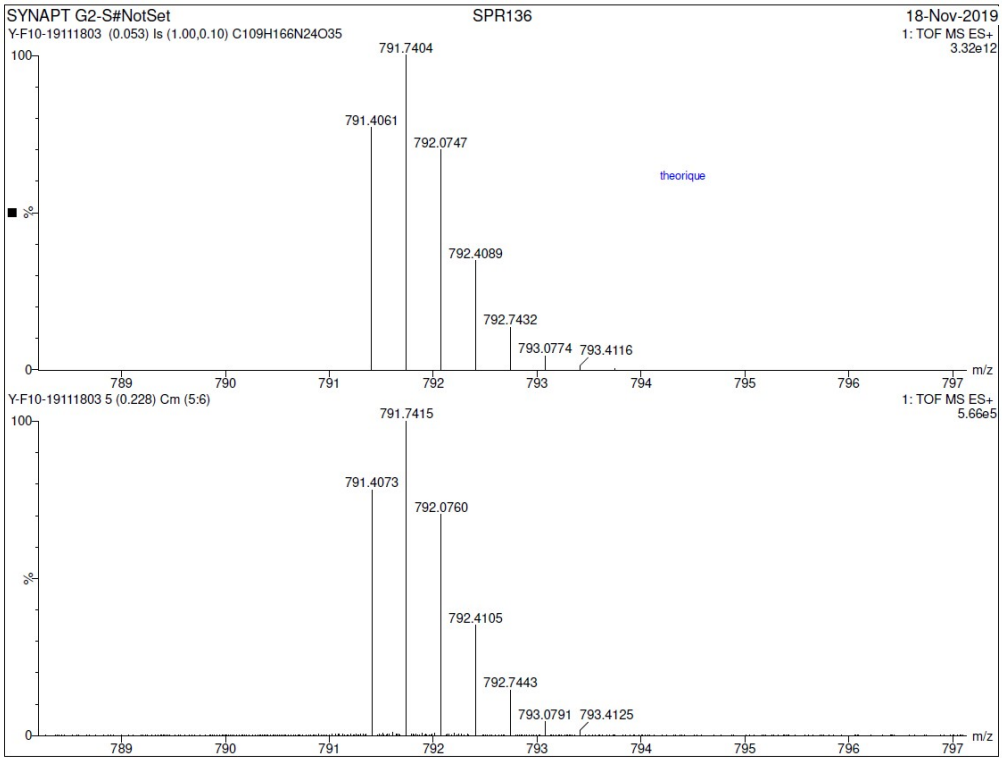


Chart S5. HRMS profile of JMV 7489 with (M+3H)/3 (M = 2371.1947, (M+3H)/3 = 791.7404.

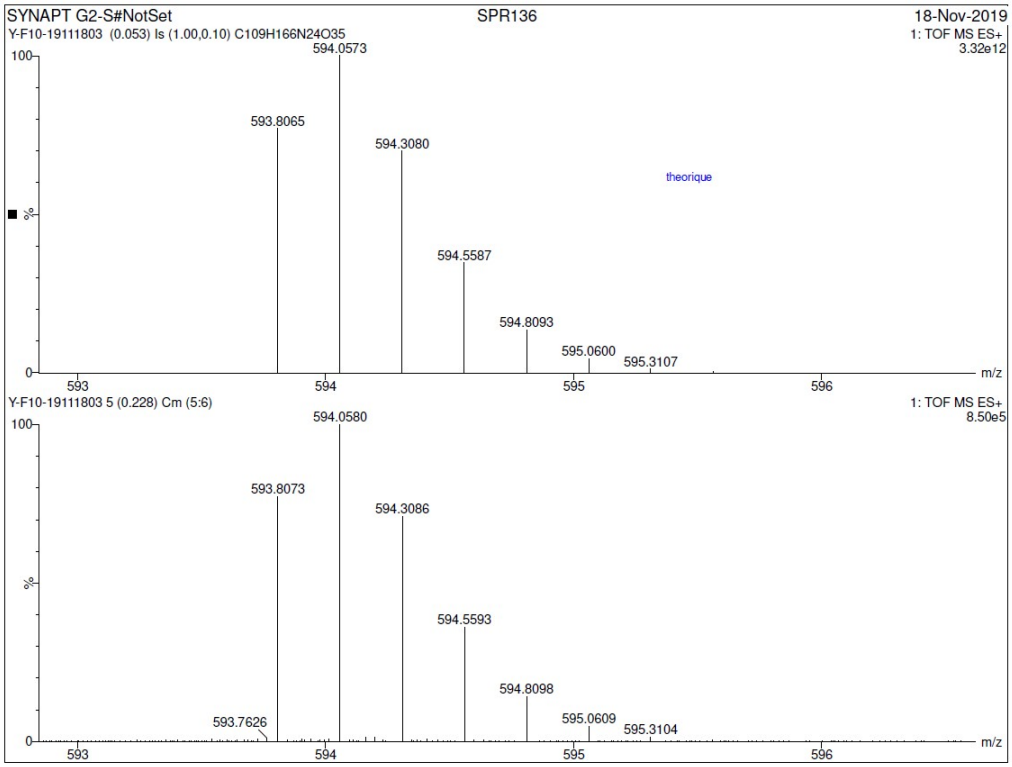
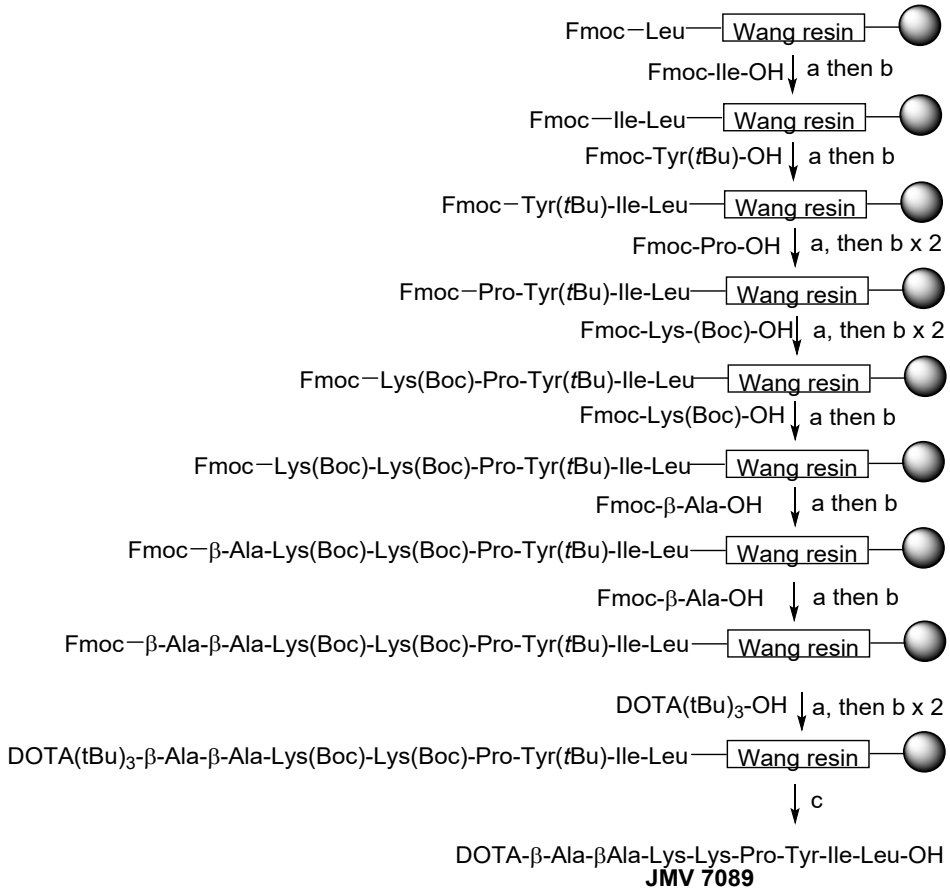


Chart S6. HRMS profile of JMV 7489 with (M+4H)/4 (M = 2371.1947, (M+4H)/4 = 594.0573).

Synthesis of JMV 7089

JMV 7089 was synthesized in SPPS starting from Fmoc-Leu-Wang resin (loading 0.8 mml/g) in a scale of 0.15 mmol. Initially, the resin (194 mg) was swollen in a 5 mL plastic syringe equipped with a Teflon filter using DCM for 20 min. Fmoc-deprotection, coupling reactions, and final cleavage were performed as already reported above for the synthesis of **JMV 7489**.

Then, volatiles were removed *in vacuo* and the desired compound was purified by preparative HPLC. Consistency: white solid. Yield: 27%. RT in HPLC system = 2.95 over 10 min. RT in LC-MS system = 0.96 min. Exact mass determined for $C_{60}H_{100}N_{14}O_{17}$: 1288.7391 [M], 645.3695 [(M+2)/2], 430.5797 [(M+3]/3], 323.1847 [(M+4)/4]; found: 645.9 [(M+2)/2], 430.7 [(M+3]/3], 323.4 [(M+4)/4]. HR-MS = determined: 1289.7463; found: 1289.7464.



a: 20% piperidine in DMF, 5 min x 3
b: Fmoc-AA-OH (4 eq.), HATU (4 eq.), DIPEA (5 eq.) in DMF, 1h
c: TFA/TIS/H_2O (95/2.5/2.5), 7h

Scheme S3. Synthesis of **JMV 7089**.

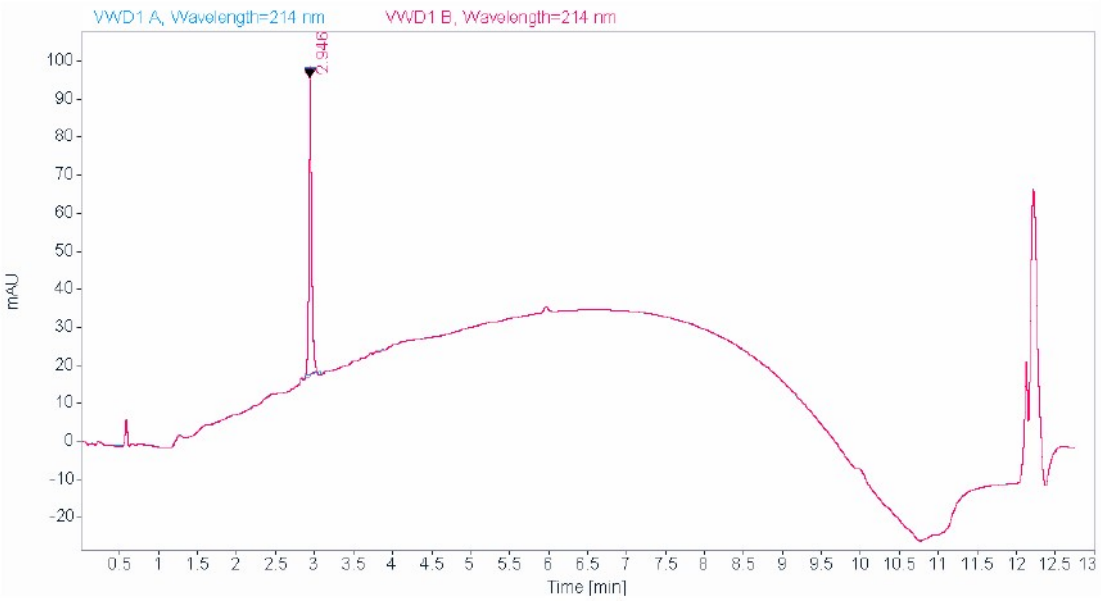
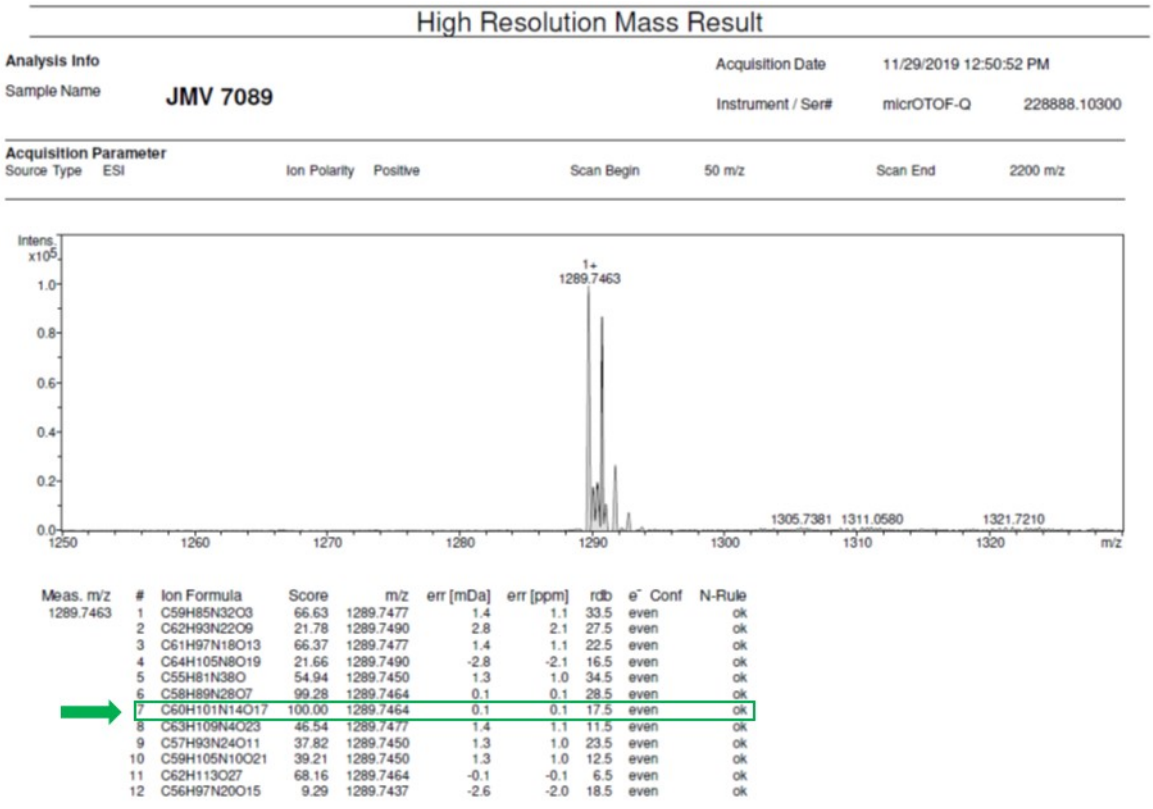
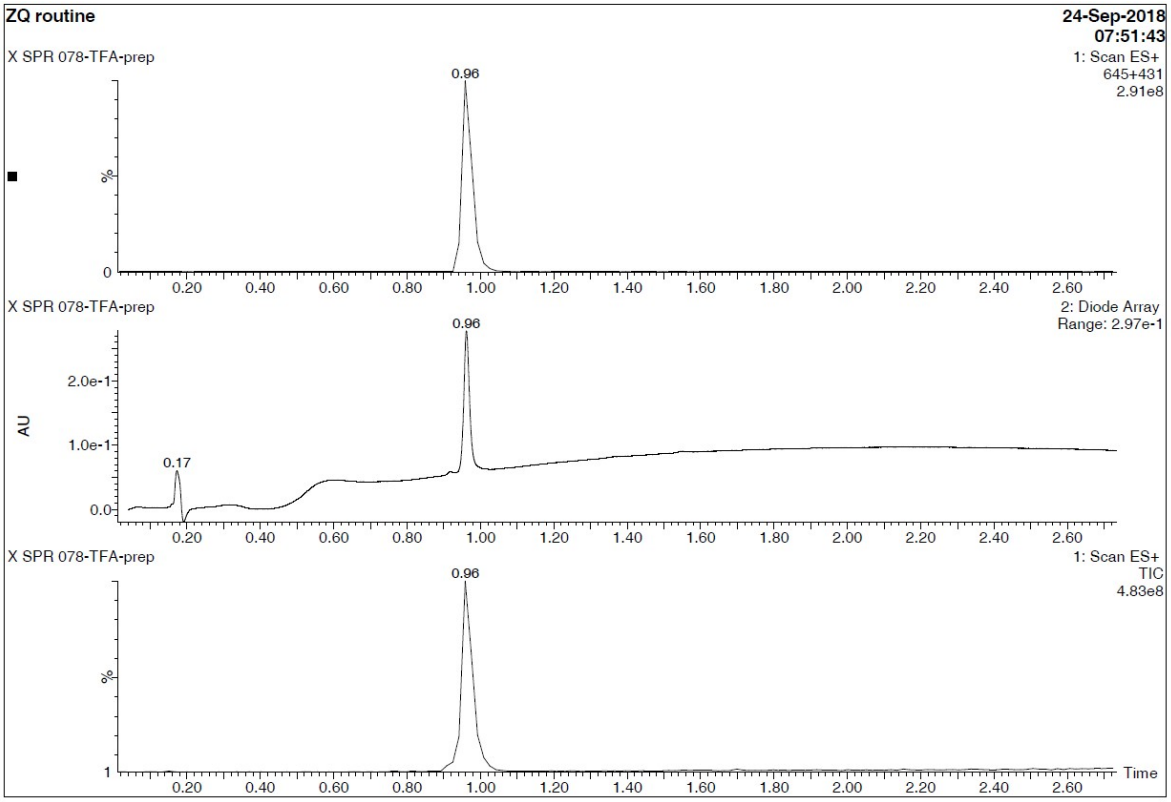


Chart S7. HPLC profile of JMV 7089.



Meas. m/z	#	Ion Formula	Score	m/z	err [mDa]	err [ppm]	rdb	e⁻ Conf	N-Rule
1289.7463	1	C59H85N32O3	66.63	1289.7477	1.4	1.1	33.5	even	ok
	2	C62H93N22O9	21.78	1289.7490	2.8	2.1	27.5	even	ok
	3	C61H97N18O13	66.37	1289.7477	1.4	1.1	22.5	even	ok
	4	C64H105N8O19	21.66	1289.7490	-2.8	-2.1	16.5	even	ok
	5	C55H81N38O	54.94	1289.7450	1.3	1.0	34.5	even	ok
	6	C58H89N28O7	99.28	1289.7464	0.1	0.1	28.5	even	ok
	7	C60H101N14O17	100.00	1289.7464	0.1	0.1	17.5	even	ok
	8	C63H109N4O23	46.54	1289.7477	1.4	1.1	11.5	even	ok
	9	C57H93N24O11	37.82	1289.7450	1.3	1.0	23.5	even	ok
	10	C59H105N10O21	39.21	1289.7450	1.3	1.0	12.5	even	ok
	11	C62H113O27	68.16	1289.7464	-0.1	-0.1	6.5	even	ok
	12	C56H97N20O15	9.29	1289.7437	-2.6	-2.0	18.5	even	ok

Chart S8. HR-MS profile of JMV 7089.



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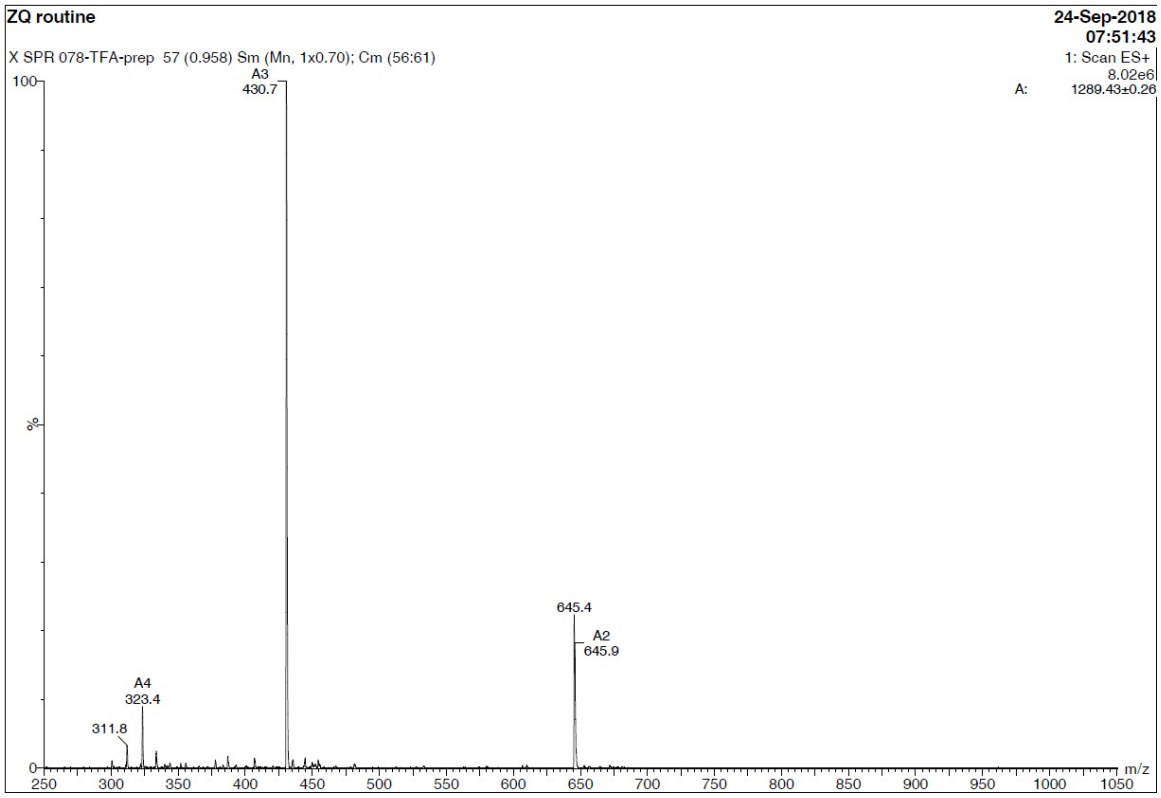

Chart S9. LC-MS profile of JMV 7089.

2. Biological evaluation

Radiolabeling

Radiolabeling was achieved using the FastLab automate cassette system (GE Healthcare). 1.1 mL of $^{68}GaCl_3$ in HCl 0.1 M (Galli Eo, IRE-EliT, Belgium) was incubated, without further purification, with 50 µg of the peptide (**JMV 7089** or **JMV 7489**) in acetate buffer (0.1M, pH 4.6, final volume 2 mL) and heated using a microwave at 90 °C for 5 min. The crude peptide was then purified by a Sep-Pak Light C_{18} cartridge (WAT023501) using 0.5 mL of ethanol and then formulated in PBS to obtain a final volume of 3 mL. Radiochemical purity was monitored with UV-radio HPLC as previously described.[1] and was > 95% for both [^{68}Ga]Ga-**JMV 7089** (Chart S10) and [^{68}Ga]Ga-**JMV 7489** (Chart S11).

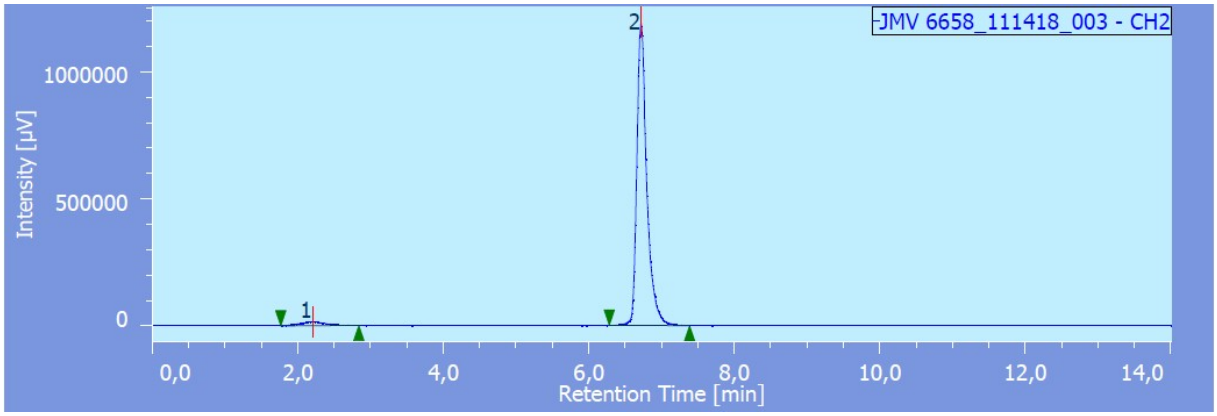

Chart S10. Radio-HPLC chromatogram of [^{68}Ga]Ga-JMV 7089.

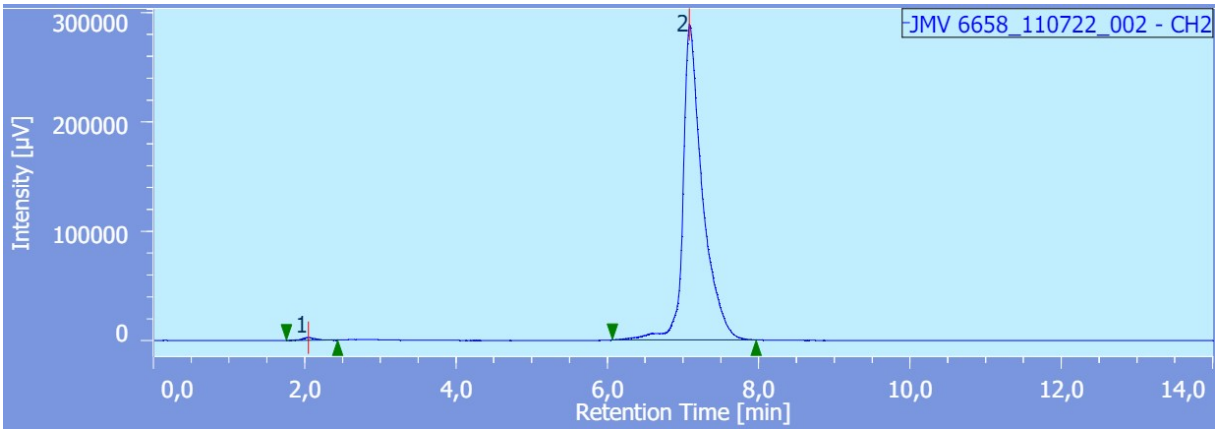

Chart S11. Radio-HPLC chromatogram of [^{68}Ga]Ga-JMV 7489.

Hydrophilicity

The hydrophilicity was assessed by the PBS–octanol distribution coefficient method. Briefly, 3.7 MBq of the radiolabelled peptide was added to a final volume of 500µL PBS and 500µL octanol. Microtubes were then vortexed and centrifuged (3 min, 4000 rpm) and after equilibrium, 100µL of each phase were carefully withdrawn and counted in an automatic gamma-counter.

Cell lines

HT29 (colon adenocarcinoma) and PC3-pip (bone metastases from grade IV prostate cancer) were grown at 37°C and 5% CO2 in RPMI supplemented with 400UI/mL penicillin, 400 µg/mL streptomycin, 10% FBS and 1% Glutamax.

Saturation binding assays

The affinity of [^{68}Ga]Ga-JMV 7489 and [^{68}Ga]Ga-JMV 7089 were studied on cells seeded at a density of 2.10^{5}cells per well in 24-well plates (Corning, area of each well was 9.5 cm^2) and incubated overnight with complete medium. Well plates were first set on ice 30 min before the beginning of the experiment. The radiopharmaceutical was then added to the medium at concentrations of 0.1, 1, 10, 100, and 500 nM, and cells were incubated (in triplicate) for 2 h at 4 °C. Incubation was stopped by removing medium and washing cells twice with ice-cold PBS. Finally, cells were treated with NaOH (1 M), and the radioactivity was measured in a gamma counter. To assess for nonspecific affinity, excess of neurotensin (to block NTS_1 binding, final concentration 10 µM) or 2-PMPA (to block PSMA binding, final concentration 10 µM) was added to selected wells. Kd was determined using non-linear regression using GraphPad Prism v6.01 (GraphPad software Inc., USA)

3. References

1. R. Fanelli, A. Chastel, S. Previti, E. Hindie, D. Vimont, P. Zanotti-Fregonara, P. Fernandez, P. Garrigue, F. Lamare, R. Schollhammer, L. Balasse, B. Guillet, E. Rémond, C. Morgat and F. Cavelier, *Bioconjug. Chem.*, 2020, **31**, 2339-2349.