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1. Abbreviation

Aa	Amino acid
Aba	4-acetamido benzoic acid
AcOH	acetic acid
AMP	antimicrobial peptide
BiFC	bimolecular fluorescence complementation
B. subtilis	*Bacillus subtilis*
Boc	*tert*-Butyloxycarbonyl
BODIPY	borondipyrromethene
br	broad (signal) (NMR)
^{o}C	Celsius
c	concentration
Cbz	benzyloxylcarbonyl
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
δ	chemical shift (NMR)
d	doublet (NMR)
dd	doublet of doublet (NMR)
DCM	dichlormethane
DIC	*N,N'*-Diisopropylcarbodiimid
DIPEA	diisopropylethylamin
DMAP	4-(Dimethylamino)-pyridin
DMF	*N,N*-Dimethylformamid
DMSO	dimethyl sulfoxide
D_2O	Deuterium water
EDT	ethane-1,2-dithiol
ε (Epsilon)	molar extinction coefficient ($M^{-1}cm^{-1}$)
eq.	equivalent
ESI	Electrospray-Ionisation (MS)
F	phenylalanine
FAM	Fluorescein amidite
Fmoc	9-Fluorenylmethoxycarbonyl
FRET	Föster Resonance Energy Transfer
g	gram
h	hour, high field/upfield (NMR)
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HATU	hexafluorophosphate azabenzotriazole

	tetramethyl uranium
HBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphat
Hdn	histidine-*N,N*-dinaphthalene
H_2O	water
HPLC	High Pressure Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry
Hz	Hertz (s^{-1})
I	isoleucine
J	Spin-Spin-Coupling
K	Kelvin, lysine
L	liter, leucine
l	low filed/downfield (NMR)
λ (Lambda)	wavelength (nm)
LB medium	lysogeny broth medium
LTQ-FT	Linear Ion Trap and Fourier Transform (MS)
m	mass (g or mg), multiplet (NMR)
M	molar mass (g/mol)
MHz	megaherz
min	minute
mL	milliliter
mmol	millimolar
MS	mass spectrometry
n	amount of substance (mol or mmol)
NaOD	deuterated sodium hydroxide
NaOH	sodium hydroxid
NEt_3	triethylamine
nm	nanometer
NMR	Nuclear Magnetic Resonance
OD	Optical Density
Oxyma	ethyl hydroxyiminoacetate
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfony
pH	acidity level
ppm	parts per million
R	arginine

R_f	retention factor (TLC)
ρ (Rho)	density (g/cm^3)
RT	room temperature
RP-HPLC	Reversed Phase - High Pressure Liquid Chromatography
s	singlet (NMR)
SPPS	Solid Phase Peptide Synthesis
t	triplet (NMR)
TFA	trifluoroacetic acid
TLC	Thin-layer Chromatography
TNBS	2,4,6-Trinitrobenzene-1-sulfonic acid
UV	ultraviolet light
V	volume
W	tryptophane

2. Materials and Methods

All steps were prepared after script [1] and modified when needed.

2.1 Organic Synthesis – Synthesis of 6-carboxylfluorescein

The reaction protocol was proceeded according to the work of HAMMERSHØJ *et al.* [2], modified when needed.

Method:

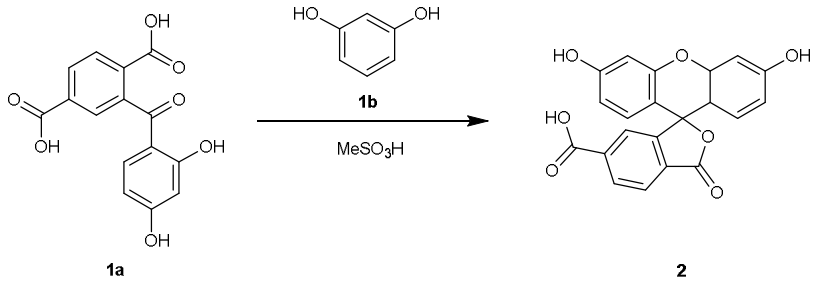


*Figure 1. Reaction equation of the 6-carboxylfluorescein 6-FAM **2** synthesis.*

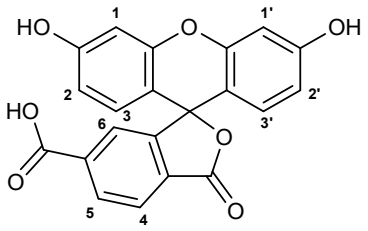
Table 1. Used educts and solvent for the synthesis of 6-FAM

Educts and solvent	M [g/mol]	eq	n [mmol]	m [mg]	V [mL]
2-(2,4-Dihydroxybenzoyl) terephthalic acid **1a**	302.2	1.00	0.66	200.0	
Resurcinol **1b**	110.1	1.10	0.73	81.0	
$MeSO_3H$					5.0

A solution of 2-(2,4-Dihydroxybenzoyl)terephthalic acid **1a** (200.0 mg, 1.00 eq, 0.66 mmol) in methanesulfonic acid (5.0 mL) and resorcinol **1b** (81.0 mg, 1.10 eq, 0.73 mmol) is added in a 25.00 mL round flask. The reaction mixture was stirred at room temperature overnight (18 h), then added to ice–water (50.0 mL), which is stirred in an ice bath with salt (-15°C) for 1 h. The precipitate is filtered via vacuum filtration, the crude is dissolved in an ice-cooled 2 M sodium hydroxide NaOH aqueous solution (40.0 mL), then precipitated with an ice-cooled 2 M hydrochloride acid HCl aqueous solution and filtered. The crude compound was re-precipitated by dissolving the solid completely in ethanol EtOH (10.0 mL), then the solvent was removed in vacuo. The crude product is dissolved in EtOH (2.0 mL), then added into ice water (50.0 mL) until precipitation. After cooling the suspension on ice for 1 h, the product is precipitated and then filtered. This re-precipitation step is repeated with ice-cooled 2 M NaOH aqueous solution (40.0 mL) and ice-cooled 2M HCl aqueous solution (40.0 mL). During the final filtration, the product is washed with H_2O to remove possible sodium chlorid NaCl. After drying in vacuo, the product is obtained as a yellow-to-reddish solid.

The reaction was control using TLC method (10% MeOH in DCM) before and after workup. The final product was analysed by NMR using 50.0 µL of 1 mM NaOD in 0.6 mL D_2O.

Analysis:



TLC Rf (10% MeOH in DCM) before workup**:** 0.3

Rf (10% MeOH in DCM) after workup**:** 0.24

Yield: 69%

^{1}H NMR (300 MHz, D_2O/NaOD, 300 K) δ = 7.95–7.92 (d, ^3J = 8.05 Hz, 1H, H_5), 7.71–7.68 (m, 2H, H_4, H_6), 7.01–6.98 (m, 2H, H_1, $H_{1'}$), 6.44–6.39 (m, 4H, H_2, $H_{2'}$, H_3, $H_{3'}$).

^{13}C NMR (75 MHz, D_2O/NaOD, 300K) δ = 180.58, 174.85, 174.30, 158.59, 158.19, 141.80, 137.15, 131.46, 130.30, 129.69, 128.11, 122.87, 112.24, 103.59.

HR-MS ESI$^-$: (m/z): $C_{21}H_{11}O_7$ $[M]^{-1}_{calc}$ = 375.0501 g/mol; $[M]^{-1}_{found}$ = 375.0501 g/mol

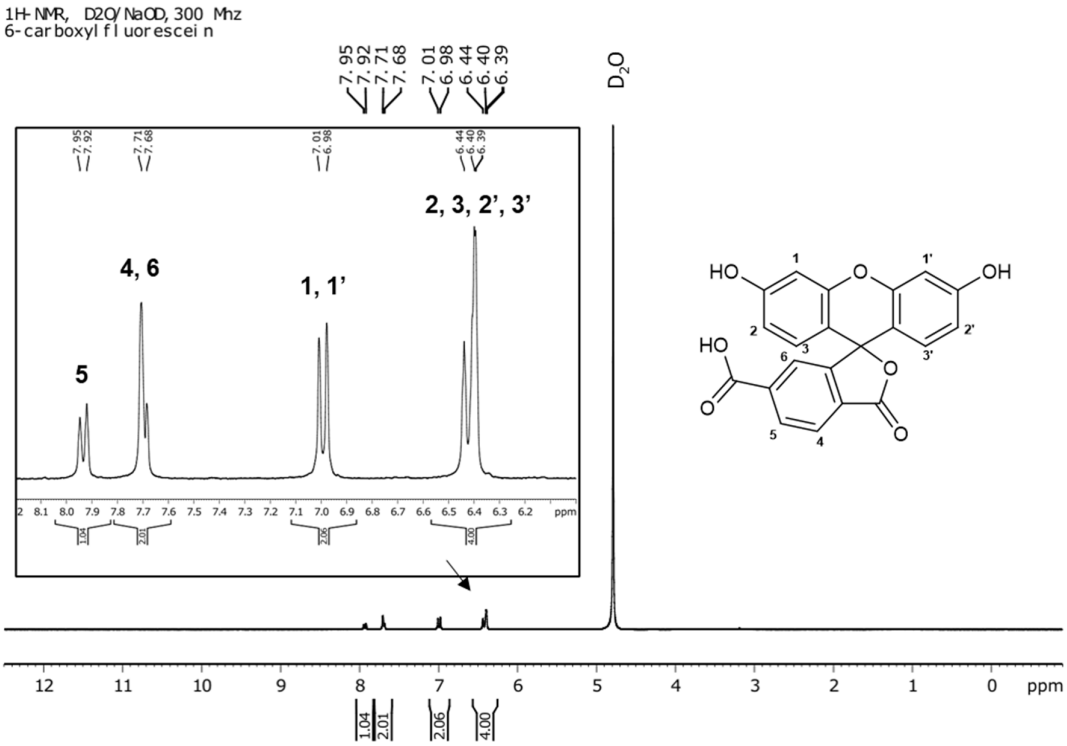


Figure 2. ^{1}H-NMR of 6-carboxylfluorescein at 300 MHz, 300 K in D_2O/NaOD.

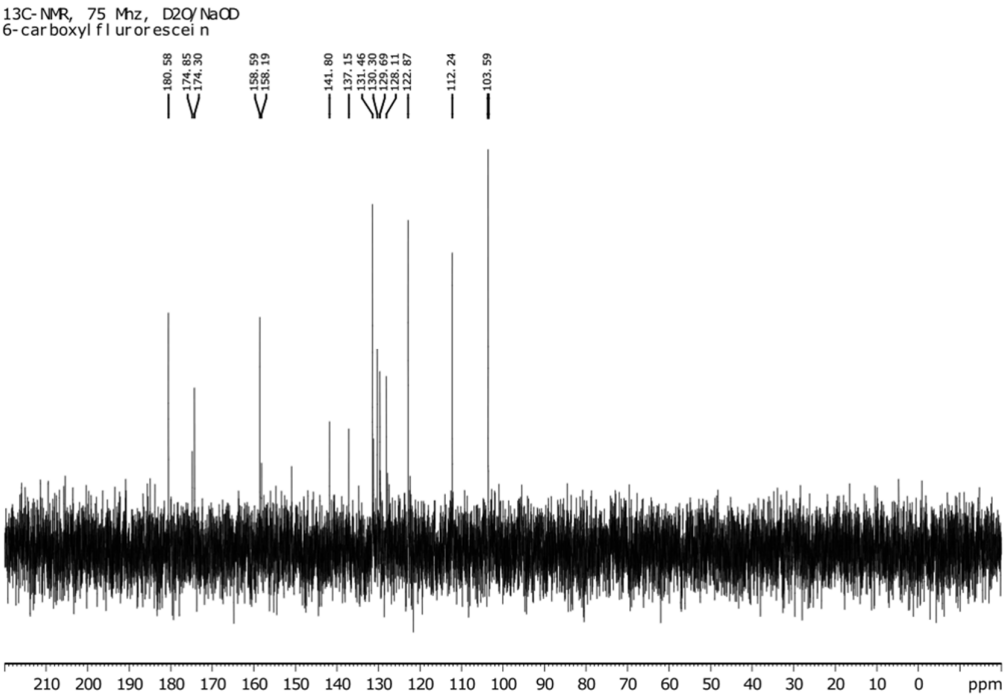


Figure 3. ^{13}C-NMR of 6-carboxylfluorescein at 75 MHz, 300 K in D_2O/NaOD.

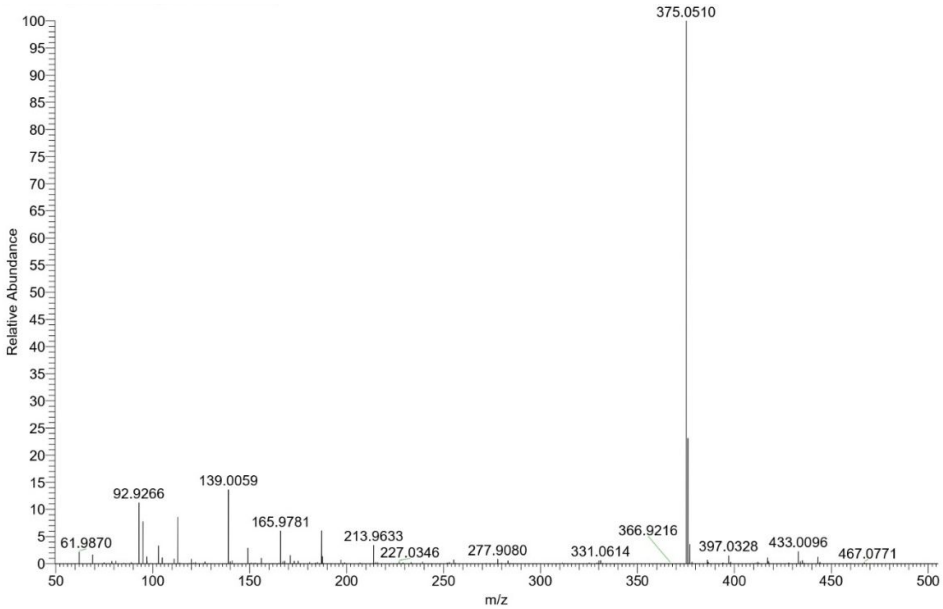


Figure 4. Full mass spectrometry spectrum of 6-carboxylfluorescein.

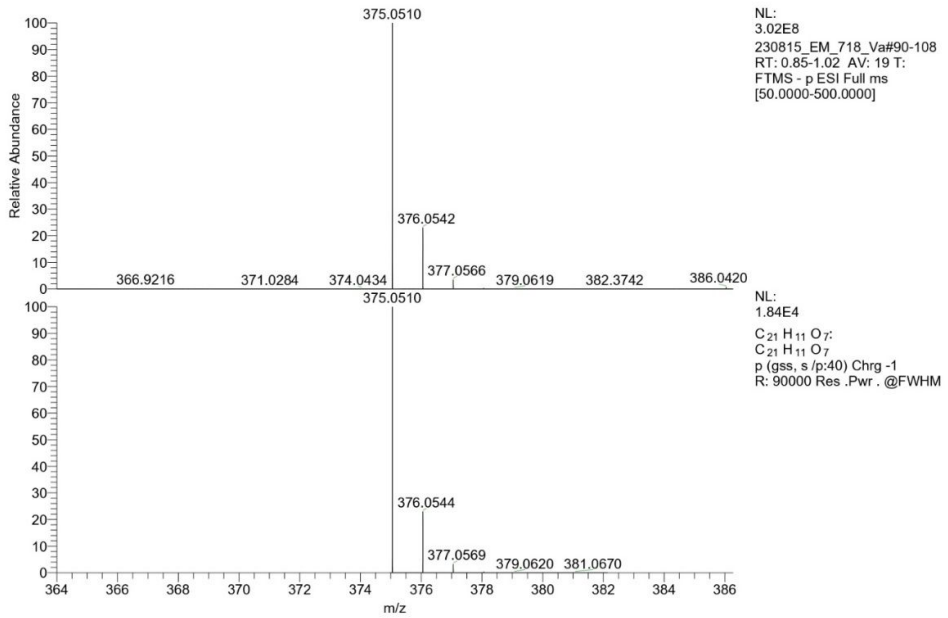


Figure 5. Calculated mass (above) and found exact mass (below) spectrometry spectrum of 6-carboxylfluorescein.

2.2 pH Dependence of Spectroscopic Properties from Fluorescein

Method:

Six buffer solutions were prepared according to GOMORI [3] (see Table 2) and their pH was measured with a pH-electrode. 6-FAM **2** (2.2 mg) was dissolved in dimethylsulfoxide DMSO (1222 µL) and 6-Naph **2d** (2.1 mg) in DMSO (1000 µL) to obtain a stock solution with a concentration of 5 mM.

Table 2. Preparation of buffer solutions according to GOMORI [3]

pH	Buffer solution	Educts and solvent
1	HCl/KCl	0.2 M KCl (125.0 mL), 0.2 M HCl (242.5 mL), diluted up to 500.00 mL solution with H_2O
3	Citrate buffer	0.1 M citric acid (199.0 mL), 0.2 M dibasic sodium phosphate (51.0 mL), diluted up to 500.0 mL solution with H_2O
5	Acetate buffer	0.2 M AcOH (74.0 mL), 0.2 M NaOAc (176.0 mL), diluted up to 500.0 mL solution with H_2O
7	Phosphate buffer	0.2 M monobasic sodiumphosphate (97.5 mL), 0.2 M dibasic sodium phosphate (152.5 mL), diluted up to 500.0 mL solution with H_2O
9	Tris/HCl buffer	0.2 M Tris (125.0 mL), 0.2 M HCl (12.5 mL), diluted up to 500.0 mL solution with H_2O
10	Carbonate/Bicarbonate buffer	0.2 M sodium carbonate (68.8 mL), 0.2 M sodium bicarbonate (56.3 mL), diluted up to 500.0 mL solution with H_2O

- Absorption:

A blank sample of buffer (3992 µL) was measured from 200 nm to 600 nm. Then the fluorescein stock solution (8.0 µL) was added, mixed and measured. After measurement, the cuvette was washed three times with MeOH and H_2O, then dried with nitrogen for the next measurement.

- Fluorescence emission:

A blank sample of buffer (3992 µL) was measured from 495 nm to 750 nm, with the exciting wavelength at 485 nm. The fluorescein stock solution (8.0 µL) was added, mixed and measured. After measurement, the cuvette was washed three times with MeOH and H_2O, then dried with nitrogen for the next measurement.

All absorption and emission spectra at the different pHs were calculated and plotted on Excel and GraphPad Prism (see Fig. 6, Fig. 7)

<u>Analysis:</u>

- Absorption:

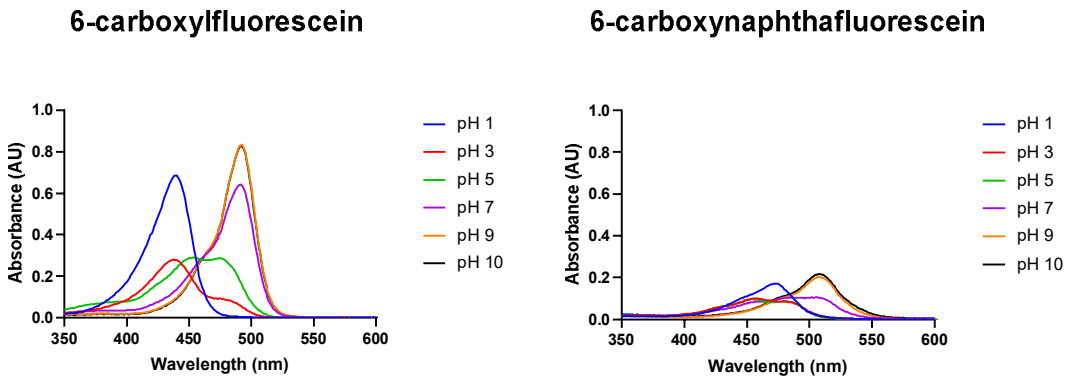


Figure 6. Absorbance spectrum of 6-carboxyfluorescein (left) and 6-carboxylnaphthafluorescein (right) at wavelengths from 350 nm to 600 nm in different buffer with pH values at 1, 3, 5, 7, 9 and 10.

- Fluorescence:

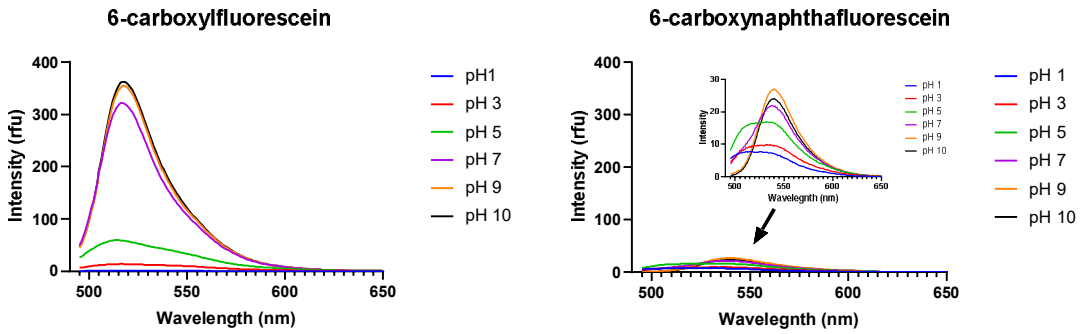


Figure 7. Fluorescence spectrum of 6-carboxyfluorescein (left) and 6-carboxylnaphthafluorescein (right) at wavelengths from 490 nm to 650 nm in different buffer with pH values at 1, 3, 5, 7, 9 and 10.

2.3 Solid phase peptide synthesis (SPPS)

Fmoc-protected natural and modified amino acids were coupled using *N,N'*-Diisopropylcarbodiimide (DIC) and ethyl cyanohydroxyiminoacetate (Oxyma) as coupling reagents on Tentagel rink amide resin. The final *N*-terminal coupling was performed with 4-acetamido benzoic acid (Aba) using Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (HATU) and *N,N*-Diisopropylethylamine (DIPEA) as coupling reagents.

<u>Method:</u>

The decapeptide PCB1-2 with the sequence Aba-K-Hdn-RR-Hdn-IRWL-$CONH_2$ (see Fig. 6) was synthesized in this experiment according to the script. [1] The first 3 amino acids at *C*-terminal (leucine L, tryptophane W, arginine R) were already bonded to the available Rink-Harz. After the first coupling of isoleucine, the 2,4,6-Trinitrobenzolsulfonsäure (TNBS) test was performed to quantitate the free amino groups.

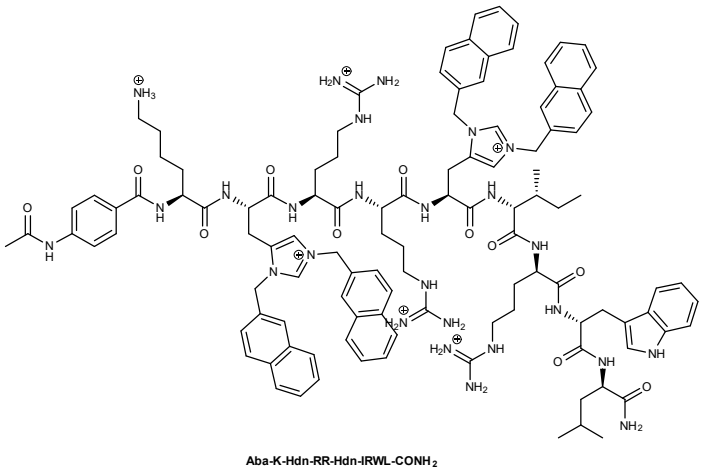


Figure 8. Molecule structure of decapeptide PCB1-2 Aba-K-Hdn-RR-Hdn-IRWL-$CONH_2$

Table 3. Preparation of SPPS reagents [1].

Reagents	Educts and solvent
20% piperidine in DMF	2.0 mL piperidine, diluted up to 10.0 mL with DMF
6% 2,6-lutidine/ 5% Ac_2O in DMF	0.6 mL lutidine + 0.5 mL Ac_2O, diluted up to 10.0 mL with DMF
Dimethyl formaldehyde (DMF) for coupling	20.0 mL
DMF for washing	250.0 mL
Dichloromethane (DCM) for washing	250.0 mL
TNBS solution A (10% DIPEA in DMF); TNBS solution B (1 % picryl sulfonic acid in DMF)	provided from assistant
Cleavage cocktail	3.8 mL trifluoracetic acid (TFA), 0.1 mL ethane-1,2-dithiol (EDT), 0.1 mL Milli-Q H_2O, 0.04 mL triisopropysilane (TIPS)

- Swelling:

The peptide will be synthesized in a 10.00 µmol scale using Tentagel resin, which is pre-coupled with leucine L, tryptophane W, arginine R, with a loading of 0.15 mmol/g. The resin (67.2 mg) was into the syringe reactor and incubated with DMF (1000 µL) for 30 minutes on a rocker, then the supernatant was discarded.

- Deprotection of Fmoc:

20 % piperidine in DMF (500.0 µL) was added into the reactor, the syringe needle was replaced with a cap and shaken for 5 minutes. This step was repeated twice. After deprotection, the resin was washed five times each with DMF, DCM and DMF (500.0 µL each time).

- Coupling of Fmoc amino acids:

Fmoc-amino acid-OH and Oxyma were weighted in a 1.5 mL microcentrifuge tube, then dissolved in DMF (100.0 µL) before adding DIC. The tube was then pre-activated for 1 minute, then centrifuged at 14 000 rpm for 1 minute to create the coupling solution. The syringe needle was removed, the tip of the reactor was tapped against paper towel to remove solvent residues. A new needle was placed on the reactor to withdraw the coupling solution, then replaced with a clean cap. The reactor was shaken in 45 minutes.

Before the first coupling of isoleucine, the cap from washing step was not replaced. For the next couplings, new syringe and cap were used before withdrawing the coupling solution into the reactor.

Table 4. Preparation of Fmoc amino acids for SPPS for each coupling [1].

Educts and solvent	M [g/mol]	eq	n [µmol]	m [mg]	V [µL]
Fmoc-Ile-OH	353.4	4.00	40.00	14.0	
Fmoc-Arg(Pbf)-OH	648.8	4.00	40.00	26.0	
Fmoc-Lys(Boc)-OH	468.5	4.00	40.00	19.0	
Fmoc-Hdn-OH	694.2	2.00	20.00	14.0	
Aba	179.2	10.00	100.00	18.0	
Oxyma	142.1	4.00	40.00	6.0	
DIC	126.2	4.00	40.00	6.2	
HATU	380.2	10.00	100.00	38.0	
DIPEA	129.3	20.00	200.00		34.0
DMF					100.0

- TNBS test:

This test was only performed once after the first coupling of isoleucine, since Hdn presence in the peptide could show a false positive result. Using a pipette tip, a few resin beads were removed from reactor and transferred into a 0.5 mL microcentrifuge tube. 2 drops of TNBS solution A and 1 drop of TNBS solution B were added to the tube, the solution was mixed by flicking. After 5 minutes, the staining of beads was checked to be negative.

- Capping:

6% 2,6-lutidine/ 5% Ac_2O in DMF (500.0 µL) was added to the resin, mixed well before replacing the syringe needle with a cap, the reactor was shaken for 5 minutes. The supernatant was discarded, the resin was washed five times each with DMF, DCM and DMF (500.0 µL each time).

- Coupling of Aba:

Aba and HATU were weighted in a 1.5 mL microcentrifuge tube, then dissolved in DMF (200.0 µL) before adding DIPEA. The tube was then pre-activated for 3 minutes, then centrifuged at 14 000 rpm for 1 minute to create the coupling solution. The syringe needle was

removed, the tip of the reactor was tapped against paper towel to remove solvent residues. A new needle was placed on the reactor to withdraw the coupling solution, then replaced with a clean cap. The reactor was shaken in 45 minutes.

- Cleavage of peptide from Harz:

After the final coupling with Aba, the resin was washed five times with DMF and DCM (500.0 µL each time), then dried in vacuo for 15 minutes. The cleavage cocktail (2.0 mL) was added to the dried resin and the reactor was shaken for 3 hours. The supernatant was added into a 15.0 mL Falcon tube. The resin was then washed with TFA (500.0 µL), the supernatant was also added to the same Falcon tube. The cleavage solution was then dried using nitrogen gas down to 0.5 mL. Cold diethyl ether Et_2O kept at -15^oC was added to the evaporated solution to precipitate the light-yellow peptide. The Falcon tube was centrifuged at 8000 rpm in 5 minutes, the supernatant was discarded and the solid peptide was precipitated again with cold Et_2O. This step was repeated one time before the Falcon tube with peptide was air-dried overnight.

- Purification with HPLC:

Before purification, the crude peptide was first analysed by assistant using HPLC method. After that, all three peptide samples of the same sequence PCB1-2 were diluted in 920.0 µL MeCN/Milli-Q H2O 1:1 + 1% TFA.

1. Preparative HPLC:

A diluted sample of the crude peptide used for an analytical HPLC-MS measurement on an Agilent 1260 Infinity II instrument, with the HPLC column *Waters XBridge Peptide* BEH C18 OBD Prep Column, 300 Ã, 5 µm. The linear gradient from 35% to 75% MeCN to H_2O was used with detection from 220 nm and 260 nm by UV-absorption. The two fractions F1 and F2 were selected and gathered for the purification.

2. Analytical/ Purification HPLC:

The OD of 2 peptide fractions was measured at 220 nm, with F1 at 91.8 au and F2 at 70.4 au. These two fractions were diluted to 36 au in 1.0 µL with MeCN/Milli-Q H_2O 1:1 + 1% TFA. 1.0 µL from each diluted fraction was purified by HPLC on a Waters instrument with a linear gradient from 5% to 95% TFA/MeCN to H_2O at 220 nm every 30 minutes. The fractions containing the product were lyophilized.

The crude fractions were twice purified using the same parameter. The second fraction after two purification steps was used for the bioassay experiment and other analytics.

- Other analytics:

Yield: 4.5%

HR-MS ESI$^-$: (m/z) $C_{112}H_{138}N_{26}O_{11}$ = $[M+H]^{+3}_{calc}$ = 675.0341 g/mol; $[M+H]^{+3}_{found}$ = 675.0377 g/mol

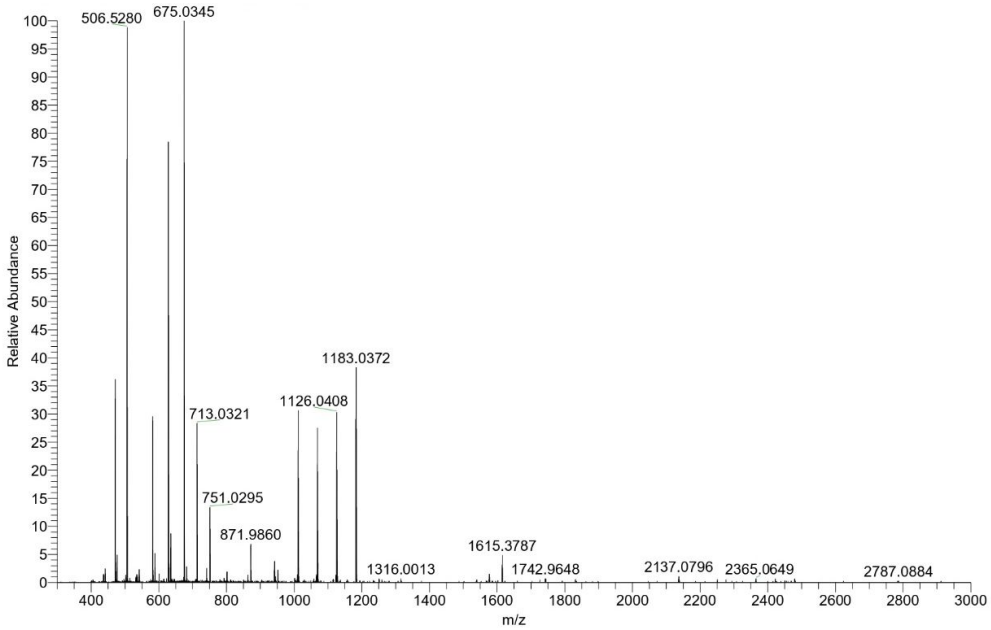


Figure 9. Full mass spectrometry spectrum of decapeptide PCB1-2 Aba-K-Hdn-RR-Hdn-IRWL-CONH$_2$

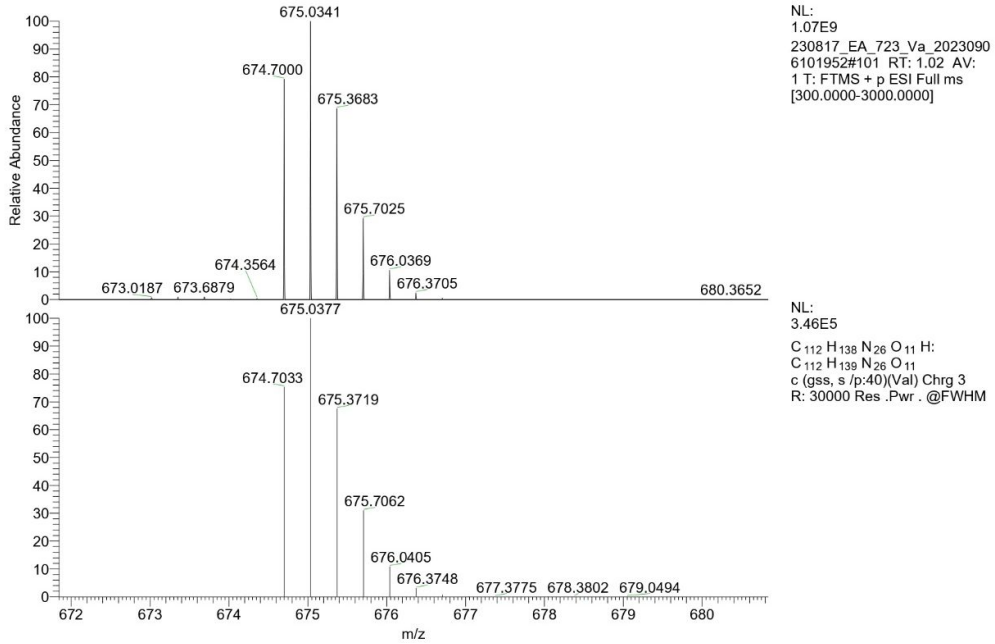


Figure 10. Calculated mass (above) and found exact mass (below) spectrometry spectrum of decapeptide PCB1-2 Aba-K-Hdn-RR-Hdn-IRWL-CONH$_2$.

Analysis:

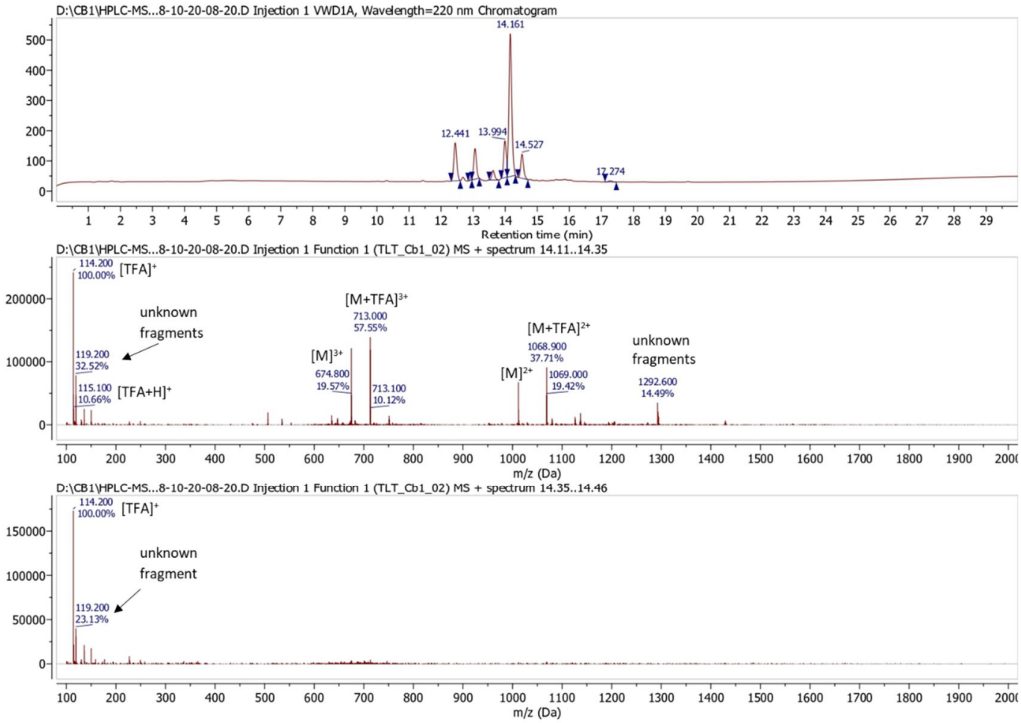


Figure 11. (above) Full HPLC-MS spectrum, (below) at R_t 14.16 minutes spectrum at 220 nm from crude peptide PCB1-2 before purification, in gradient of 35%-75% MeCN to H_2O.

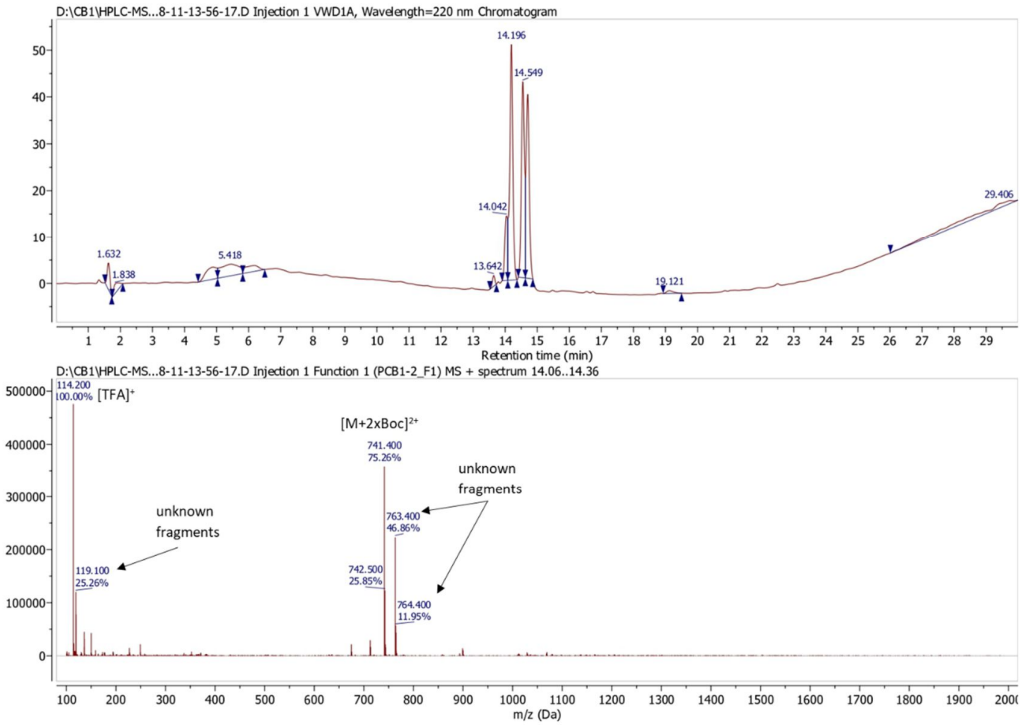


Figure 12. (above) Full HPLC-MS spectrum, (below) at R_t 14.19 minutes spectrum at 220 nm from peptide PCB1-2 after 1st purification, fraction F1, in gradient of 5% to 95% TFA/MeCN to H_2O.

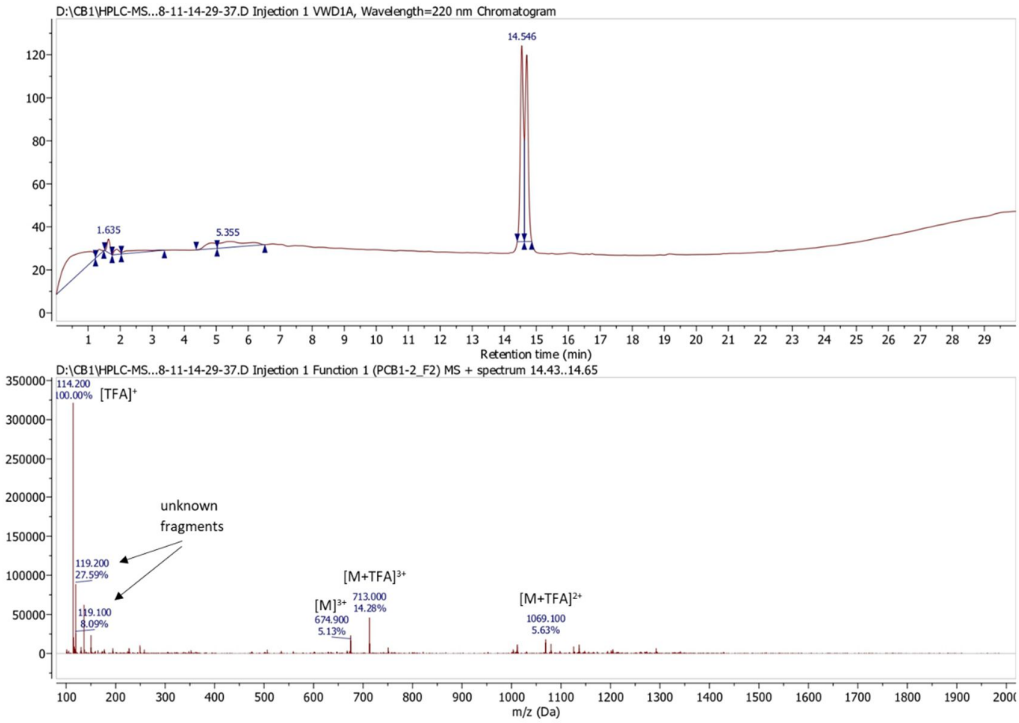


Figure 13. (above) Full HPLC-MS spectrum, (below) and at R_t 14.54 minutes spectrum at 220 nm from peptide PCB1-2 after 1st purification, fraction F2, in gradient of 5% to 95% TFA/MeCN to H_2O.

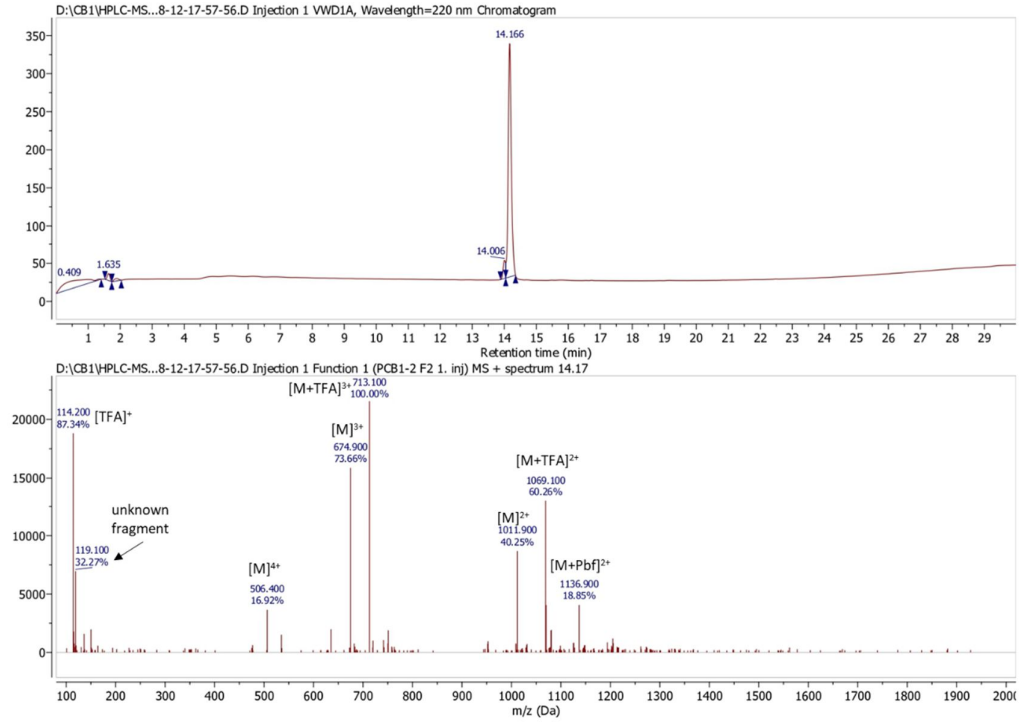


Figure 14. (above) Full HPLC-MS spectrum, (below) and at R_t 14 minutes spectrum at 220 nm from peptide PCB1-2 after 2nd purification, fraction F2, in gradient of 5% to 95% TFA/MeCN to H_2O.

2.4 Bioassay – Hemmhof Test (Agar Diffusion Assay)

Method:

A colony of each *E. coli* DH5α and *B. subtilis* 3610 was picked up from agar plate and inoculated in LB-medium to create the overnight culture overnight at 37°C. The overnight culture (100.0 µL) was diluted in LB medium (10.0 mL) and shaken in a baffled flask for 6 hours at 37°C. The OD_{600} of the cultures was measured in a diluted sample (1:10 dilution, 100.0 µL culture in 900.0 µL LB medium) in every 30 minutes up to determine the optical density between 0.3 and 0.4. At OD_{600} of 0.347 for *E. coli* and 0.401 for *B. subtilis*, each culture was diluted to an OD_{600} of 0.1 and was spread on an LB-medium agar plate. Round sterile filter papers were placed on the agar surface, then 0.01 M HCl (negative control, 2.0 µL) and 5 types of antibiotics (positive control, 2.0 µL) were pipetted on round filters. The agar plate was incubated overnight at 37°C.

Table 5. Preparation of antibiotics [1].

Antibiotics	M [g/mol]	m [mg]	Solvent	$V_{Solvent}$ [mL]
Kanamycin sulphate **[1]**	582.5	2.1	Milli-Q H_2O	1.3
Ciprofloxacin **[2]**	110.1	2.0	0.01 M HCl	2.1
Tetracycline hydrochloride **[3]**	331.3	2.1	Milli-Q H_2O	1.5
Chloramphenicol **[4]**	480.9	2.0	Milli-Q H_2O	2.2
Polymyxin B sulphate **[5]**	322.1	2.0	Milli-Q H_2O	0.5

For the contact test (Abklatsch): a sample of bacteria was taken with a pipette tip from smartphone screen, then diluted in 4.0 µL of LB-medium before plating on agar plate. The plate was incubated for 2 days at 37°C before final analysing.

Analysis:

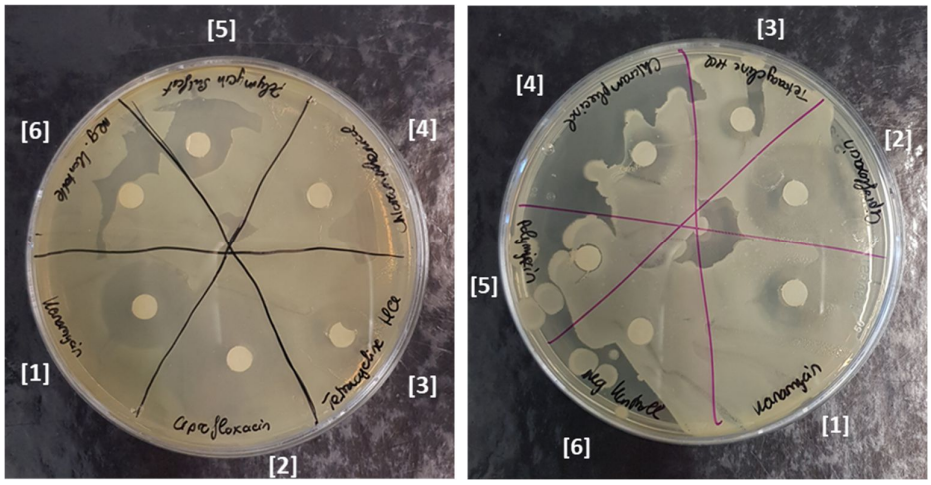


Figure 15. Agar plates with E. coli (left) and B. subtilis (right) after incubation overnight. The numbering of each portion represented each antibiotic or negative control (0.01 M HCl).

Table 6. Measurement of Hemmhof diameter after overnight incubation.

	Diameter of Hemmhof/cm (including filter paper)	
Antibiotics	***E. coli***	***B. subtilis***
Kanamycinsulfat **[1]**	2.0	1.6
Ciprofloxacin **[2]**	3.2	2.0
Tetracyclinehydrochlorid **[3]**	1.4	1.0
Chloraphenicol **[4]**	0.4	0.4
Polymyxin B sulfat **[5]**	1.5	0.5
Negative control (0.01M HCl) **[6]**	0	0

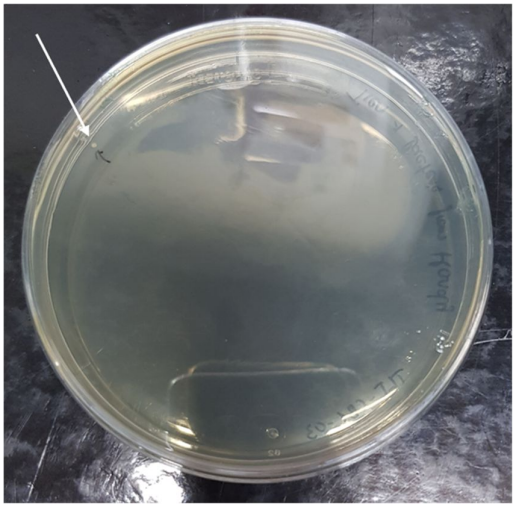


Figure 16. Contact test (Abklatsch-test) on agar plate, sample was taken from smartphone's screen.

2.5 Bioassay – Growth Curve

For this experiment, there were 3 major samples prepared: bacteria control (BO), solvent control with 0.01 M HCl (SC) and peptide samples (PCB1-4, PCB1-2, P14). Three peptide stock were diluted from the 10 µM peptide concentration (Stock 1):

Table 7. Preparation of peptide stock from HPLC purified peptide.

	PCB1-4	**PCB1-2**	**P14**
Purified peptide concentration (mM)	2.70	1.61	2.41
Stock 1 (10 µM)	1.8 µL purified peptide + 478.2 µL Milli-Q H_2O	3.0 µL purified peptide + 477.0 µL Milli-Q H_2O	2.0 µL purified peptide + 478.0 µL Milli-Q H_2O
Stock 2 (5 µM)	160.0 µL Stock 1 + 160.0 µL Milli-Q H_2O		
Stock 3 (1 µM)	40.0 µL Stock 1 + 360.0 µL Milli-Q H_2O		

Table 8. Preparation of test samples and solutions for 96-well plates (see script [1])

	Blank	**BO (bacteria control)**	**SC (solvent control)**	**P (Peptide sample)**
Test sample	-	LB-Medium (25.0 µL)	0.01 M HCl (25.0 µL)	Peptide stock P1, P2, P3 (25.0 µL)
LB-Medium	250.0 µL	219.0 µL	219.0 µL	219.0 µL
Bacteria (*E. coli, B. subtilis*)	-	6.0 µL	6.0 µL	6.0 µL

A day culture of *E. coli* and *B. subtilis* was diluted to an OD_{600} of 0.1. On a 96-well plate, test samples of bacteria, solvent and peptide were added with LB-medium and bacteria culture according to script. [1] The plate was shaken with 250 rpm at 37°C and the OD was measured every 30 minutes in a plate reader at 600 nm.

The OD values were then calculated and plotted with Excel and GraphPad Prism.

Analysis:

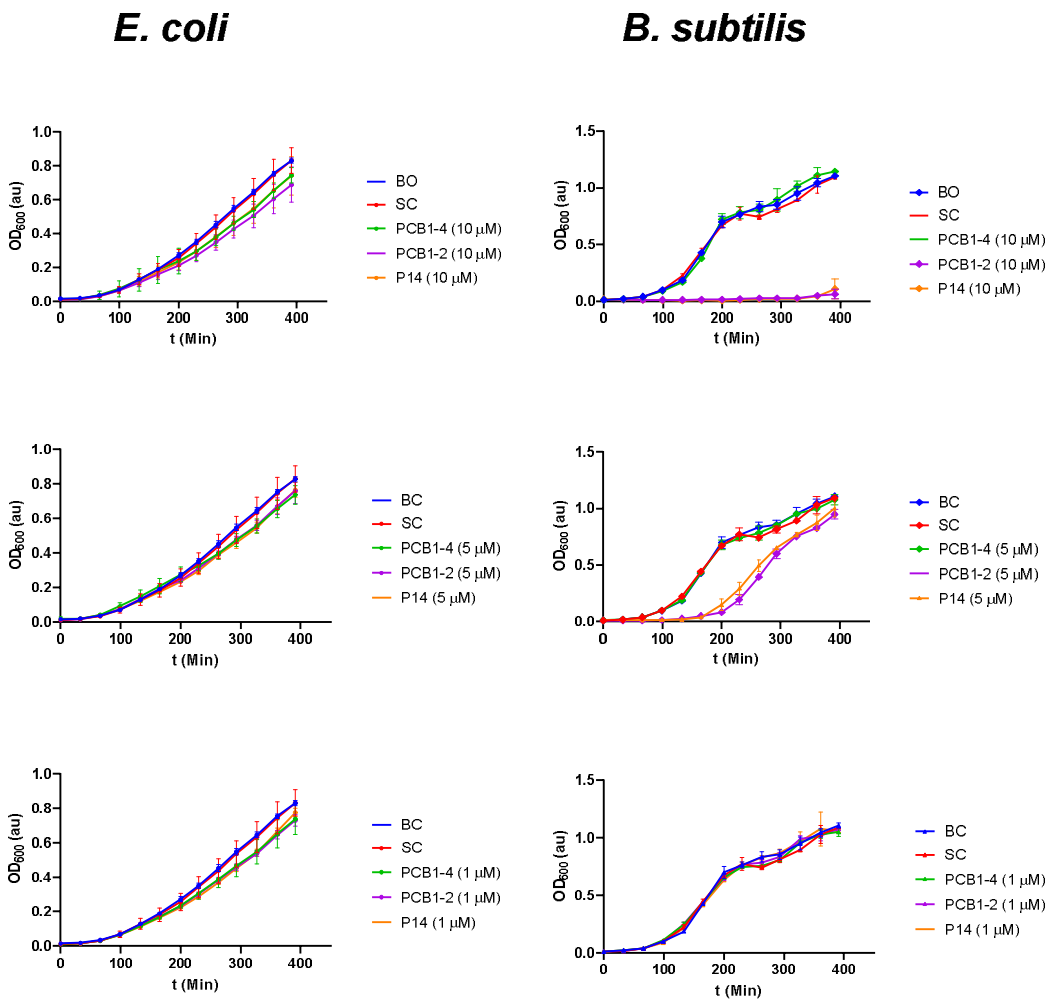


Figure 17. Growth curves of two bacteria types E. coli (left) and B. subtilis (right) in presence of antimicrobial peptides PCB1-4, PCB1-2 and P14. The inhibiting effect was compared in different peptide concentrations (10, 5 and 1 µM).

3. References

[1] V. T. Trinh, V. Viereck, *Script of Practical Course CB1- Chemical Biology SS2023*, Phillips University Marburg, Faculty Chemistry (FB 15), **2023**, 2, 8–9.

[2] P. Hammershøj, E. K. P. Kumar, P. Harris, T. L. Andresen, M. H. Clausen*, Eur. J. Org. Chem.*, **2015**, 7301–7309.

[3] G. Gomori, *Meth. Enzymol.*, **1955**, *1*,138–146.