Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Site-Specific Grafting on Titanium Surfaces with Hybrid Temporin Antibacterial Peptides

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CONTENT

1. General Information

All reagents and solvents were from AlfaAesar and Acros and were used without further purification. Protected amino acids and HBTU were purchased from Iris Biotech. All reactions and washes were conducted at ambient temperature unless otherwise noted.

The following abbreviations were used: DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N*-*N'*-dimethylformamide; HBTU, Tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, *O*-(Benzotriazol-1-yl)- -tetramethyluronium hexafluorophosphate; HPLC, High Performance Liquid Chromatography; LC/MS, Tandem Liquid Chromatography/ Mass Spectroscopy; NMP, *N*-methylpyrrolidone; SPPS, Solid Phase Peptide Synthesis; TFA, trifluoroacetic acid; THF, Tetrahydrofuran. Other abbreviations used were those recommended by the IUPAC-IUB Commission (*Eur. J. Biochem*. 1984, 138, 9-37).

2. Synthesis of hybrid peptides 1-5

Hybrid peptides **1-5** were obtained using a standard Fmoc/tBu SPPS protocol. Peptides were synthesized using the Liberty (CEM) Microwave peptide synthesizer coupled to a Discover (CEM) microwave. The introduction of the dimethylhydroxysilyl chain at the *N*-ter was realized after Fmoc deprotection (Figure S1, Pathway A). For chain introduction into the sequence and at *C*-ter, one Alloc protected aminoacid was introduced into the sequence (Figure S1, Pathway B). After the Alloc group deprotection, dimethylhydroxysilyl moiety was introduced on the free amine group.

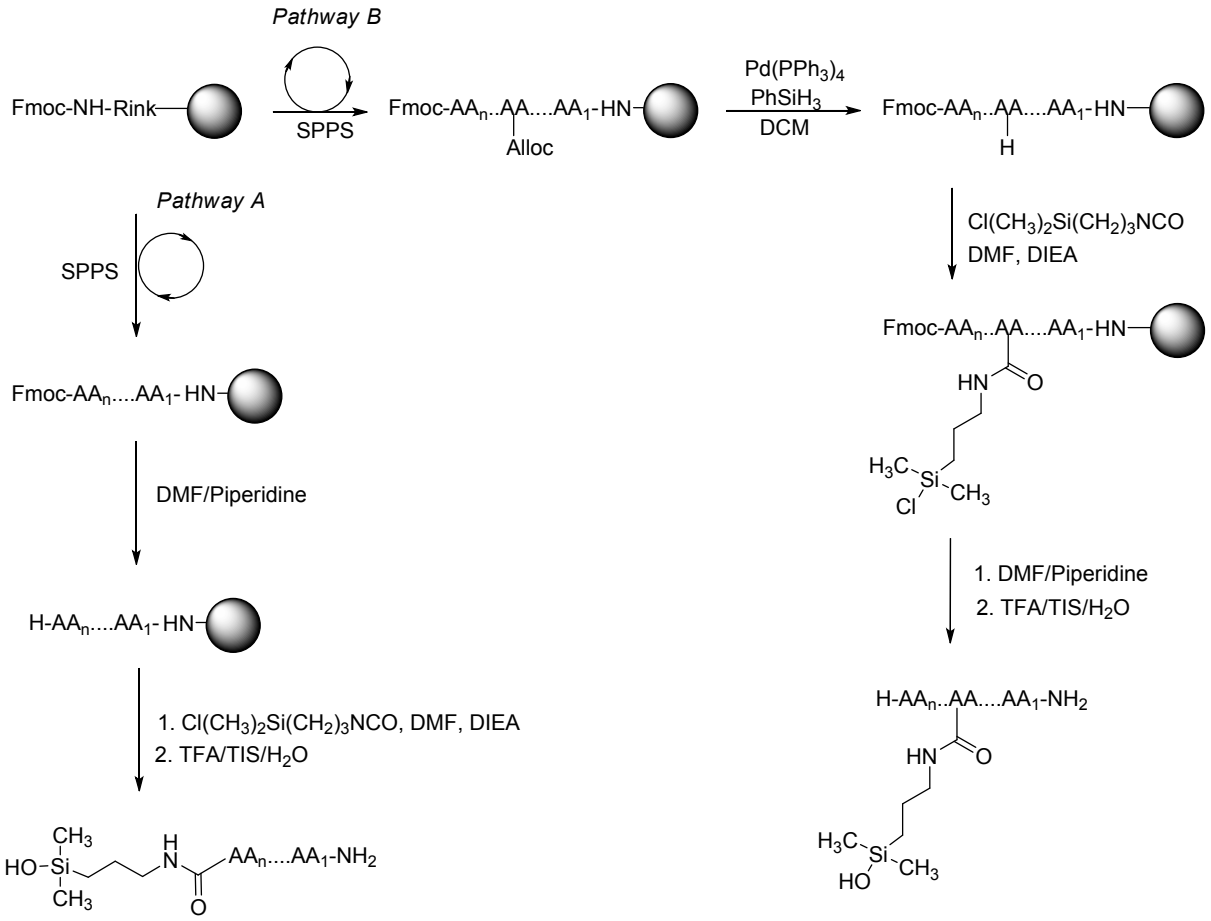

Figure S1. General strategies for the synthesis of hybrid peptides **1**-**5**.

- *Anchoring and deprotection steps*

Fmoc-Rink amide ChemMatrix resin (0.25 mmol, loading: 0.49 mmol.g^{-1}) was swollen for 15 min in DCM. Fmoc deprotection was carried out with 20% piperidine in DMF under microwave irradiation twice (40 Watts at 75 °C for 30 seconds and 35 Watts at 70 °C for 3 minutes). The beads were washed with DMF three times.

- *Coupling step*

0.2 M solution of Fmoc-amino acid in DMF (5 equiv), 0.5 M solution of HBTU in DMF (5 equiv) and 2 M solution of DIEA in NMP (10 equiv) were successively added to the resin. The coupling was carried out under microwave irradiation (23 Watts at 70 °C for 300 seconds, except for Val-8, which was coupled for 420 seconds). Double coupling steps were used for the amino acids that follow Val-8. The resin was washed with DMF three times.

- *Alloc deprotection*

To the resin was added a solution of $Pd(PPh_3)_4$ (29 mg, 25 µmol, 0.1 eq), phenylsilane (93 µL, 81 mg, 0.75 mmol, 30 eq) in 4 mL of dry DCM for 2h at room temperature and in the dark. The resin was washed with DMF (3x), MeOH (2x) and DCM (3x).

- *Introduction of the silyl moiety on solid support*

Peptides were silylated in DMF using 3-isocyanatopropyldimethylchlorosilane (5 eq) in the presence of diisopropylethylamine (5 eq) at room temperature overnight. The resin was washed with DMF (3x), DCM (3x), MeOH (1x) and DCM (1x).

- *Cleavage from the Rink amide resin*

The supported protected hybrid peptide on Rink amide resin was deprotected and cleaved from the resin concomitantly, using a TFA/TIS/H_2O (95/2.5/2.5 v/v/v) cocktail for 2 hours at room temperature. After filtration, the solution was concentrated under reduced pressure. The hybrid peptide was precipitated in diethyl ether, filtered and dried under vacuum. Hybrid peptides were purified by preparative RP-HPLC and then freeze-dried.

Compound **1**: H-Phe-Leu-Ser-Gly-Ile-Val-Gly-Met-Leu-Gly-Lys-Leu-Phe-Orn(CONH$(CH_2)_3Si(CH_3)_2OH$)-NH_2

MS (ESI) m/z: calcd for $C_{78}H_{133}N_{18}O_{17}SSi$ $[M+H]^+$ 1654.0, found $[M+2H]^{2+}$ 827.5, $[M-H_2O+2H]^+$ 819.1, $[M+3H]^{3+}$ 552.1, $[M-H_2O+3H]^{3+}$ 546.2; t_R = 1.56 min.

Compound **2**: H-Phe-Leu-Ser-Gly-Ile-Val-Lys(CONH$(CH_2)_3Si(CH_3)_2OH$)-Met-Leu-Gly-Lys-Leu-Phe-NH_2

MS (ESI) m/z: calcd for $C_{77}H_{132}N_{17}O_{16}SSi$ $[M+H]^+$ 1611.0, found $[M+2H]^{2+}$ 806.9, $[M-H_2O+2H]^+$ 797.4, $[M-H_2O+3H]^{3+}$ 532.0; t_R = 1.59 min.

Compound **3**: H-Dab(CONH$(CH_2)_3Si(CH_3)_2OH$)-Phe-Leu-Ser-Gly-Ile-Val-Gly-Met-Leu-Gly-Lys-Leu-Phe-NH_2

MS (ESI) m/z: calcd for $C_{77}H_{131}N_{18}O_{17}SSi$ $[M+H]^+$ 1639.9, found $[M+2H]^{2+}$ 820.5, $[M-H_2O+2H]^+$ 811.6, $[M-H_2O+3H]^{3+}$ 541.6; t_R = 1.62 min.

Compound **4**: $HO(CH_3)2Si(CH_2)_3NHCO$-Phe-Leu-Ser-Gly-Ile-Val-Gly-Met-Leu-Gly-Lys-Leu-Phe-NH_2

MS (ESI) m/z: calcd for $C_{73}H_{123}N_{16}O_{16}SSi$ $[M+H]^+$ 1539.9, found $[M+H]^+$ 1540.2, $[M+2H]^{2+}$ 770.6, $[M-H_2O+2H]^+$ 761.6; t_R = 1.97 min

Compound **5**: H-Phe-Leu-Ser-Gly-Ile-Val-Gly-Met-Leu-Gly-Lys($CONH(CH_2)_3Si(CH_3)_2OH$)-Leu-Phe-NH_2

MS (ESI) m/z: calcd for $C_{73}H_{123}N_{16}O_{16}SSi$ $[M+H]^+$ 1539.9, found $[M+2H]^{2+}$ 771.2, $[M-H_2O+2H]^+$ 761.7; t_R = 1.66 min

3. Supplementary figures

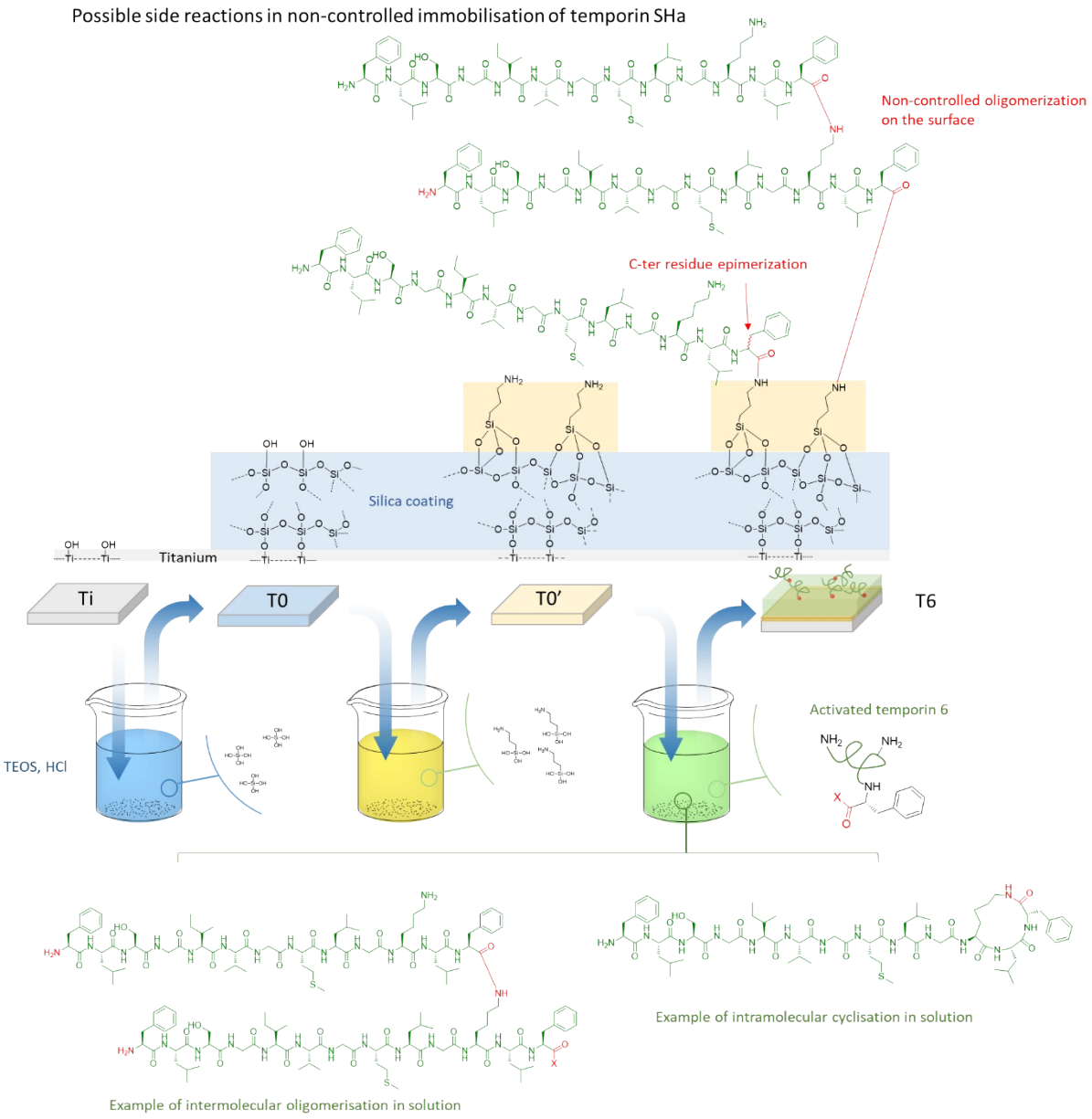

Figure S2. Possible side reactions during non-controlled immobilization of temporin **6** through *C*-ter activation.

Sample	WCA (°)
Ti	57.3 ± 1.1
T0 (TEOS)	78.5 ± 1.4
T0' (APTES)	94.7 ± 1.5
T1	79.0 ± 1.1
T2	67.4 ± 1.8
T3	66.5 ± 1.7
T4	78.7 ± 1.4
T5	78.4 ± 2.8
T6	62.7 ± 1.6

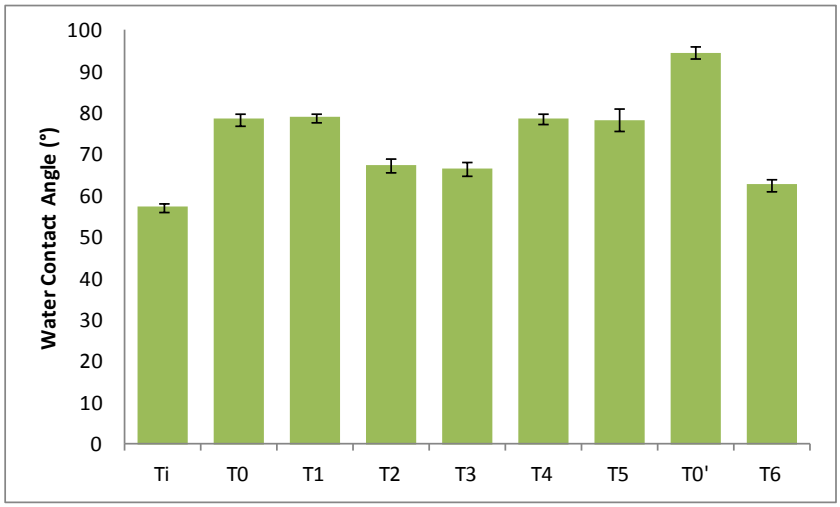

Figure S3. Sessile Drop Water contact angle (WCA) results in degree obtained on the different surfaces. WCA data obtained with 1 µL milliQ water sessile drop and bare titanium surface (Ti), premodified surfaces (TEOS and TEOS+APTES) and for the 6 temporin-grafted surfaces (T1-T6). These tests were done in duplicates on each type of sample and 3 drops were measured on each sample. The uncertainty attached to these results follows from the statistical analysis of these repeated experiments.

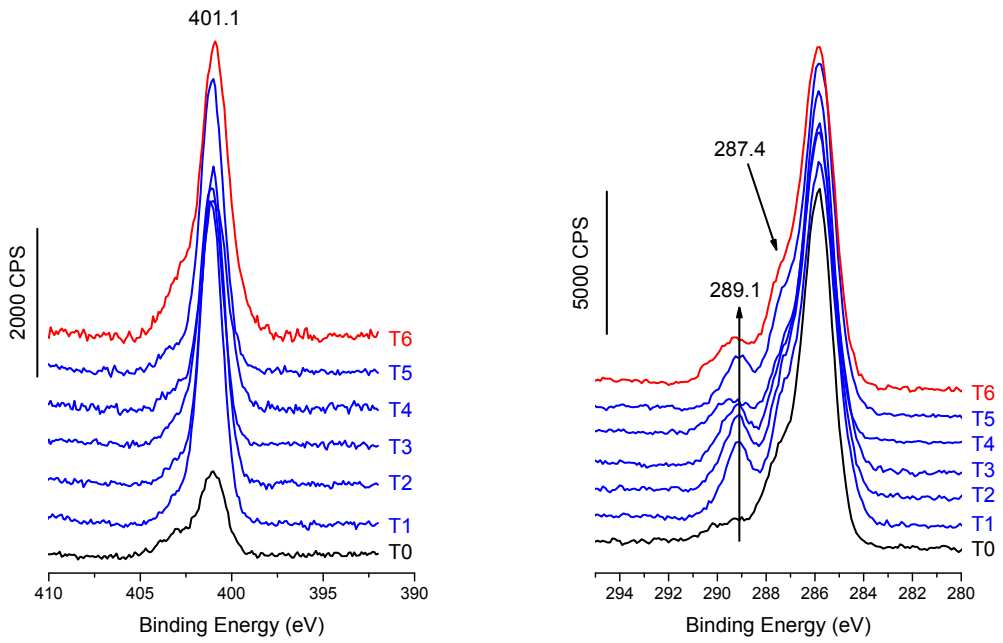

Figure S4: XPS high resolution spectra for N1s (left) and C1s (right) of Titanium surface grafted with the hybrid peptides 2 (T1 to T5) and SHa peptide (T-) compared to non grafted surface (Ti-TEOS).

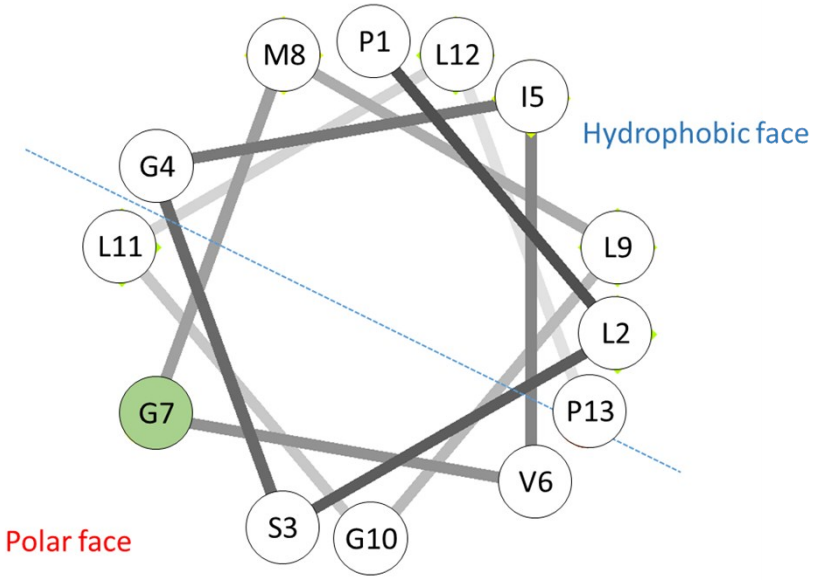

Figure S5: Schematic helical wheel projection of Temporin SHa. Adapted from free software http://rzlab.ucr.edu/scripts/wheel/wheel.html

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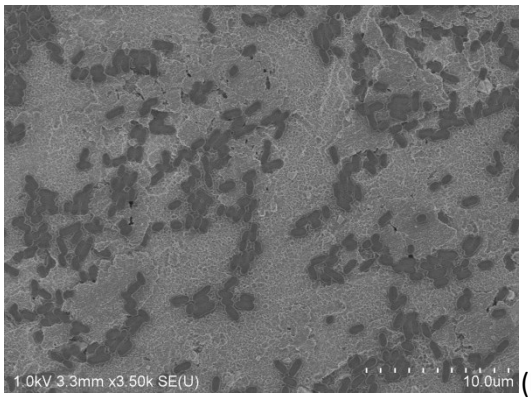
(a) 

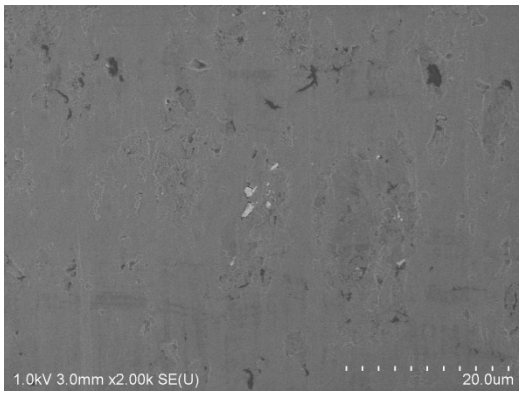
(b) 

Figure S6: SEM-FEG images of (a) titanium surface covered with bacteria after 3 hours of contact time; (b) titanium surface after removal of adhered bacteria via the sonication process.

4. Thickness evaluation

The thickness *d* of the obtained molecular layer could be estimated by means of the intensities ratio I_{Si2p}/I_{Ti2p} XPS spectra, related by the formula, by postulating that the adlayers formed are rather homogeneous:

$$\frac{ISi2p}{ITi2p} = \frac{T_{Si2p}}{T_{Ti2p}} \frac{\sigma_{Si2p}}{\sigma_{Ti2p}} \frac{\lambda_{Si2p}^{ad} C_{Si2p}^{ad} \left[1 - exp\left(\frac{-d}{\lambda_{Si2p}^{ad} cos\theta}\right)\right]}{\lambda_{Ti2p}^{su} C_{Ti2p}^{su} exp\left(\frac{-d}{\lambda_{Ti2p}^{ad} cos\theta}\right)} \qquad [1]$$

where $cos\vartheta$ is 1 because the photoelectron collection angle θ is equal to zero. T_{C1s} and T_{Cu2p} are the relative sensitivity factors of C and Cu, respectively, provided by the spectrometer manufacturer. The Scofield photoionization cross sections σ are 0.682 for Si2p and 7.02 for Ti 2p. The superscripts *ad* and *su* designate the adsorbed layer and the Titanium substrate, respectively. C_x^y *and* λ_x^y represent the

[1] Tanuma, S.; Powell, C. J.; Penn, D. R. Calculations of Electron Inelastic Mean Free Paths (IMFPs). 6. Analysis of the Gries Inelastic Scattering Model and Predictive IMFP Equation. *Surf. Interface Anal.* **1997**, *25*, 25-35.

concentration and the mean free path of the element x in the matrix y, respectively. The electron inelastic mean free paths λ were calculated using the Quases program based on the TPP2M formula[1].

The number of peptide per square centimeter n_{pep} could be estimated by using the following equation where N_A is the Avogadro number and ρ is the estimated density of the grafted peptide. In the present case, the 6 different adalyers are composed of peptides bearing very similar sequence, hence the density is estimated to be similar, making the thickness and surface density quantitatively comparable.

$$n_{pep} \quad \left(molecules \,.cm^{-2}\right) = \frac{\rho_{pep} \quad d \quad \times N_A}{M_{pep}}$$

5. Bacterial killing assays

Table S1 presents the values of the %killing presented in Figure 3, as well as the mean values of CFU counting, normalized in cfu/mL.

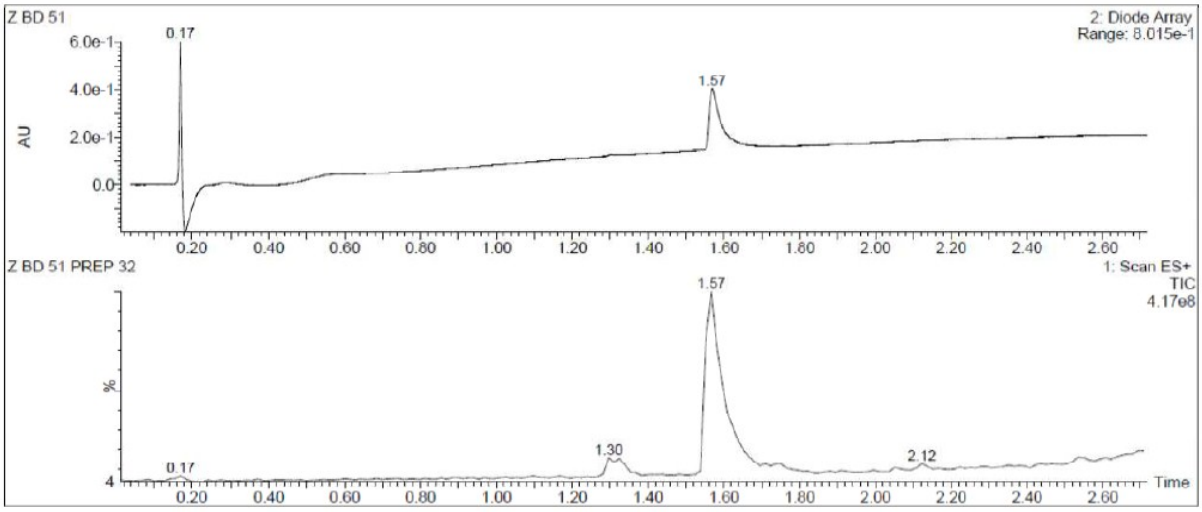
Surface	titanium		gold	titanium				gold	
bacteria	*S. epidermidis*	*E. coli*	*E. coli*	*S. epidermidis*		*E. coli*		*E. coli*	
	mean equivalent cfu/ml			mean killing	SD	mean killing	SD	mean killing	SD
inoculum	$2.15x10^8$	$1.73x10^8$	$1.68x10^8$						
Au			$1.77x10^8$						
T0	$2.70x10^8$	$3.44x10^8$							
T1	$1.91x10^8$	$2.32x10^8$		29.3	5.0	32.6	6.1		
T2	$1.47x10^8$	$1.31x10^8$	$0.98x10^8$	45.6	1.1	61.9	3.2	44.8	9.6
T3	$2.01x10^8$	$1.95x10^8$		25.9	0.7	43.3	4.9		
T4	$1.75x10^8$	$2.51x10^8$		35.3	9.0	26.9	9.2		
T5	$1.88x10^8$	$2.37x10^8$		30.2	1.7	31.0	6.9		
T0'	$1.74x10^8$	$1.66x10^8$							
T6	$1.13x10^8$	$1.36x10^8$	$1.09x10^8$	34.9	5.6	18.0	4.1	38.0	9.9

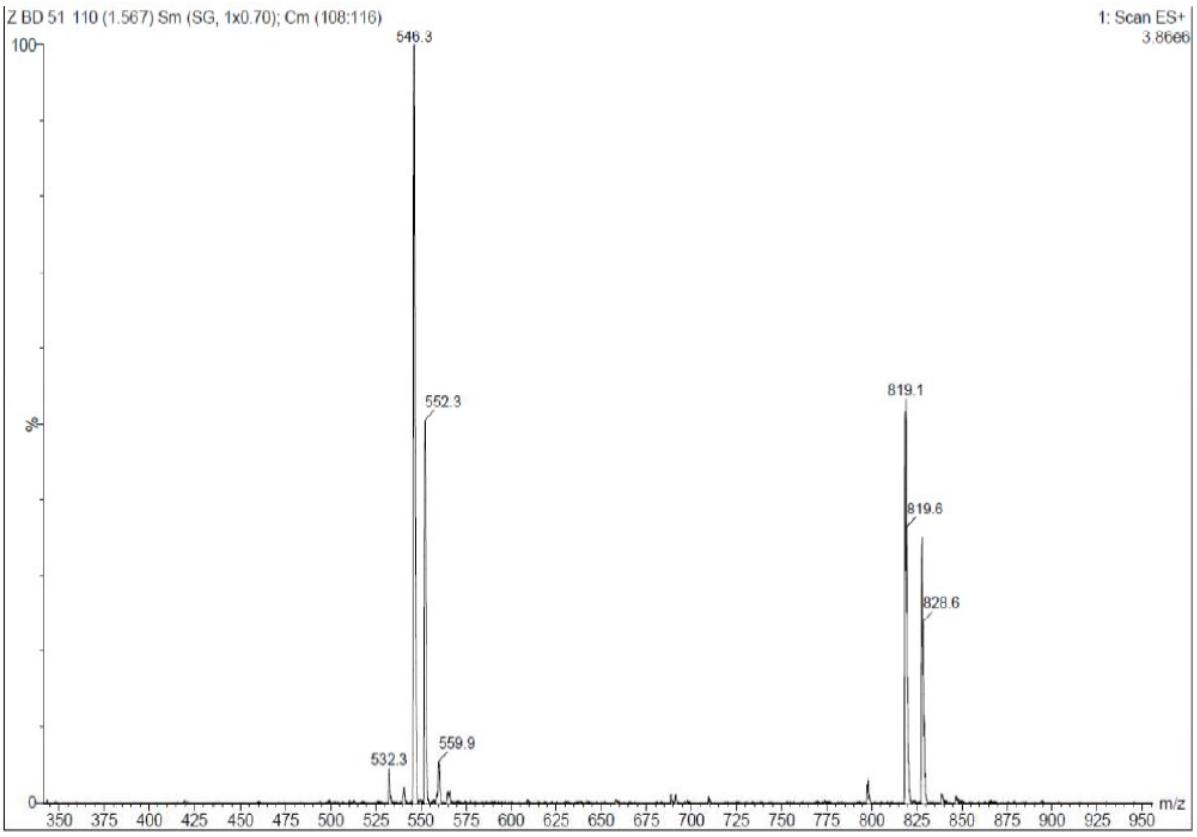
6. Statistical analysis

All the data values were expressed as **mean ± standard deviation/√n**, where n is the number of inputs used to calculate the mean value. Statistically significant value was set as $p > 0.05$ based on two-tailed t-tests.

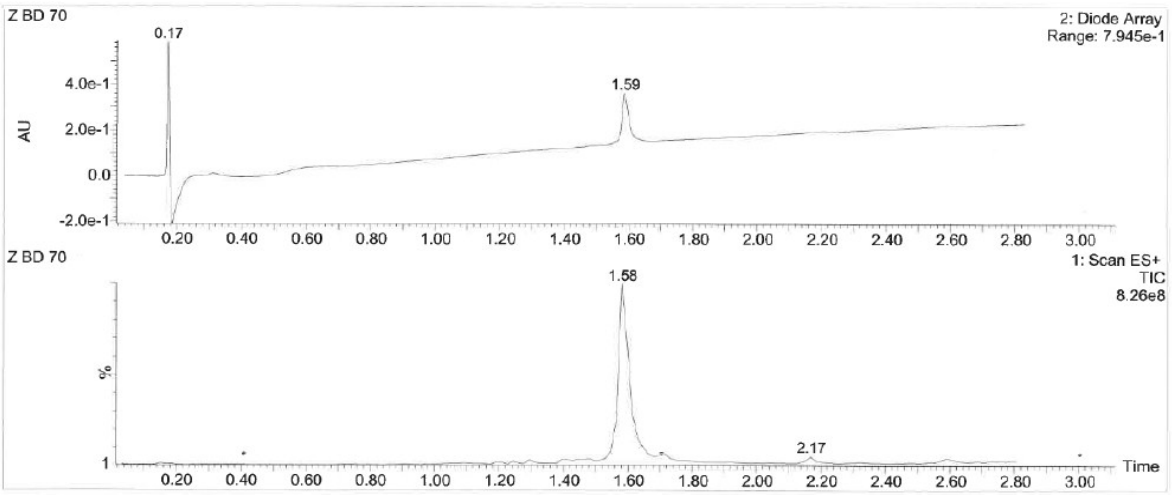
7. LC-MS analyses of compounds

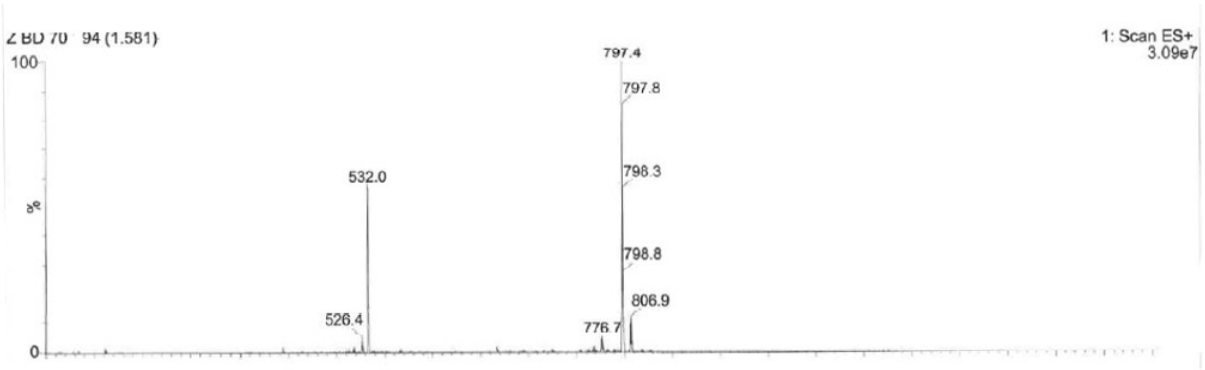
Compound **1**



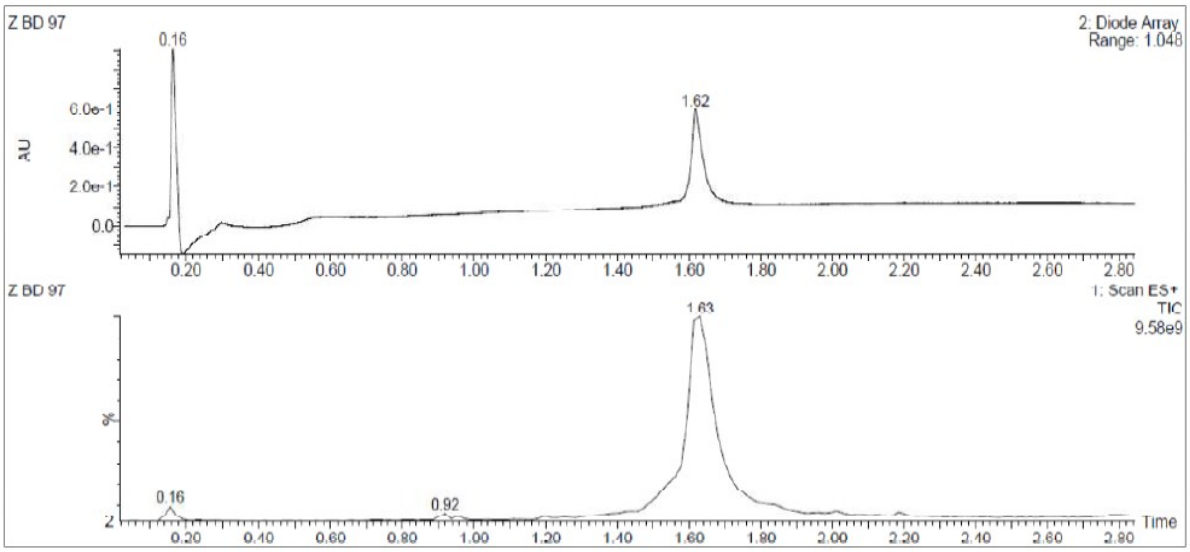

Z BD 51 110 (1.567) Sm (SG, 1x0.70); Cm (108:116)
1: Scan ES+
3.86e6
100
%
0
546.3
552.3
819.1
819.6
828.6
532.3
559.9
m/z
350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 725 750 775 800 825 850 875 900 925 950

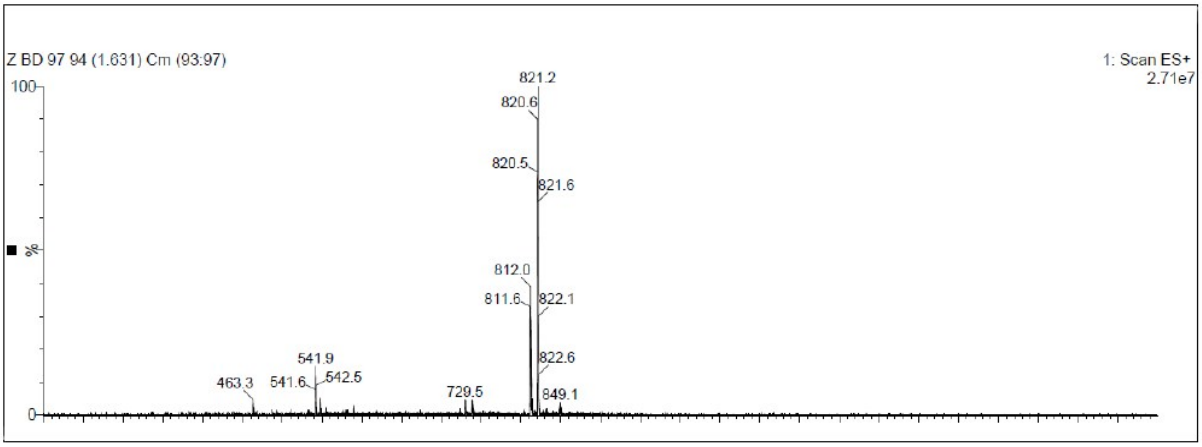
Compound **2**


Z BD 70
2: Diode Array
Range: 7.945e-1
0.17
1.59
AU
4.0e-1
2.0e-1
0.0
-2.0e-1
0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80 3.00
Z BD 70
1: Scan ES+
TIC
8.26e8
1.58
2.17
%
1
Time
0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80 3.00

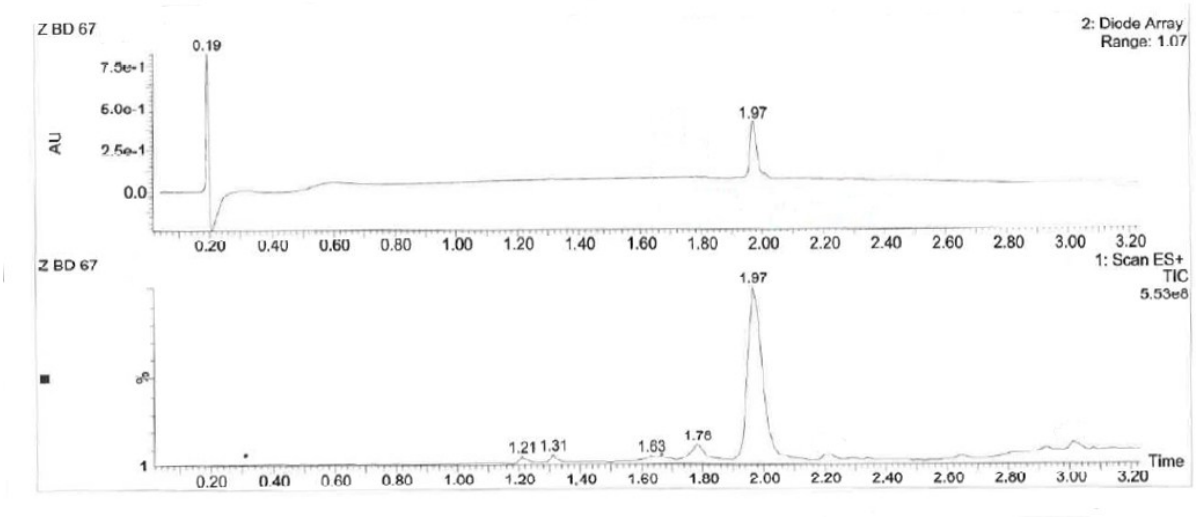

Z BD 70 94 (1.581)
1: Scan ES+
3.09e7
100
%
0
797.4
797.8
798.3
798.8
806.9
532.0
526.4
776.7

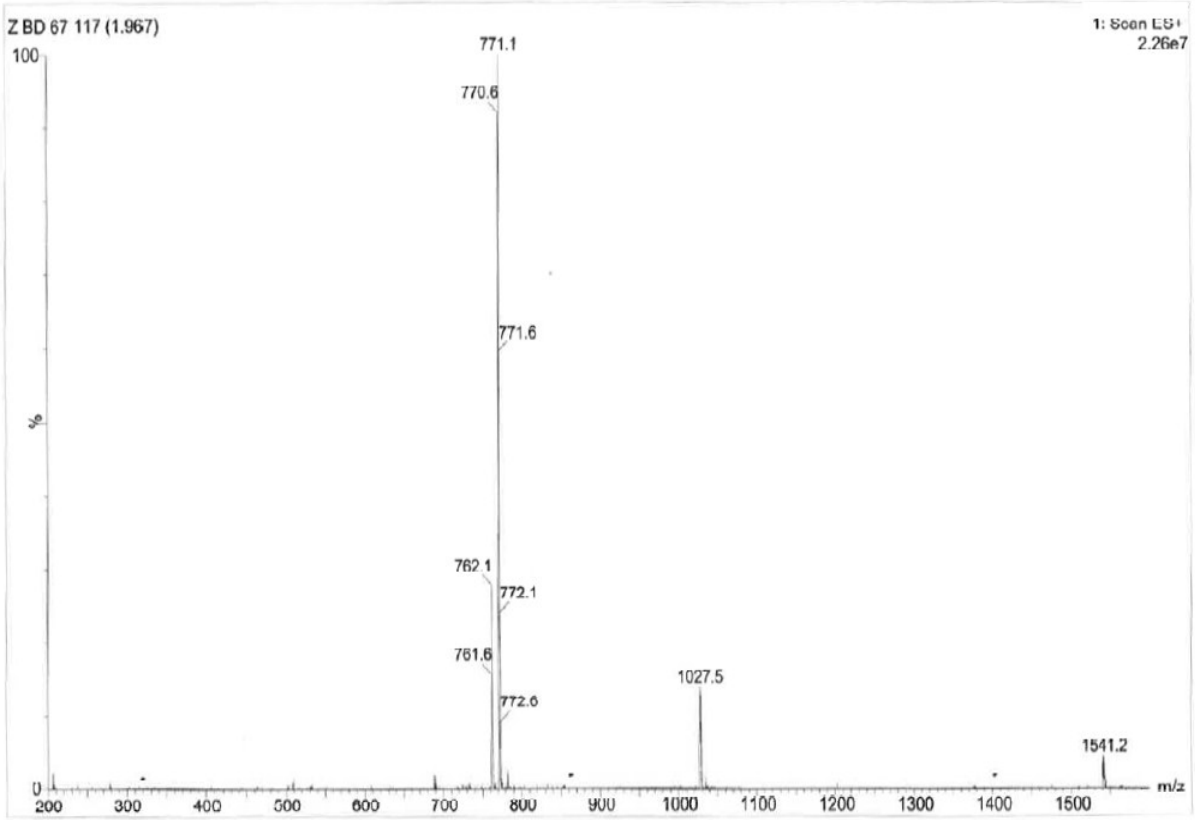
Compound **3**


Z BD 97
2: Diode Array
Range: 1.048
0.16
1.62
AU
0.0e-1
4.0e-1
2.0e-1
0.0
0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80
Z BD 97
1: Scan ES+
TIC
9.58e9
1.63
%
0.16
0.92
Time

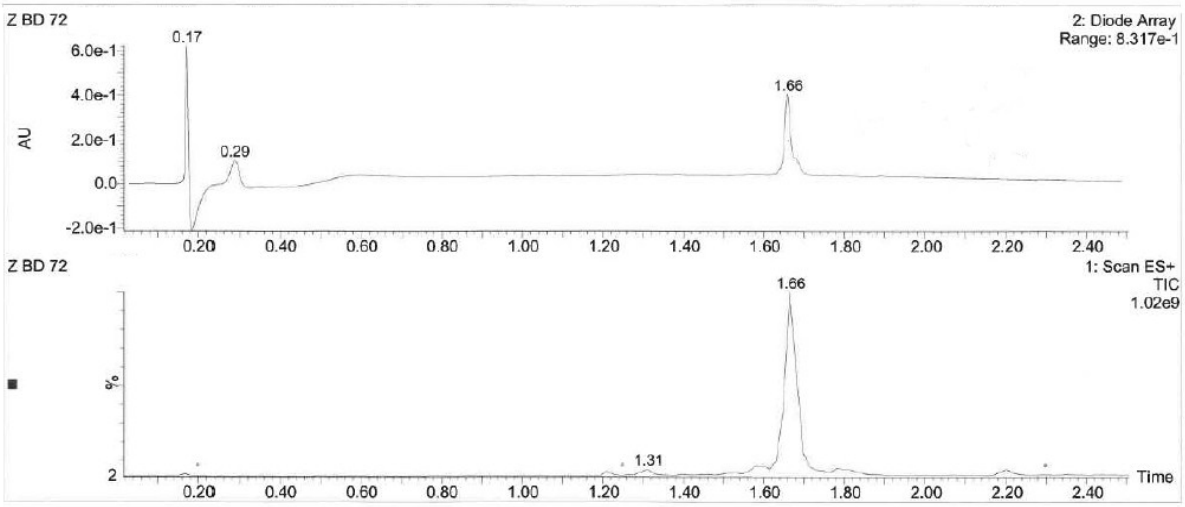

Z BD 97 94 (1.631) Cm (93:97)
1: Scan ES+
2.71e7
100
%
0
821.2
820.6
820.5
821.6
812.0
811.6
822.1
822.6
849.1
541.9
542.5
541.6
463.3
729.5

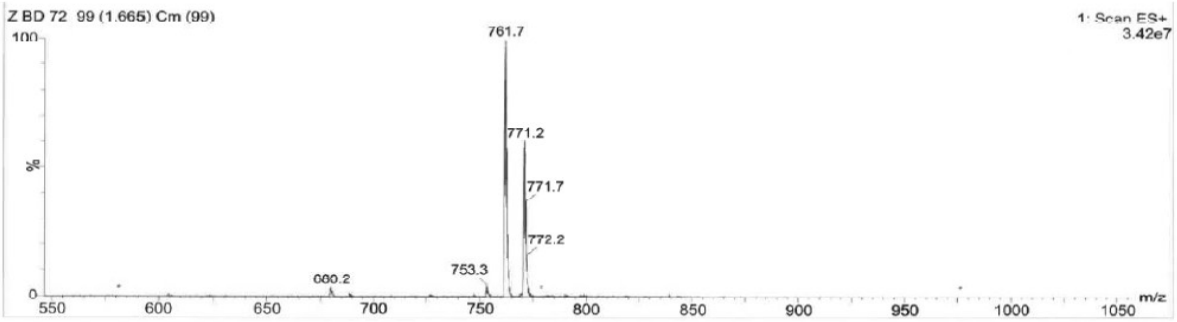
Compound **4**


Z BD 67
2: Diode Array
Range: 1.07
0.19
1.97
AU
7.5e-1
6.0e-1
2.5e-1
0.0
0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80 3.00 3.20
Z BD 67
1: Scan ES+
TIC
5.53e8
1.97
%
1.21 1.31
1.63
1.76
1
Time
0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80 3.00 3.20

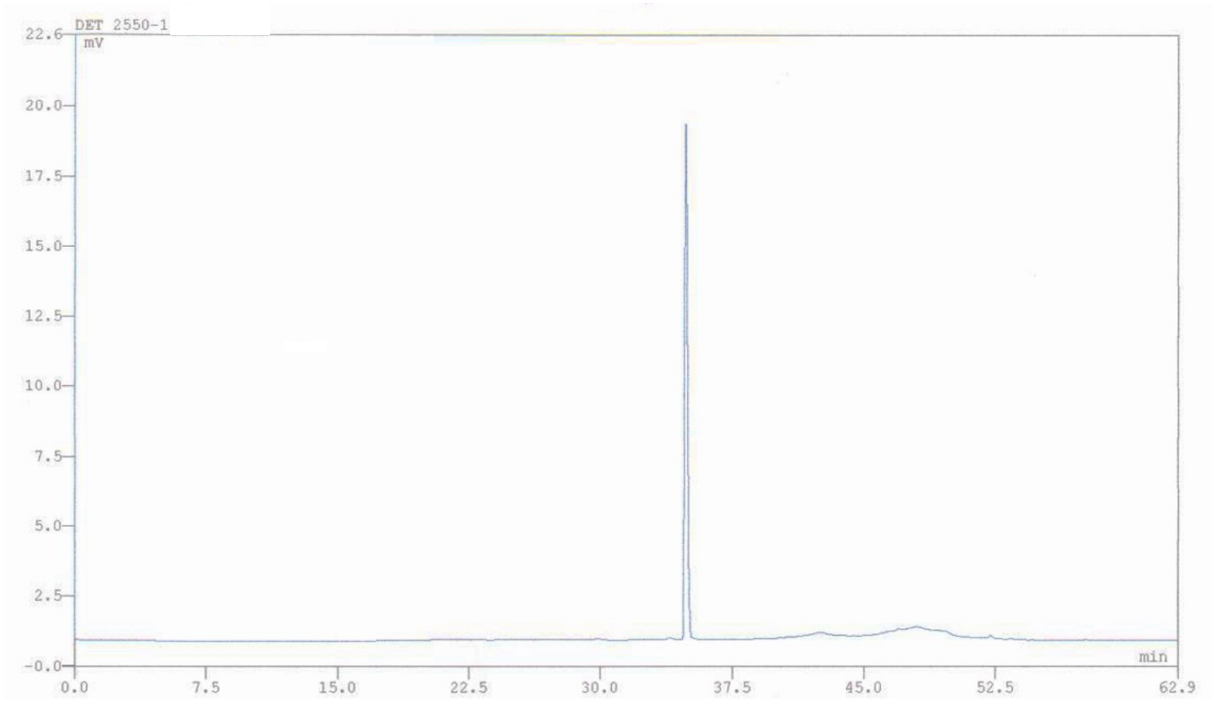

Z BD 67 117 (1.967)
1: Scan ES+
2.26e7
100
771.1
770.6
771.6
%
762.1
772.1
761.6
1027.5
772.6
1541.2
0
m/z
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500

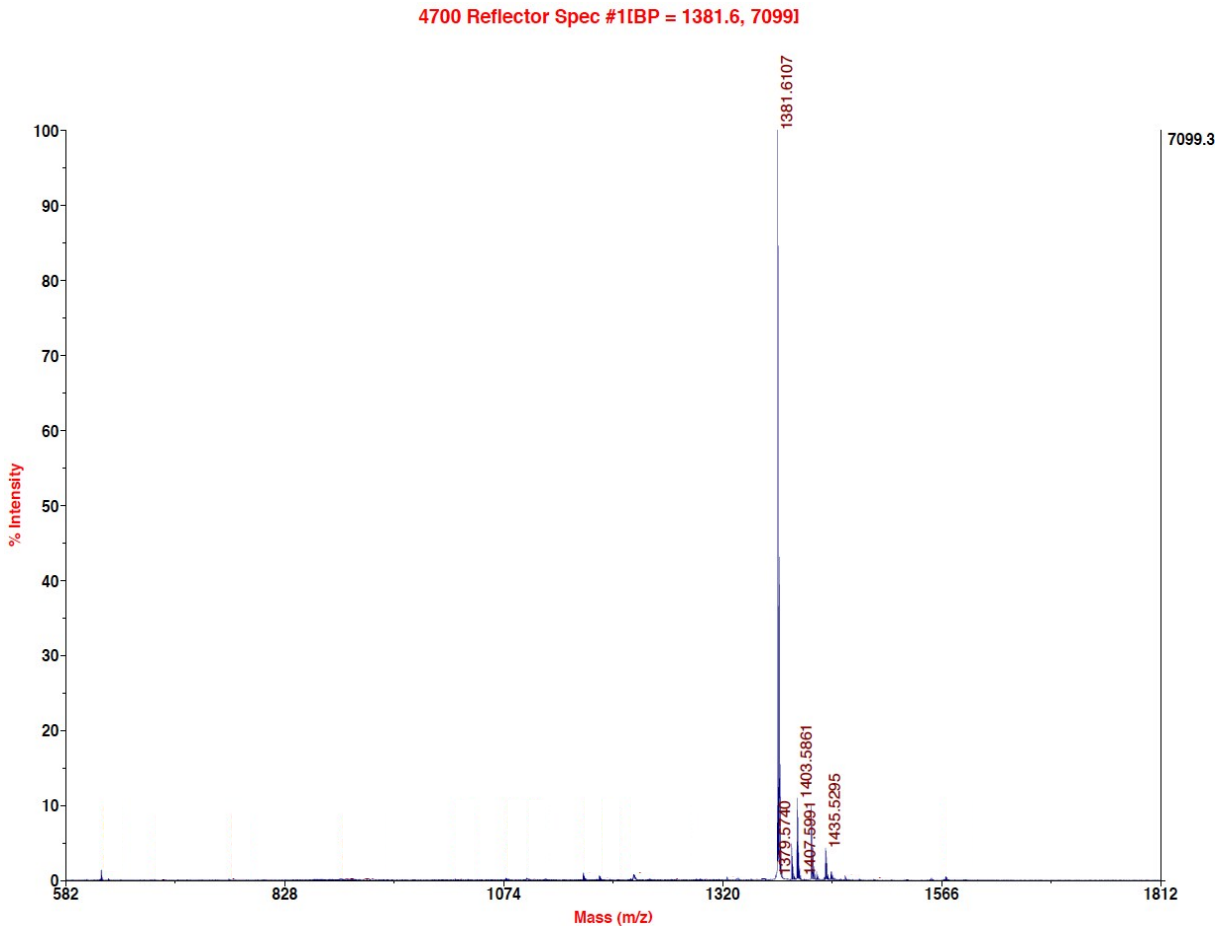
Compound **5**


Z BD 72
2: Diode Array
Range: 8.317e-1
0.17
0.29
1.66
AU
6.0e-1
4.0e-1
2.0e-1
0.0
-2.0e-1
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20
2.40
Z BD 72
1: Scan ES+
TIC
1.02e9
1.66
1.31
%
2
Time


Z BD 72 99 (1.665) Cm (99)
1: Scan ES+
3.42e7
100
761.7
771.2
771.7
772.2
753.3
660.2
%
0
550
600
650
700
750
800
850
900
950
1000
1050
m/z

Compound **6**


DET 2550-1
mV
22.6
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
-0.0
min
0.0
7.5
15.0
22.5
30.0
37.5
45.0
52.5
62.9


4700 Reflector Spec #1[BP = 1381.6, 7099]
1381.6107
7099.3
1403.5861
1435.5295
1379.5740
1407.5991
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
582
828
1074
1320
1566
1812