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Exploration of α/β/γ-Peptidomimetics Design for BH3 Helical Domain

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Contents

Materials

Fmoc α-amino acids and resin for solid phase peptide synthesis were purchased from Novabiochem or Chem-Impex, and coupling reagents and additives, O-benzotriazole -N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU), O-(7-azabenzotriazol-1yl)-N, N, N, N'-tetramethyluronium hexafluorophosphate(HATU), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), hydroxybenzotriazole (HOBt), 1-hydroxy-7 azabenzotoriazole) (HOAt), were purchased from Chem-Impex. 6-((4,4-Difluoro-1,3 dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2- propionyl)amino)hexanoic acid, succinimidyl ester (BODIPY-TMR-X-SE) was purchased from Invitrogen. Other reagents and solvents were purchased from Sigma Aldrich. Fmoc β^3-amino acids, were purchased from Chem-Impex. Acyclic γ-amino acids were purchased from Polypeptide group. Fmoc β- and γ- amino acids were prepared according to previous reports.[1]

Peptides synthesis and purification

All the α- and α/β/γ-peptides described in the present work were synthesized on Nova PEG rink amide resin (25-50 μmol scale) by microwave-assisted solid-phase method using a CEM MARS microwave reactor. [2-4] NovaPEG Rink Amide resin was swelled in DMF for 30 minutes prior to the sequential amino acid coupling reactions. For α- and cyclic/acyclic β-amino acids coupling reactions, 4 equiv of Fmoc-protected α- or β-amino acids, 3.95 equiv of HBTU, 8 equiv of diisopropylethylamine (DIEA), and 4 equiv of HOBT (0.1 M) were dissolved in DMF (1 mL DMF per 25 μmol resin scale) in a separate vial 1-2 minutes prior to the coupling reaction to pre-activate the amino acids. The amino acid solution was added to resin in a fritted syringe, which was then subjected to microwave irradiation: 2 minutes ramp to 70 °C, 4 minutes or 12 minutes hold at 70 °C for α- or β-amino acids, respectively. For acyclic γ-amino acid coupling reactions, the conditions used for β-amino acid coupling were employed, but DIEA was added just before the start of microwave irradiation in order to avoid the cyclization side-reaction of the γ-amino acid. Cyclic γ-amino acid coupling reactions were performed using 4 equiv of Fmoc-amino acid, 4 equiv of EDCI, 8 equiv of DIEA and 4 equiv of HOAt (0.1 M at final) in DMF (1 mL DMF per 25 μmol resin). The amino acid solution was added to the resin and allowed to nutate for 14 hours at room temperature. Fmoc deprotection reactions were carried out using 20 % (v/v) piperidine in DMF under microwave irradiation (2 min ramp to 80 °C, 2 min hold at 80 °C). The resin was washed with 3-5 resin volumes of DMF after each coupling and Fmoc deprotection reactions. The N-terminal amino group of the final residue was acetylated by stirring the resin in 8:2:1 (e.g., 1.6 mL:0.4 mL:0.2 mL for 25-50 μmol resin scale) DMF:DIEA:acetic anhydride solution for 10 minutes at room temperature. The resin was washed thoroughly (3×DMF, 3×CH_2Cl_2, 3×MeOH) and then dried under vacuum.

Peptides were globally deprotected and cleaved from the resin by suspending the resin in cleavage cocktail (95% trifluoroacetic acid (TFA), 2.5 % water, and 2.5% triisopropylsilane)

for 4-5 hours at room temperature. After the filtration, the crude peptide in TFA filtrate was concentrated under a stream of nitrogen, precipitated by addition of cold diethyl ether. The mixture was then centrifuged, decanted, and the remaining solid was drided under a stream of nigrogen. The crude peptides were purified using a preparative reverse-phase HPLC with a C4 or C18 column (Vydac, Anaheim, CA), and eluted with gradients of MeCN w/0.1% TFA (solvent B) in water w/0.1% TFA (solvent A). The peptide identity was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The purity of the peptides was evaluated by analytical reverse-phase HPLC. In all cases, the purity was over 95 %. The concentration of each peptide stock in DMSO was determined by measuring absorbance at 280 nm using UV spectroscopy. The molar extinction coefficeint for each peptide was calculated based on the presence of Trp (ε = 5690 $M^{-1}cm^{-1}$) and Tyr (ε = 1280 $M^{-1}cm^{-1}$).

Synthesis of fluorescently labeled peptides

BODIPY^{TMR}-Bak (BODIPY^{TMR}-GQVGRQLAIIGDDINR-NH_2)

Flu-Bim (6-carboxyfluorescein-WIAQELRRIGDEFNA-NH_2)

Flu-8 (6-carboxyfluorescein-IWIAγ_{cyc}LRZIGγ^4DFNXKY-NH_2)

Peptides Bak, Bim and α/β/γ-peptide **8** were prepared as described above and labeled with BODIPY-TMR or Fluorescein using previously reported methods.[5] For labeling peptides with Fluorescein, the Fmoc-deproteced α/β/γ-peptide **8** or α-Bim 15-mer was coupled with three equiv of 6-carboxyfluorescein as a last step for 12 hours at room temperature with the same coupling condition used for the α-amino acid couplings. The peptides were subsequently deprotected, cleaved, and purified. For BODIPY labeling, Bak was synthesized with N-terminus as free amine, cleaved, purified and lyophilized. Bak (2 mg/mL) was dissolved in 0.1 M $NaHCO_3$ at pH 8.3, and added with 15 equiv of BODIPY-TMR-SE (10 mM DMSO stock). The mixture was reacted for 8 hours on a mechanical shaker at dark. The peptide was purified by reverse-phase HPLC using a C18 column, and the identity and the purity was confirmed by MALDI-TOF-MS and analytical HPLC, respectively.

Fluorescence Polarization (FP) assays

Bcl-xL (plasmid: human His-EK-Bcl-xL, residue 1-196) was expressed in BL21(DE3) *E. coli* cells, and purified using Ni-NTA agarose (Qiagen) column and FF Q column (GE healthcare) as previously described.[5] FP assays were conducted at room temperature in 384-well, black polystyrene plates (Costar). Competitive and direct binding FP assays were conducted as reported previously.[6] Briefly, for direct binding assays were carried out by titrating a fixed concentration (10 nM) of tracer (BODIPY^{TMR}-Bak, Flu-Bim) with increasing concentrations (0.019 – 625 nM) of Bcl-xL in assay buffer (50 mM NaCI, 16.2 mM Na_2HPO_4, 3.8 mM KH_2PO_4, 0.15 mM NaN_3, 0.15 mM EDTA, 0.5 mg/mL Pluoronic-F68, pH 7.5) in a total volume of 50 µL per well. The binding reaction was allowed to equilibrate in for ~5 hours,

and analyzed on an Envision 2100 plate reader.

For competition FP assays the final concentrations of BODIPY-TMR-Bak tracer and Bcl-xL was 3 nM and 2 nM, respectively in FP buffer, and was added 2 μL aliquots of serial dilutions of α- or α/β/γ- Bim mimic peptides in DMSO with final concentrations ranging from 1.7 nM to 100 μM. The binding reaction was allowed to equilibrate in for ~5 hours, and plates were analyzed on an Envision 2100 plate reader.

Not all compounds showed complete inhibition at the highest peptide concentration. Thus, for the data normalization (normalized anisotropy, %), the standard top (tracer full bound) values were from the assay without competitors and the standard bottom (tracer fully displaced) values were adopted from 15mer and 18mer α-Bim (control competitors) within the given dataset. The curves from the data were plotted by non-linear dose response with variable slope using Graphpad Prism.

Circular dichroism spectroscopy

Elipticity spectra of wavelength scans (260 nm - 190 nm) were recorded in a 1 mm quartz cell with an averaging time of 6 sec for each step (1 nm step size) in pH 7.5 PBS buffer and in 50 % methanol/50% PBS buffer at 20 °C (AVIV circular dichroism spectrometer model 420). Peptides were prepared as 25-100 μM for the CD measurements. The CD data for wavelengths at which the dynode voltage was <400 V were used. Mean residue ellipticity (θ) was calculated using the following equqtion, where δ_S = sample signal, δ_R = reference signal, n = # of amides in the backbone, l = path length (in cm), and c = sample concentration (in dmol·cm^{-3}).

$$[\theta] = [(\delta_S - \delta_R)/(100nlc)] / 1000$$

Proteolysis assay

100 μM peptide stock solutions and 25 μg/mL proteinase K stock solution were prepared in TBS buffer, pH 7.5. Proteolysis reactions were performed in 50 μL TBS buffer containing 50 μM peptide with 5 μg/mL proteinase K at room temperature. The reactions were quenched at the desired time point by adding 100 μL of 1% TFA in 50:50 MeCN/H_2O. The proteolysis process was monitored at 220 nm by injecting 125 μL of the resulting solution onto an analytical reverse-phase HPLC column.

Molecular Modelling (Peptide conformational search)

Molecular models of 18-mer Bim and α/β/γ-peptide **13** energy conformers were generated with MacroModel. The X-ray crystallographic structure of 25-mer Bim bound Bcl-xL (PDB: 3FDL) was prepared as the initial starting structure. Waters beyond 5 Å from hetero groups were deleted. The 18-mer Bim was prepared by deleting respective residues from the 25-mer Bim structure. With the Bcl-x_L protein frozen, the prepared 18-mer Bim was minimized using the MMFFs and GB/SA water solvation to result a minimization converged model. The

minimized model was then searched for energy conformers with conformational search module using the MMFFs force field and GB/SA water solvation with Bcl-x_L protein frozen and no restraints on the minimized 18-mer Bim model. Relative conformers within 4 kJ/mol from the lowest-energy for 18-mer Bim was overlayed. For α/β/γ-peptide **13,** the same steps were followed. The residue overlay is from the lowest energy conformer found for 18-mer Bim and and α/β/γ-peptide **13**. The four key residues were extracted and overlayed for RMSD calculation. 35 atoms out of 35 atoms were used.

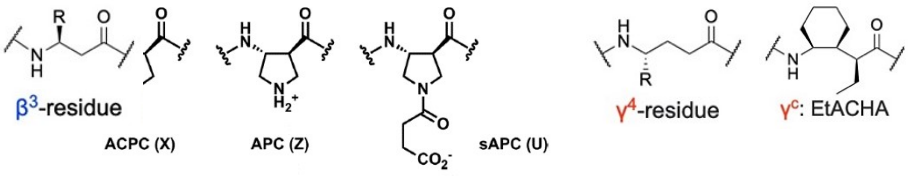
Table S1. List of α/β/γ peptides and mass spectroscopic data measured by MALDI-TOF-MS.

		Mass expected	Mass measured
1	Ac–W I A(Z) E L γ⁴hA I G β³hD E F γ⁴hA–NH_2	1557.7	1557.7
2	Ac–W I A(Z) E L(γcyc) I G β³hD E F(γcyc)–NH_2	1693.7	1693.7
3	Ac–W I A(Z) E L(γcyc) I G β³hD E F γ⁴hA–NH_2	1626.0	1626.0
4	Ac–W I A(Z) E L γ⁴hA I G β³hD E F(γcyc)–NH_2	1626.5	1626.5
5	Ac–W I A γ⁴hA L R(Z) I G γ⁴hD F N(Z)–NH_2	1596.8	1596.8
6	Ac–W I A(γcyc) L R(Z) I G γ⁴hD F N(Z)–NH_2	1664.9	1664.8
7	Ac–W I A(γcyc) L R(Z) I G γ⁴hD F N(X)–NH_2	1664.0	1663.7
8	Ac–I W I A(γcyc) L R(Z) I G γ⁴hD F N(Z) K Y–NH_2	2069.2	2069.2
9	Ac–I(X) I A(γcyc) L R(Z) I G γ⁴hD F N(Z) K Y–NH_2	1994.2	1994.1
10	Ac–I(X) I A(γcyc) L R(Z) I G γ⁴hD F N(X) K Y–NH_2	1993.2	1993.3
11	Ac–I(X) I A γ⁴hA L R(Z) I G γ⁴hD F N(X) K Y–NH_2	1925.1	1924.8
12	Ac–W I A(γcyc) L R β³hR I G γ⁴hD F N(Z)–NH_2	1723.0	1723.1
13	Ac–I(X) I A(γcyc) L R β³hR I G γ⁴hD F N(X) K Y–NH_2	2051.2	2051.1
14	Ac–I W I A(γcyc) L R β³hR I G γ⁴hD F N(X) K Y–NH_2	2126.2	2127.0
15	Ac–I(X) I A γ⁴hA L R β³hR I G γ⁴hD F N(X) K Y–NH_2	1983.2	1983.0
Flu-8	Flu–I W I A(γcyc) L R(Z) I G γ⁴hD F N(X) K Y–NH_2	2418.4	2418.4

Table S2. Susceptibility comparison for the Bim BH3 domain, selected α/β- and α/β/γ-analogues to degradation by proteinase K. Blue letters indicate β^3- or cyclic β- (when the letters are circled) residues. Red letters indicate γ^4- or cyclic γ- (when the letters are circled) residues.

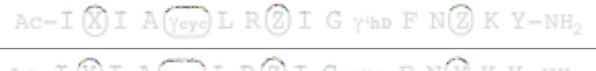
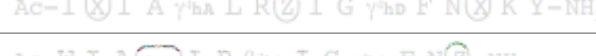
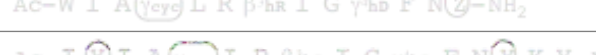
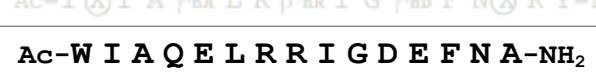
	$t_{1/2}$ (min)
Ac - I W I A Q E L R R I G D E F N A Y Y - NH_2	1.7[a]
Ac - I W I A Q E L R R I G D E F N A Y Y - NH_2	29[a]
Ac - I (X) I A Q (U) L R (Z) I G D (U) F N (X) Y Y - NH_2	210[a]
Ac - I (X) I A (γ_{cyc}) L R (Z) I G γ^4D F N (X) Y Y - NH_2	> 2700
Ac - I (X) I A (γ_{cyc}) L R R I G γ^4D F N (X) Y Y - NH_2	> 900

Residue specifications are shown below the table.



[a] Data were taken from the Table 4 in ACS Chem. Biol., 2015, 10, 1667. The procedures for the proteolytic digestion analysis are the same for all data. The $t_{1/2}$ values for the α/β/γ-analogues were based on the last minutes tested.

Table S3. IC_{50} values estimated from the FP competition assay. IC_{50} values were calculated from the normalized anisotropy (%) curves. The IC_{50} values (in parentheses) of α/β/γ-**8** and **14** were estimated from the lower concentration region data due to the aggregation problem at higher concentration.

		IC_{50} (μM)
α/β/γ 1		-
α/β/γ 2		-
α/β/γ 3		-
α/β/γ 4		-
α/β/γ 5		-
α/β/γ 6		-
α/β/γ 7		-
α/β/γ 8		(5.7)
α/β/γ 9		28
α/β/γ 10		18
α/β/γ 11		-
α/β/γ 12		10
α/β/γ 13		2.6
α/β/γ 14		(1.8)
α/β/γ 15		-
Bim 15mer	**Ac-W I A Q E L R R I G D E F N A-NH_2**	1.7
Bim 18mer	**Ac-I W I A Q E L R R I G D E F N A Y Y-NH_2**	0.11

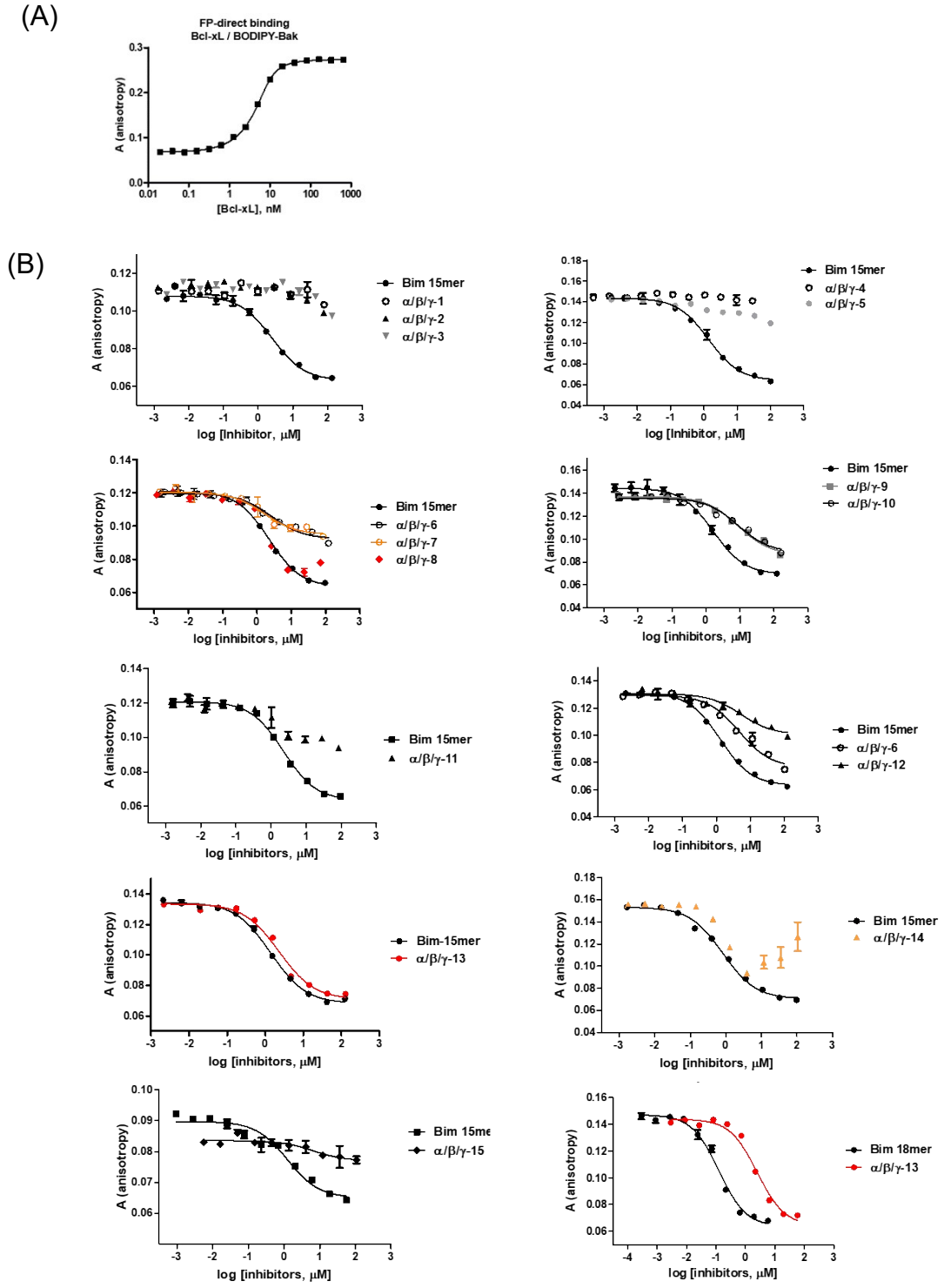


Figure S1. (A) Representative Bcl-xL direct binding FP data for BODIPY^{TMR}-labeled Bak 16-mer (Bak 16-mer: GQVGRQLAIIGDDINR).[6,7] (B) Representative Bcl-xL competition binding FP data for α-Bim and α/β/γ-peptides. [Bcl-xL]= 2 nM, [Bak-tracer]= 3 nM. Bim 18-mer IC_{50}

= 0.11±0.02 μM, Bim 15-mer IC_{50} = 1.7±0.4 μM, α/β/γ-peptide **13** IC_{50} = 2.6±0.3 μM.

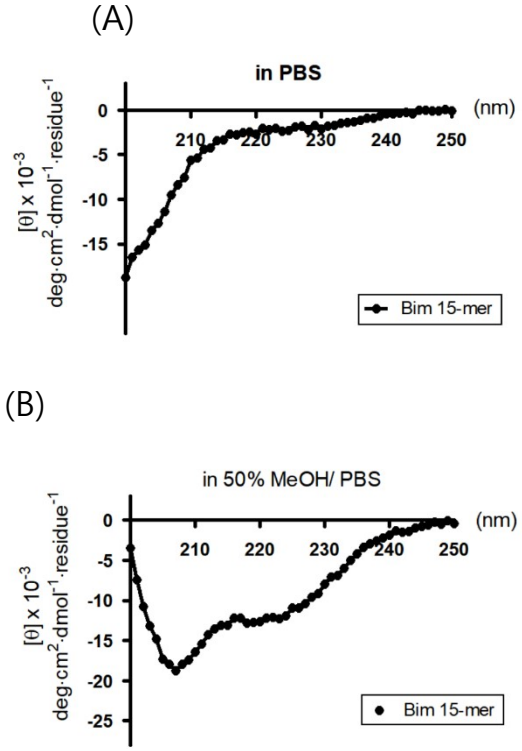


Figure S2. Circular dichroism data for α-Bim 15-mer in phosphate buffer (A) and 50 % methanol/PBS buffer (B). Peptide concentration is 100 μM. CD data for α-Bim 18-mer showed helical folding in phosphate buffer.[6] [ref 6. 2012 JACS Figure 7]

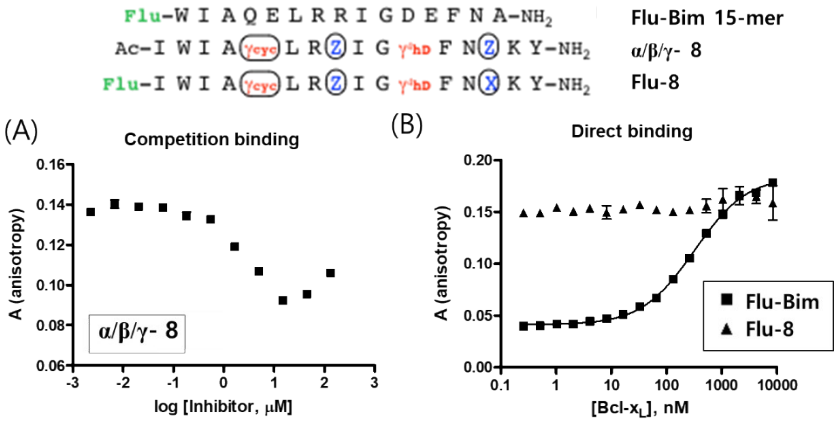


Figure S3. Sequences of Fluorescein labeled α-Bim 15mer and α/β/γ-**8** with and without Fluorescein label. (A) FP spectra of competition binding of α/β/γ-**8** (no Fluorescein label) to the Bcl-xL.(B) FP spectra of direct binding of Flu-Bim and Flu-**8** to the Bcl-xL protein.

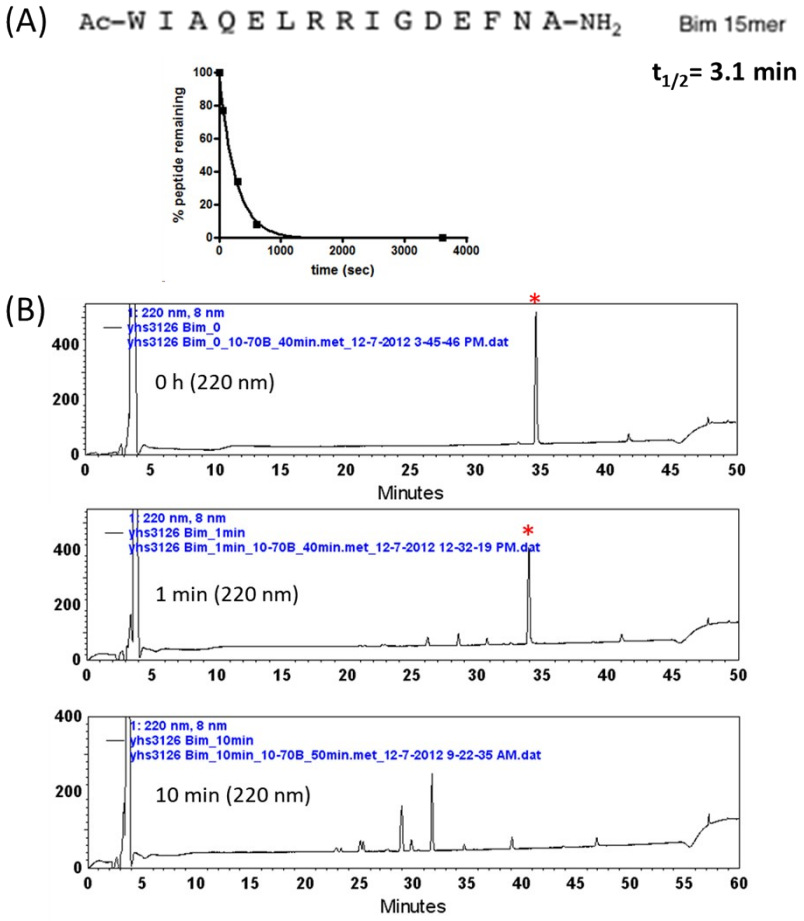


Figure S4. (A) Sequences of α-Bim 15-mer and a curve resulting from proteolysis of α-Bim 15-mer in the presence of proteinase K as a function of time; the data were fit to an exponential decay model in GraphPad Prism. (B) HPLC traces of the α-Bim 15-mer at difference time potins during incubation in proteinase K solution. (10-70% MeCN, 5 min)

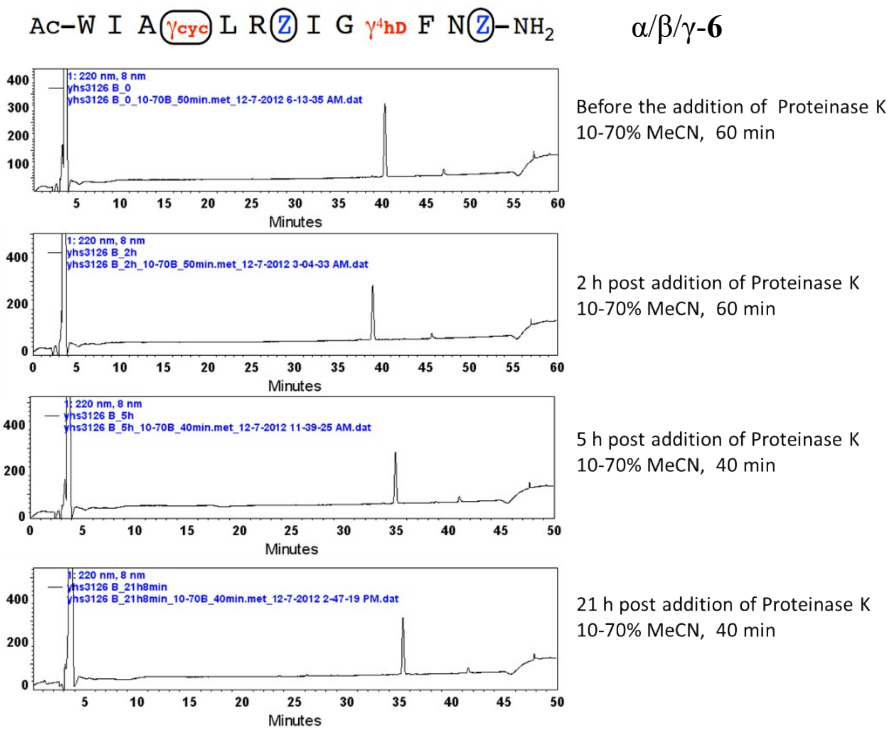


Figure S5 HPLC traces of the α/β/γ-peptide **6** at difference time points during incubation in Proteinase K solution. HPLC traces were monitored at 220 nm.

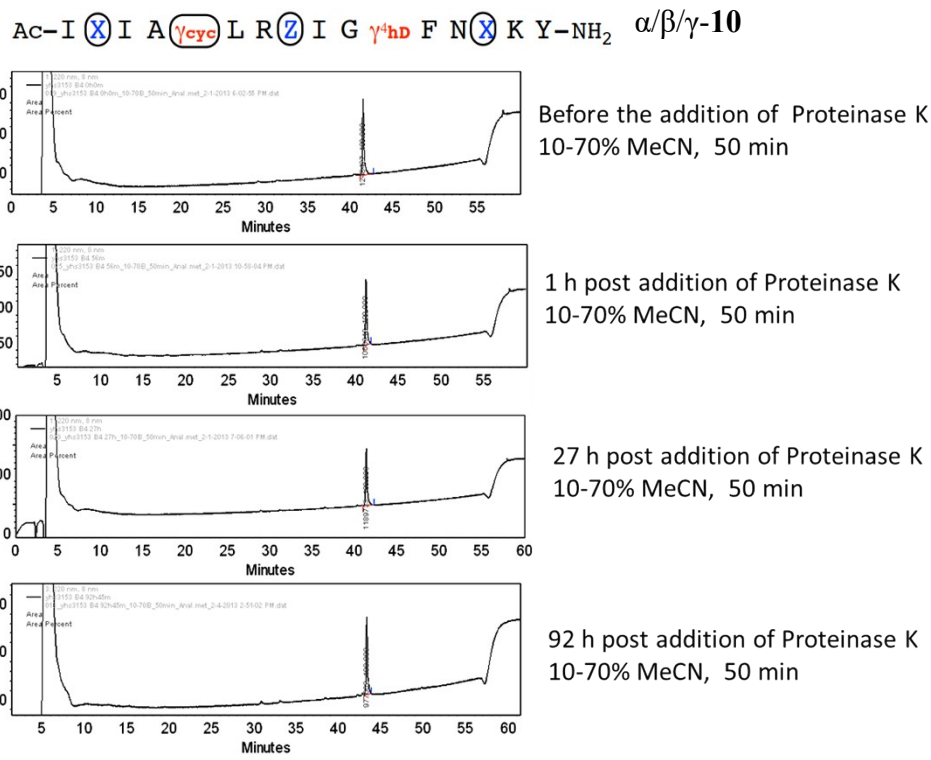


Figure S6 HPLC traces of the α/β/γ-peptide **10** at difference time points during incubation in Proteinase K solution. HPLC traces were monitored at 220 nm.

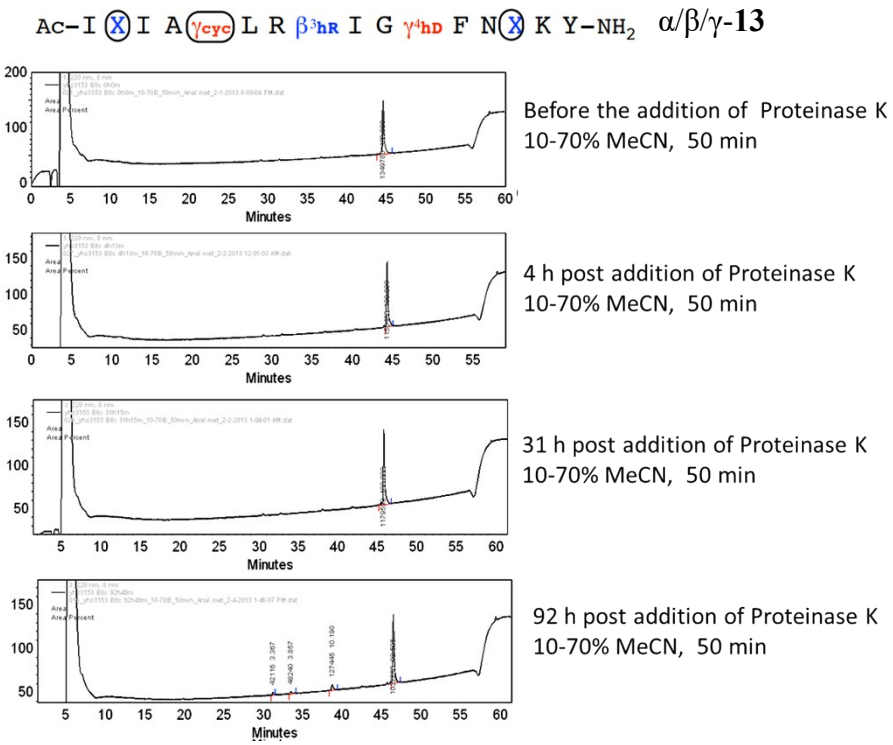


Figure S7 HPLC traces of the α/β/γ-peptide **13** at difference time points during incubation in Proteinase K solution. HPLC traces were monitored at 220 nm.

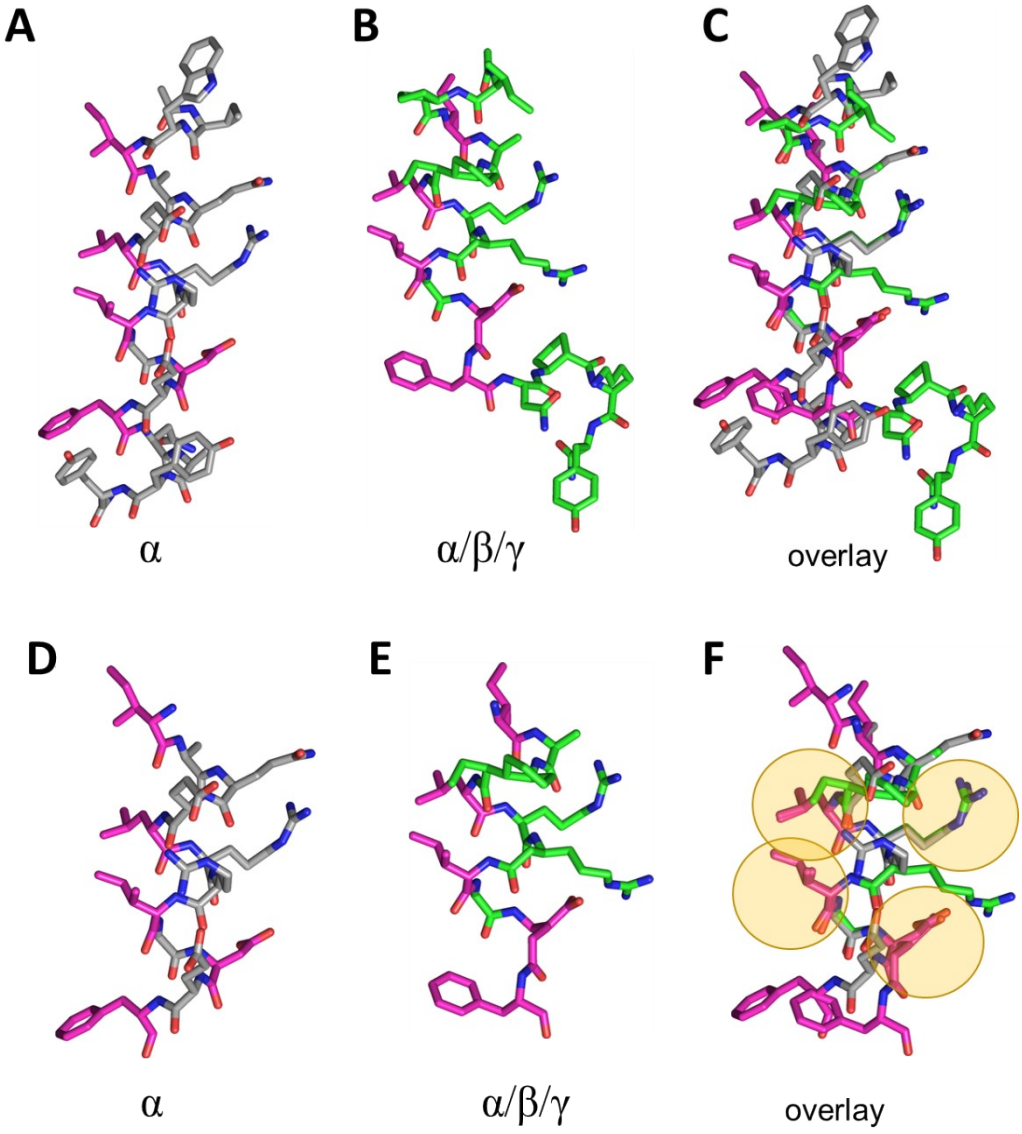


Figure S8 Molecular model of lowest-energy conformer of 18-mer Bim and α/β/γ-peptide **13**. The models were generated by conformational searching in MacroModel using the MMFFs force field and GB/SA water solvation. The initial Bcl-x_L bound structures of 18-mer Bim and α/β/γ-peptide **13** were generated by mutating and minimizing the X-ray crystallographic structure of Bim bound Bcl-x_L (PDB: 3FDL). Five key residues are represented in magenta color. The bound Bcl-x_L is removed for better visualization of the peptides.

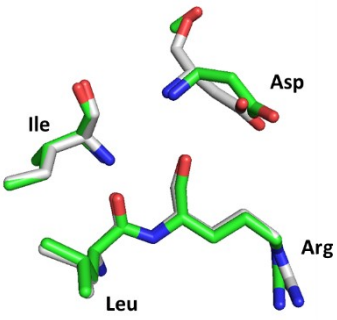


Figure S9 The four residues from 18-mer Bim (gray) and α/β/γ-peptide **13** (green) used for the RMSD calculation (35 to 35 atoms, 0.787 Å). The residue overlay is from Fig S8.

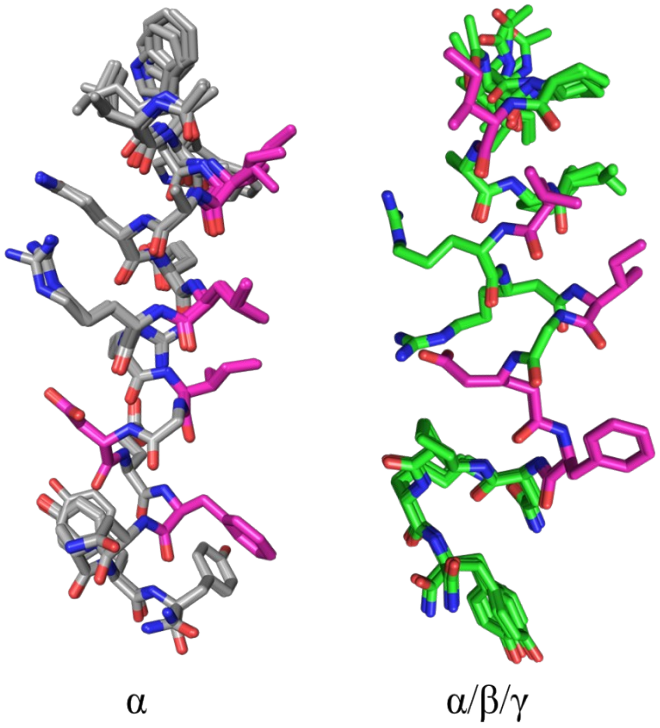
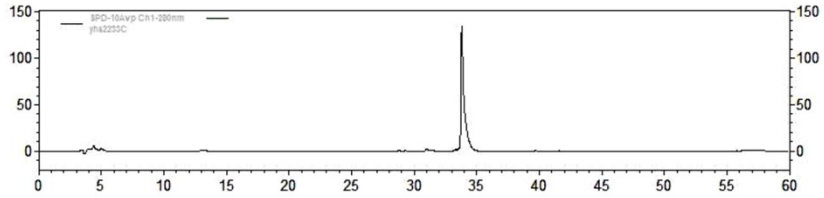


Figure S10 Molecular model of relative conformers within 4 kJ/mol from the lowest-energy for 18-mer Bim and α/β/γ-peptide **13**. The models were generated by conformational searching in MacroModel using the MMFFs force field and GB/SA water solvation. The initial Bcl-x_L bound structures of 18-mer Bim and α/β/γ-peptide **13** were generated by mutating and minimizing the X-ray crystallographic structure of Bim bound Bcl-xL (PDB: 3FDL). Five key residues are represented in magenta color. The bound Bcl-x_L is removed for better visualization of the peptides.

HLPC traces for α/β/γ-peptide purity check (1-15, Flu-8)

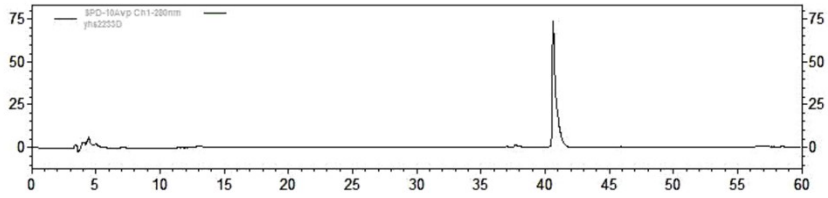
Ac–W I A(Z) E L γ⁴hA I G β³hD E F γ⁴hA–NH_2 α/β/γ-**1**

10-60 % MeCN, 50 min



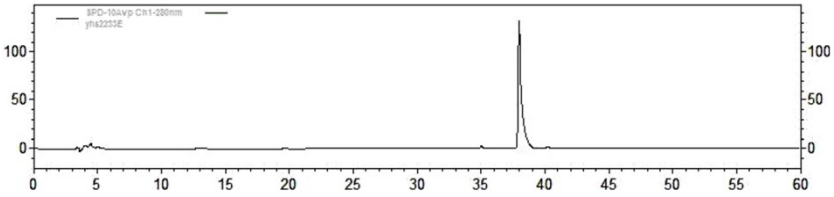
Ac–W I A(Z) E L (γcyc) I G β³hD E F (γcyc)–NH_2 α/β/γ-**2**

10-60 % MeCN, 50 min



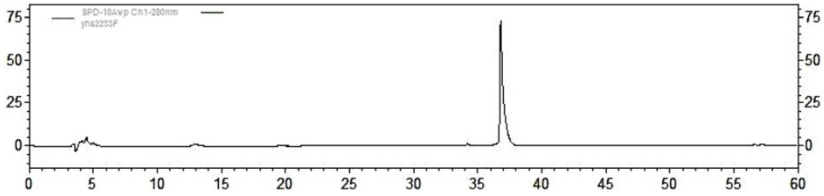
Ac–W I A(Z) E L (γcyc) I G β³hD E F γ⁴hA–NH_2 α/β/γ-**3**

10-60 % MeCN, 50 min



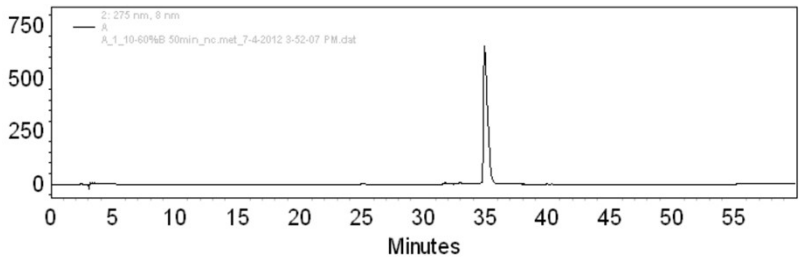
Ac–W I A(Z) E L γ⁴hA I G β³hD E F (γcyc)–NH_2 α/β/γ-**4**

10-60 % MeCN, 50 min



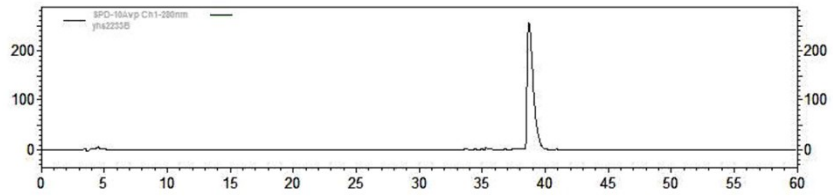
Ac–W I A γ⁴hA L R(Z) I G γ⁴hD F N(Z)–NH_2 α/β/γ-**5**

10-60 % MeCN, 50 min



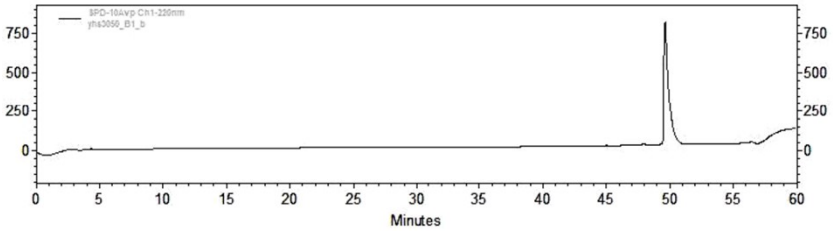
α/β/γ-**6**

10-60 % MeCN, 50 min



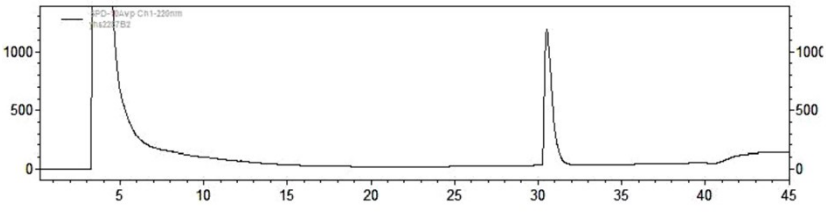
Ac–W I A(γcyc) L R(Z) I G γ⁴hD F N(X)–NH_2 α/β/γ-**7**

10-60 % MeCN, 50 min



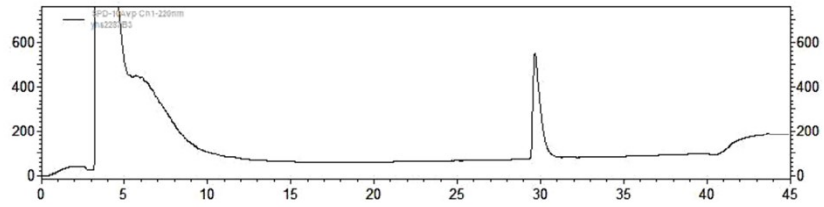
Ac–I W I A(γcyc) L R(Z) I G γ⁴hD F N(Z) K Y–NH_2 α/β/γ-**8**

10-70 % MeCN, 35 min



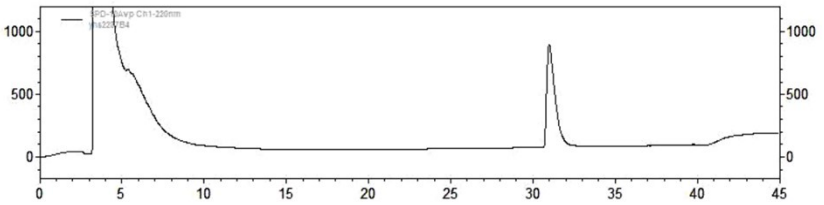
Ac–I (X) I A (γcyc) L R (Z) I G γ⁴hD F N (Z) K Y–NH_2 α/β/γ-**9**

10-70 % MeCN, 35 min



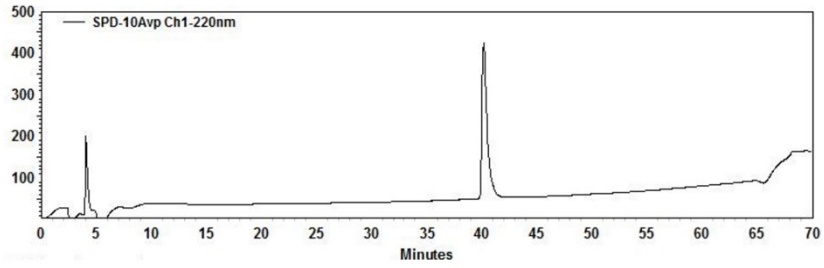
α/β/γ-**10**

10-70 % MeCN, 35 min



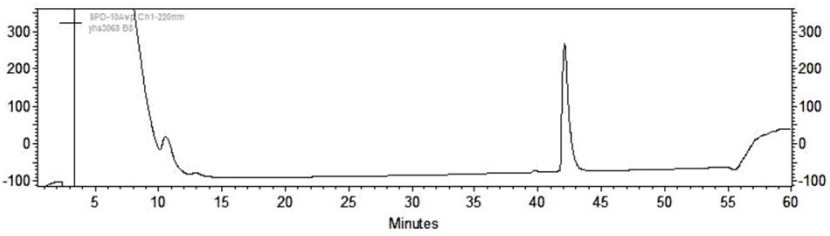
Ac–I (X) I A γ⁴hA L R (Z) I G γ⁴hD F N (X) K Y–NH_2 α/β/γ-**11**

10-90 % MeCN, 50 min



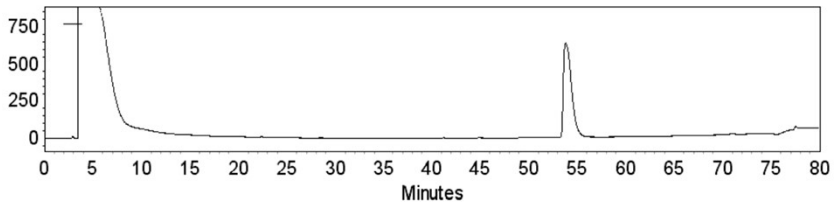
Ac–W I A (γcyc) L R β³hR I G γ⁴hD F N (Z)–NH_2 α/β/γ-**12**

10-60 % MeCN, 50 min



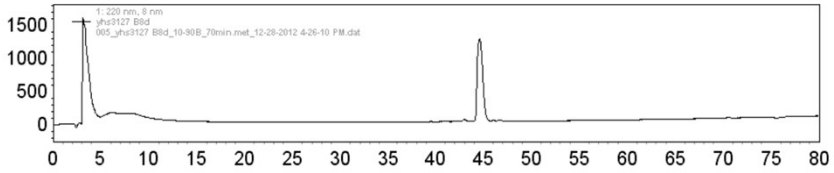
α/β/γ-**13**

10-80 % MeCN, 70 min



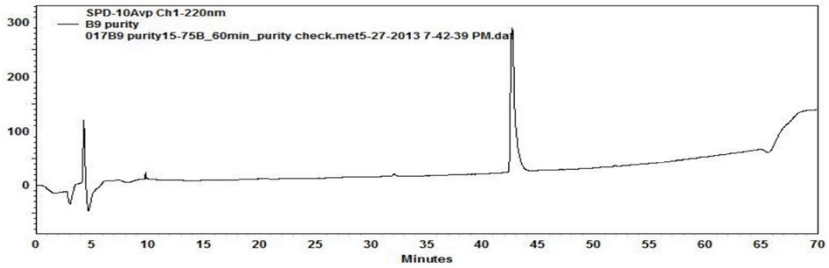
Ac–I W I A(γcyc) L R β³ʰR I G γ⁴ʰD F N(X) K Y–NH_2 α/β/γ-**14**

10-80 % MeCN, 70 min



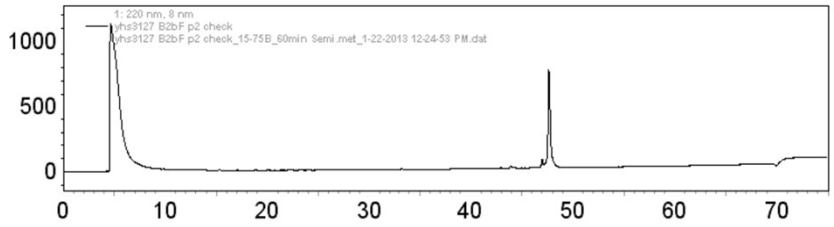
Ac–I(X) I A γ⁴ʰA L R β³ʰR I G γ⁴ʰD F N(X) K Y–NH_2 α/β/γ-**15**

15-75 % MeCN, 60 min

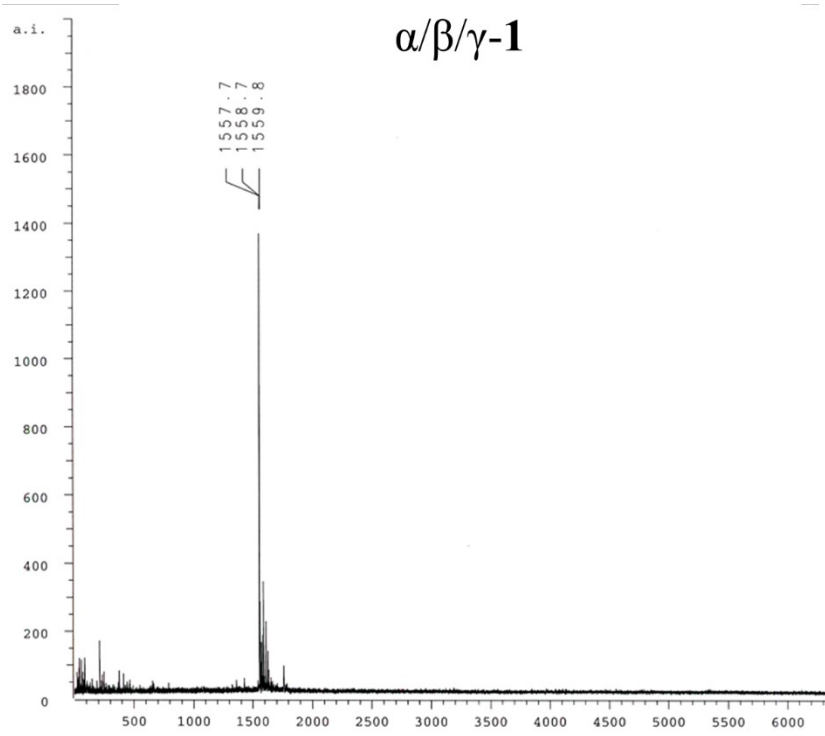


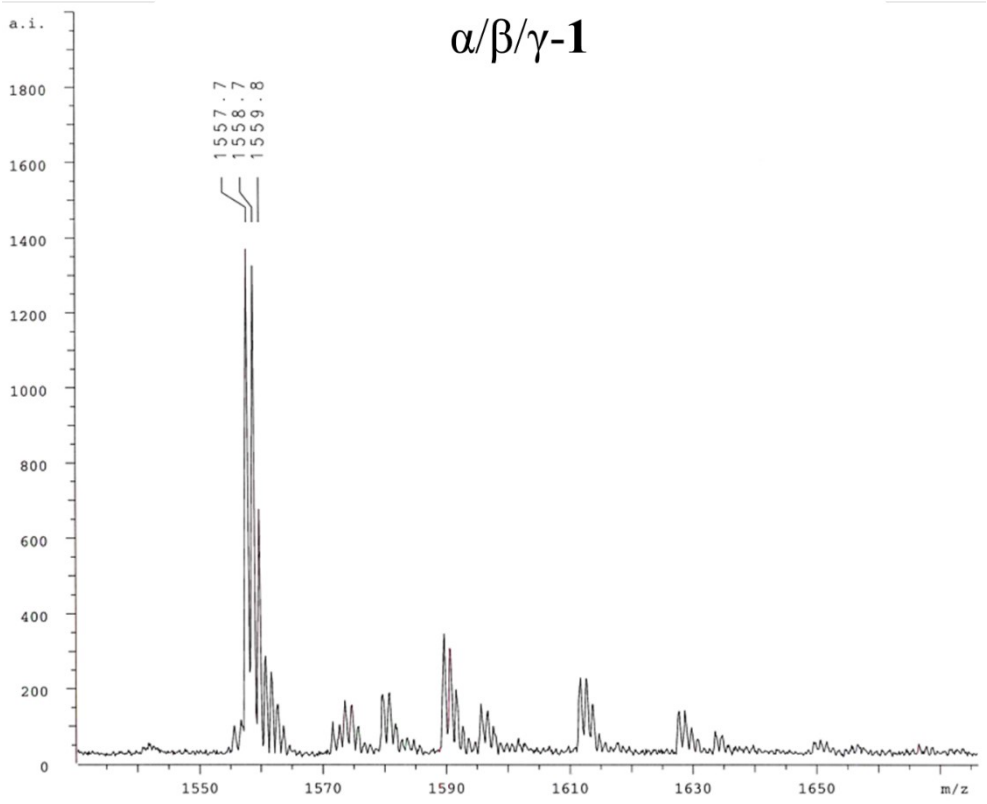
Flu–I W I A(γcyc) L R(Z) I G γ⁴ʰD F N(X) K Y–NH_2 Flu- α/β/γ-**8**

15-75 % MeCN, 60 min

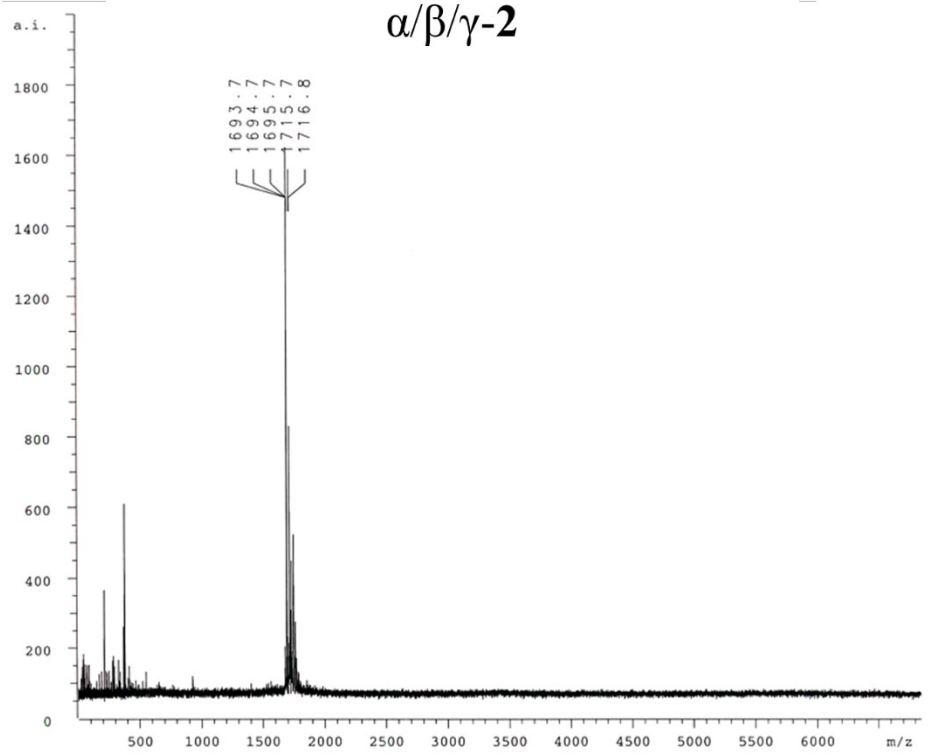


MALDI data for α/β/γ-peptides (1-15, Flu-8)

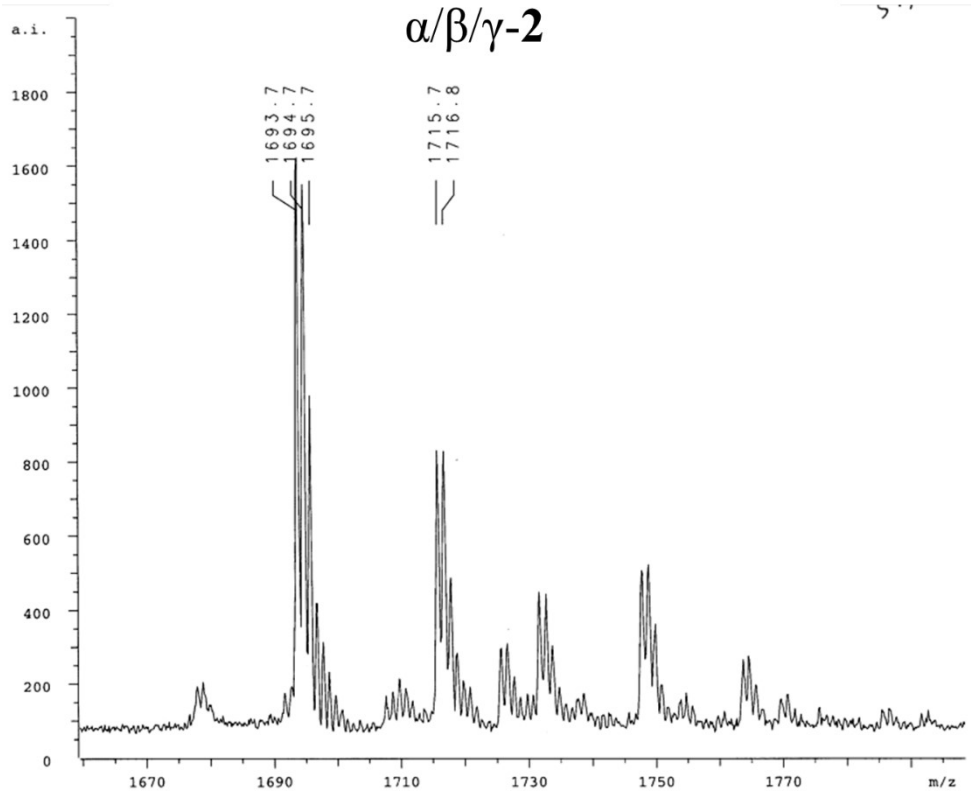


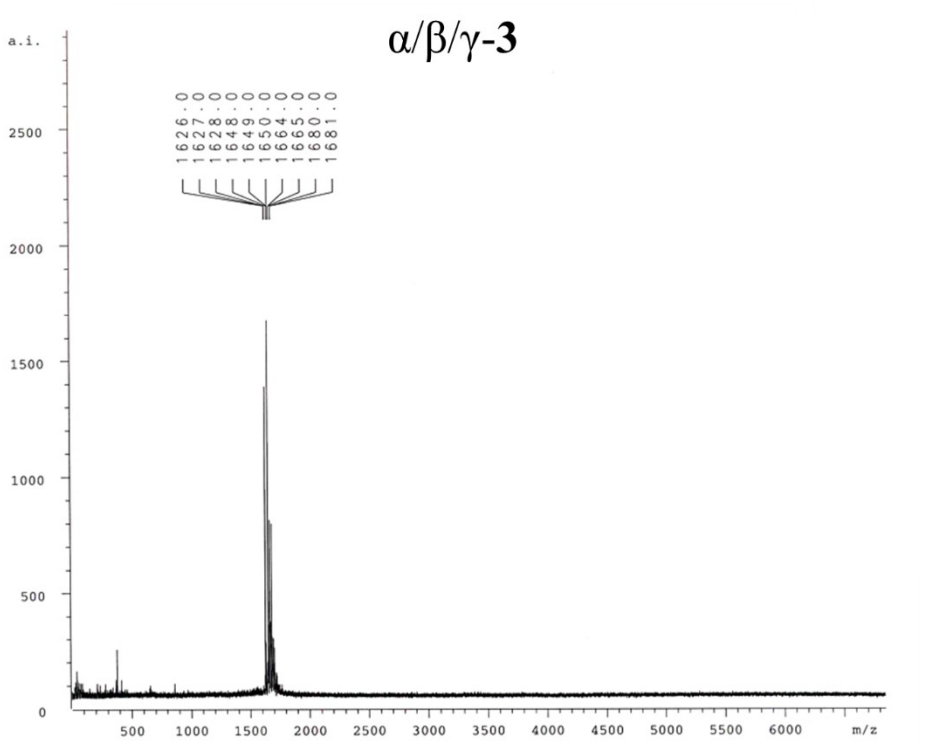


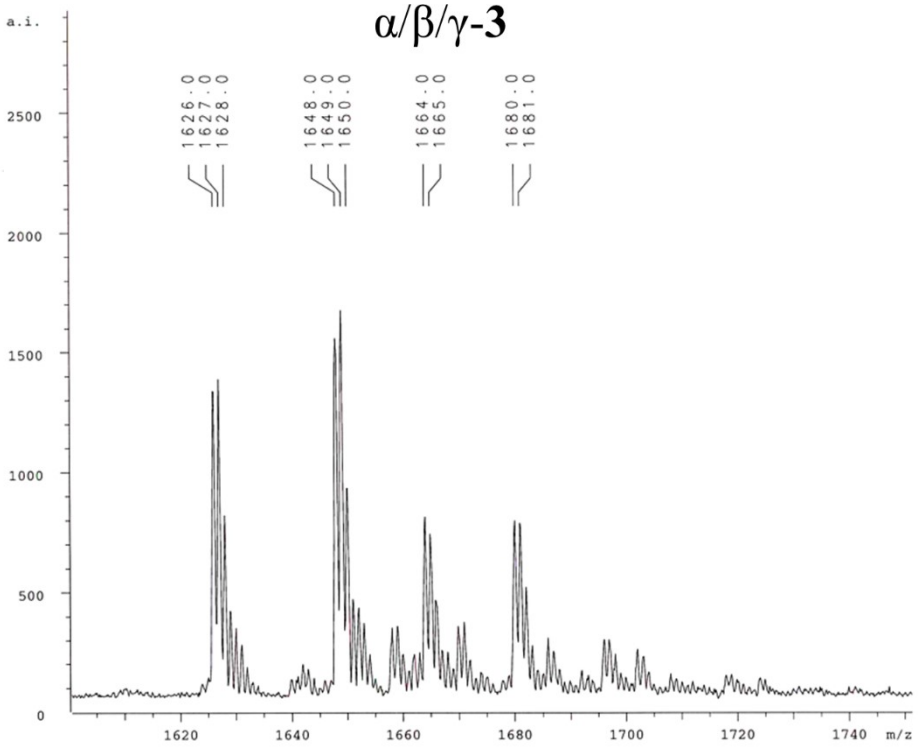
α/β/γ-**2**


a.i.
1800
1600
1400
1200
1000
800
600
400
200
0
1693.7
1694.7
1695.7
1715.7
1716.8
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z

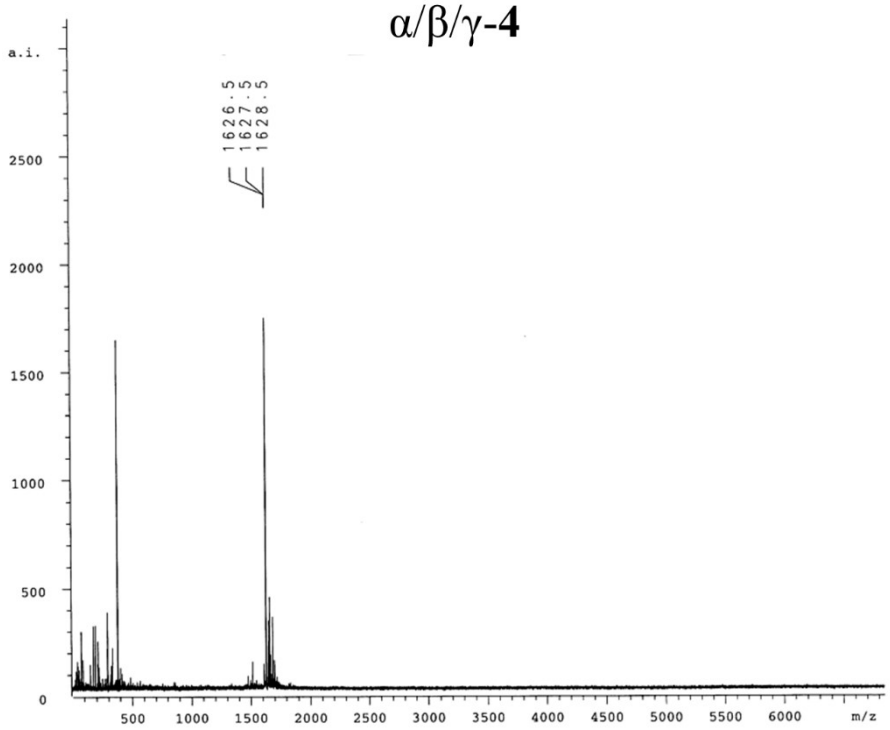
α/β/γ-**2**


a.i.
1800
1600
1400
1200
1000
800
600
400
200
0
1693.7
1694.7
1695.7
1715.7
1716.8
1670
1690
1710
1730
1750
1770
m/z

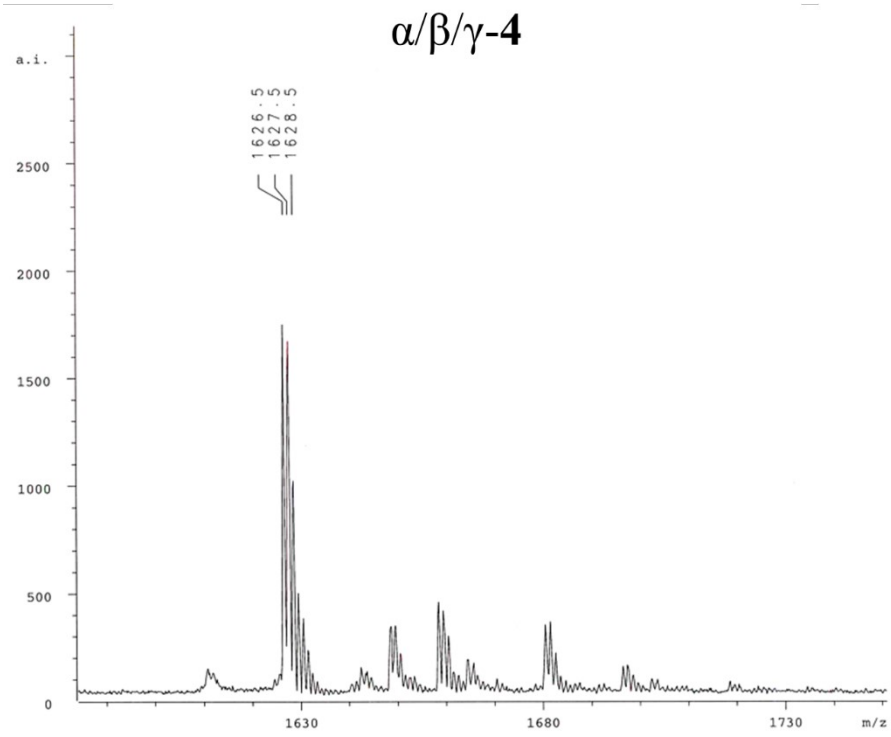

α/β/γ-3
a.i.
2500
2000
1500
1000
500
0
1626.0
1627.0
1628.0
1648.0
1649.0
1650.0
1664.0
1665.0
1680.0
1681.0
500 1000 1500 2000 2500 3000 3500 4000 4500 5000 5500 6000 m/z


α/β/γ-3
a.i.
2500
2000
1500
1000
500
0
1626.0
1627.0
1628.0
1648.0
1649.0
1650.0
1664.0
1665.0
1680.0
1681.0
1620 1640 1660 1680 1700 1720 1740 m/z

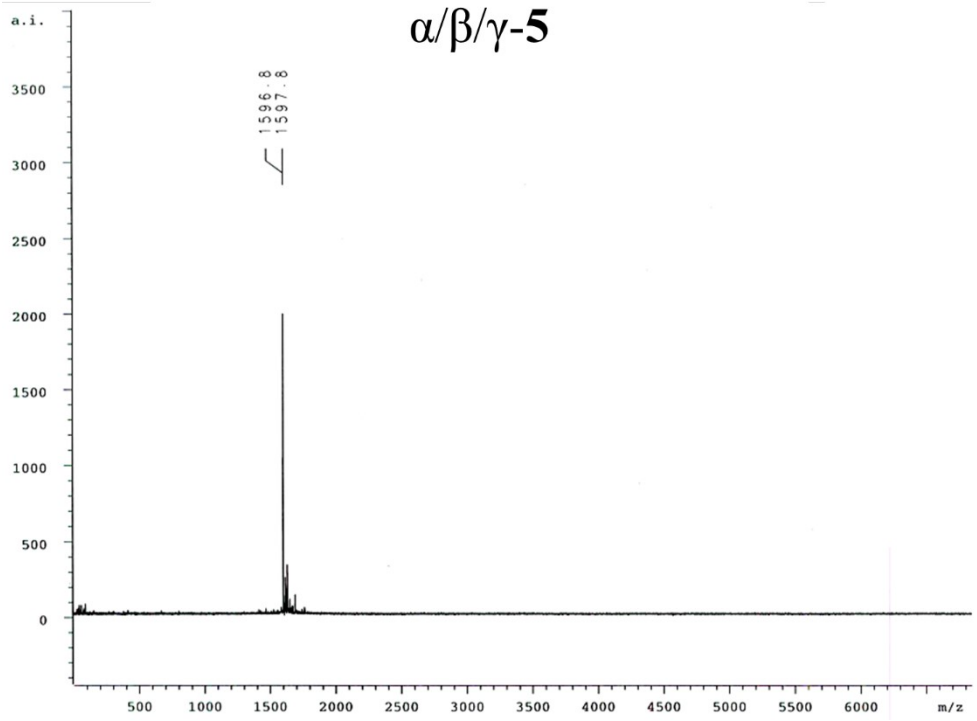
α/β/γ-**4**


a.i.
1626.5
1627.5
1628.5
2500
2000
1500
1000
500
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z

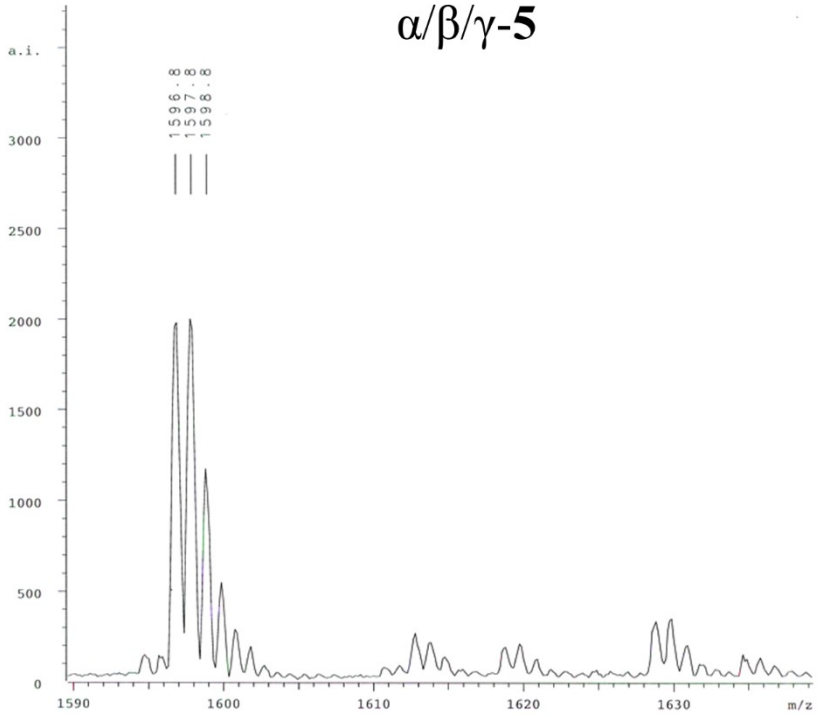
α/β/γ-**4**


a.i.
1626.5
1627.5
1628.5
2500
2000
1500
1000
500
0
1630
1680
1730
m/z

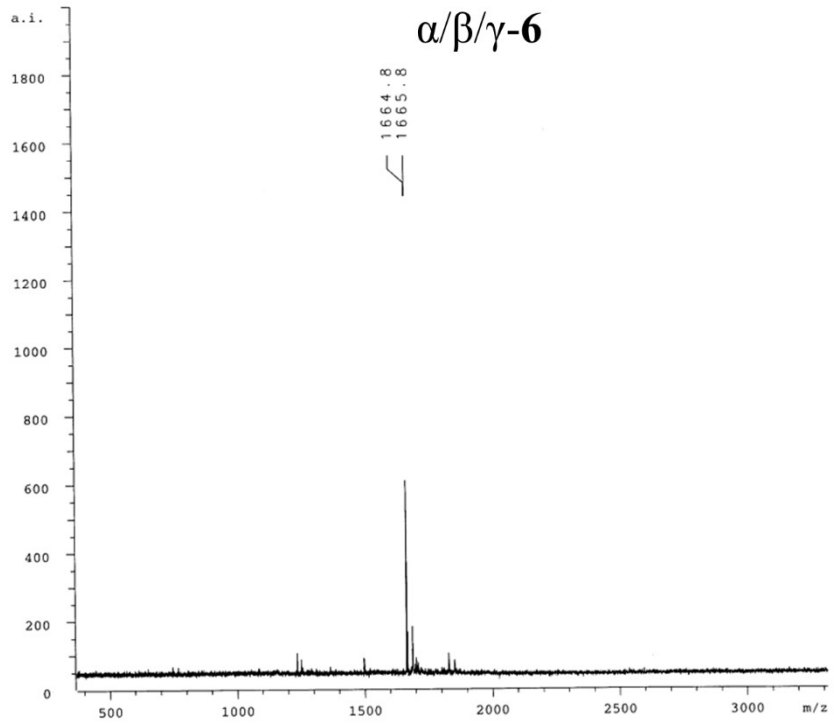
α/β/γ-**5**


a.i.
1596.8
1597.8
3500
3000
2500
2000
1500
1000
500
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z

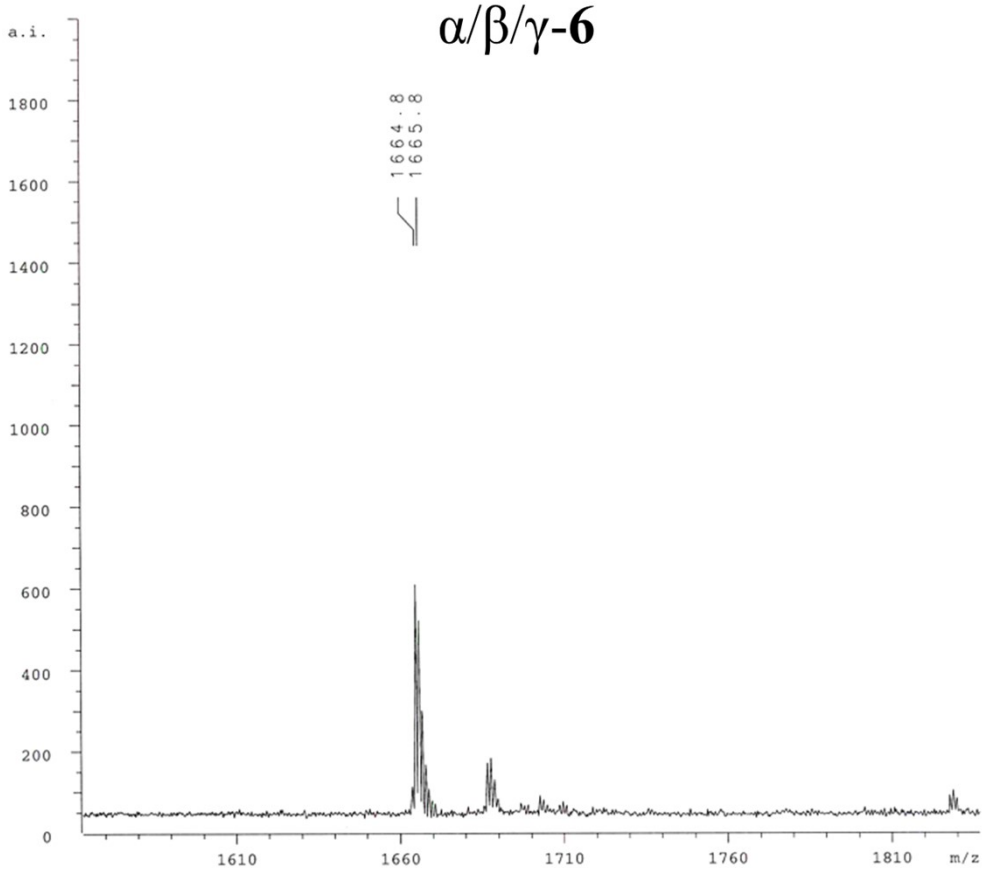
α/β/γ-**5**


a.i.
1596.8
1597.8
1598.8
3000
2500
2000
1500
1000
500
0
1590
1600
1610
1620
1630
m/z

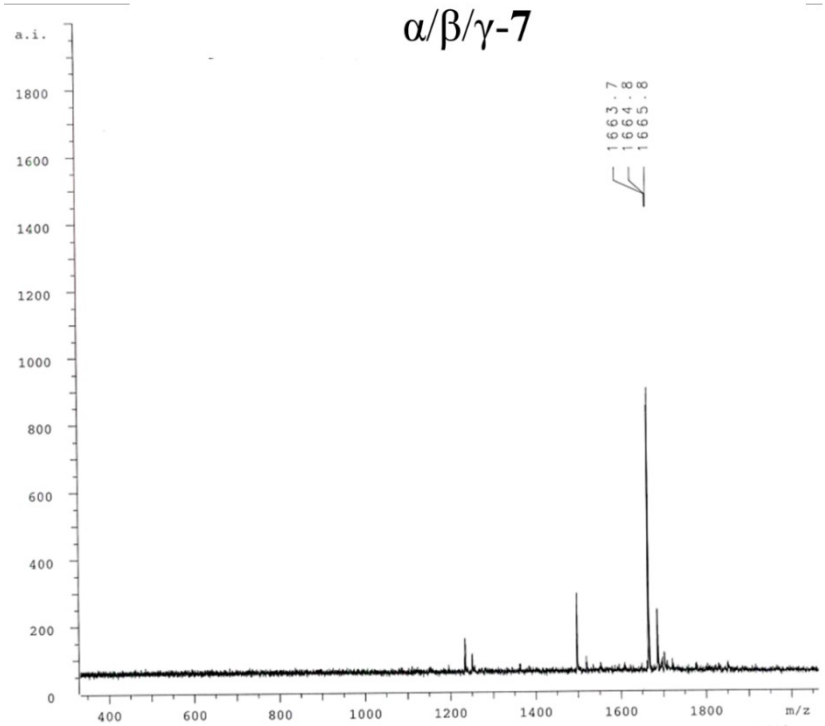
α/β/γ-**6**


a.i.
1800
1600
1400
1200
1000
800
600
400
200
0
1664.8
1665.8
500
1000
1500
2000
2500
3000
m/z

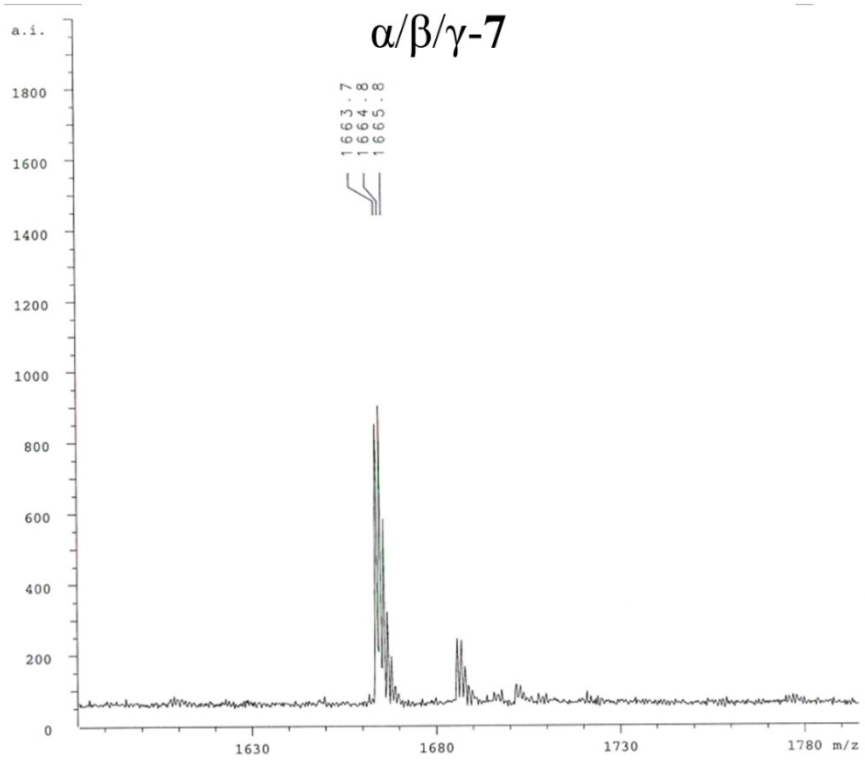
α/β/γ-**6**


a.i.
1800
1600
1400
1200
1000
800
600
400
200
0
1664.8
1665.8
1610
1660
1710
1760
1810
m/z

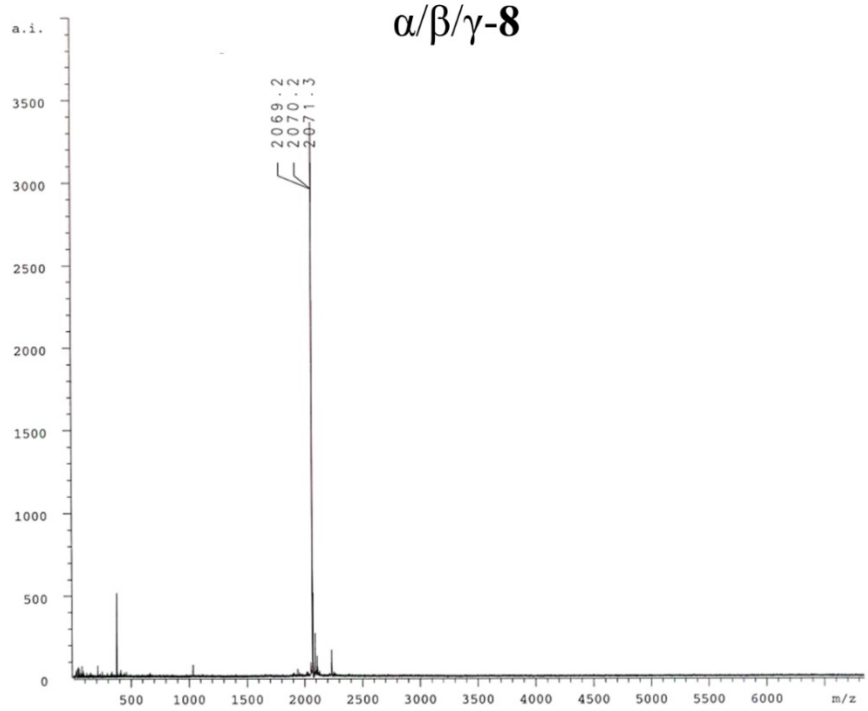
α/β/γ-7


a.i.
1800
1600
1400
1200
1000
800
600
400
200
0
1663.7
1664.8
1665.8
400
600
800
1000
1200
1400
1600
1800
m/z

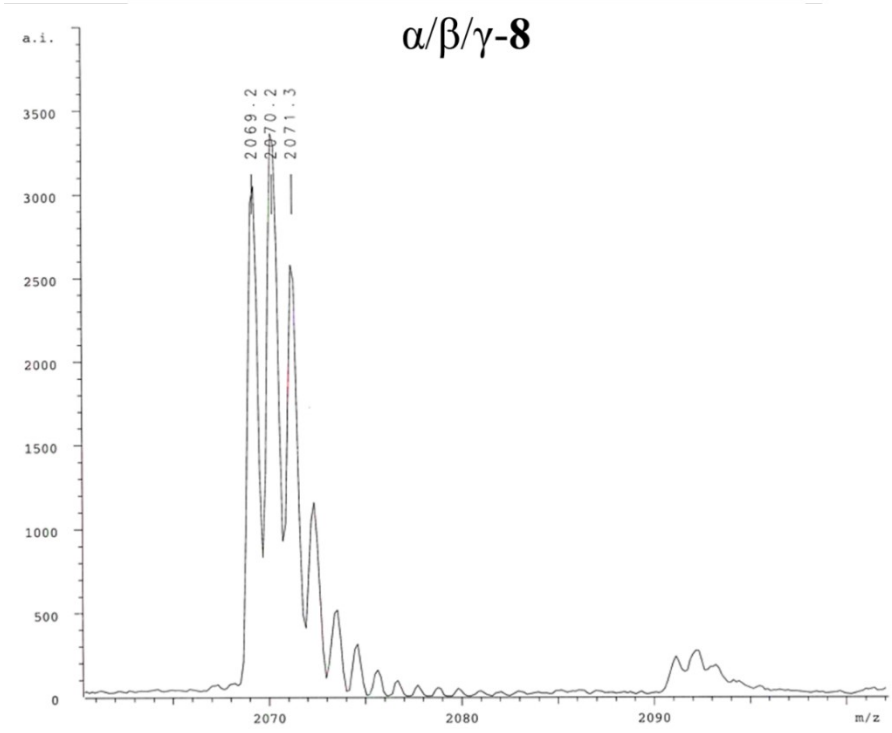
α/β/γ-7


a.i.
1800
1600
1400
1200
1000
800
600
400
200
0
1663.7
1664.8
1665.8
1630
1680
1730
1780
m/z

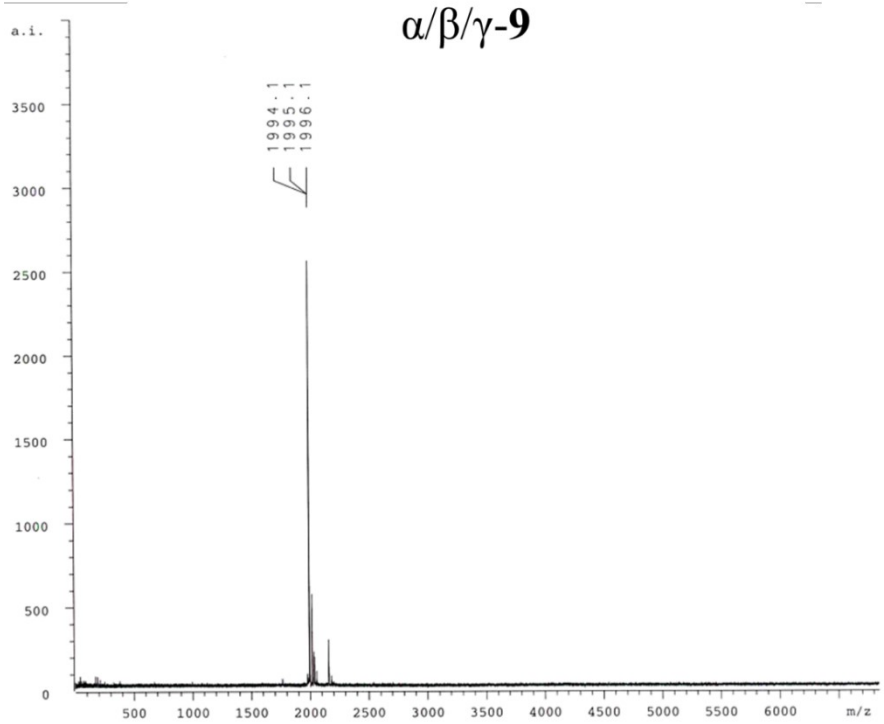
α/β/γ-**8**


a.i.
3500
3000
2500
2000
1500
1000
500
0
2069.2
2070.2
2071.3
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z

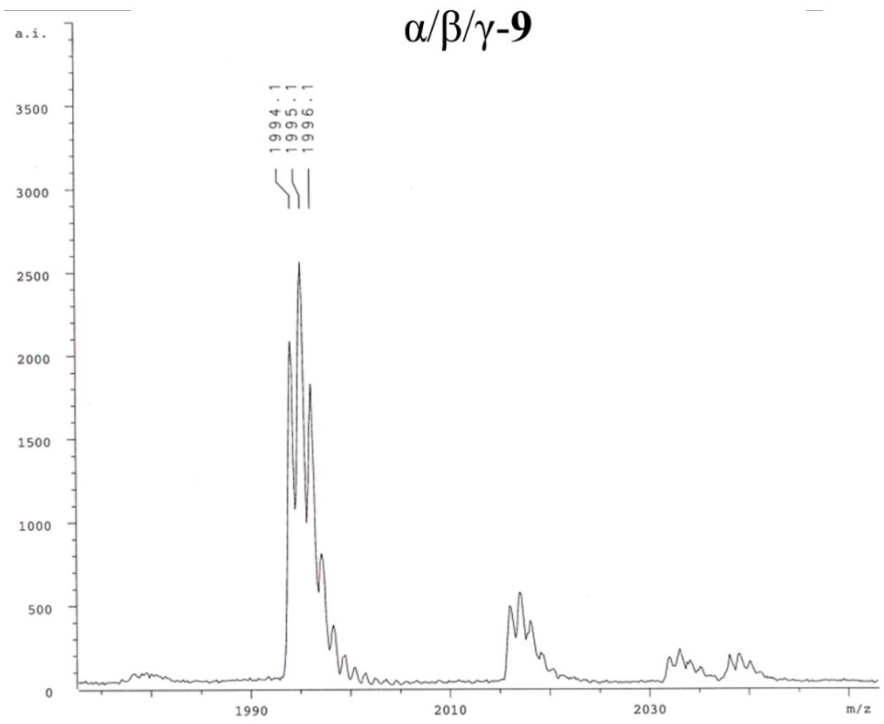
α/β/γ-**8**


a.i.
3500
3000
2500
2000
1500
1000
500
0
2069.2
2070.2
2071.3
2070
2080
2090
m/z

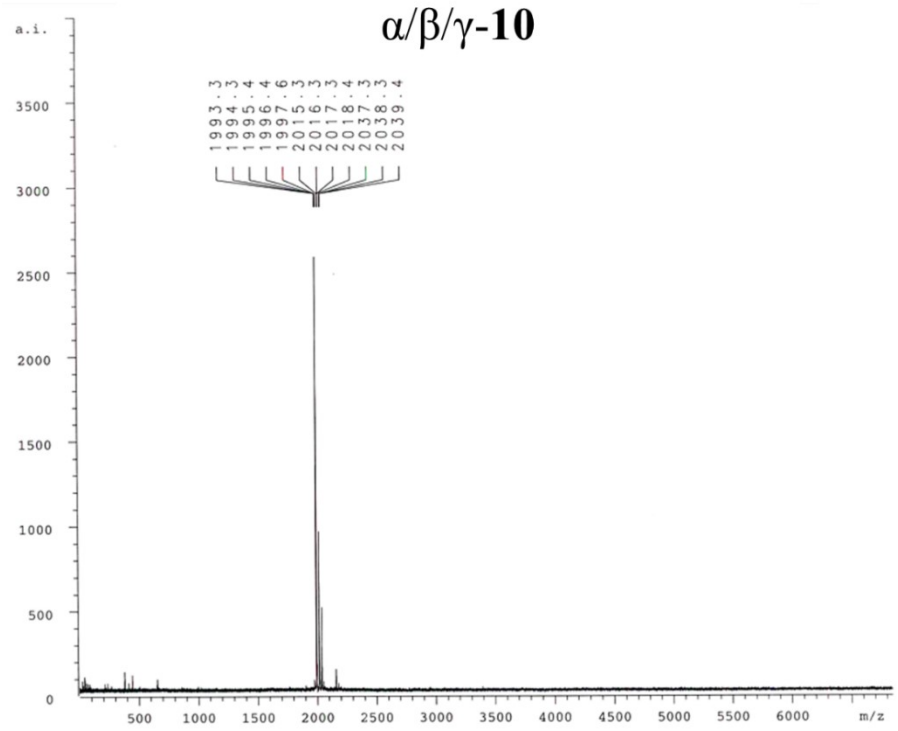
α/β/γ-**9**


a.i.
3500
3000
2500
2000
1500
1000
500
0
1994.1
1995.1
1996.1
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z

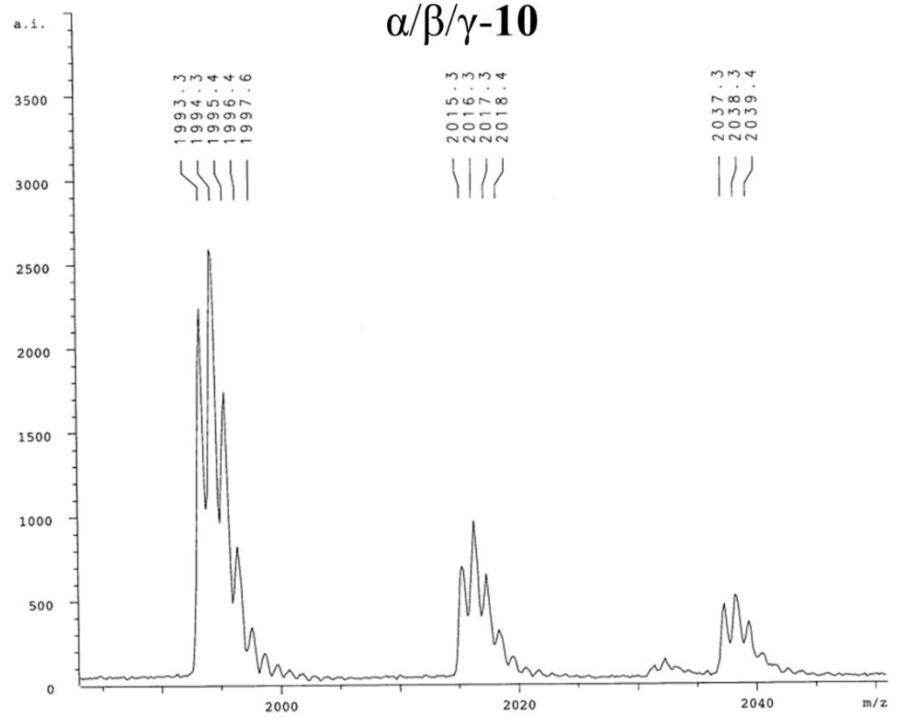
α/β/γ-**9**


a.i.
3500
3000
2500
2000
1500
1000
500
0
1994.1
1995.1
1996.1
1990
2010
2030
m/z

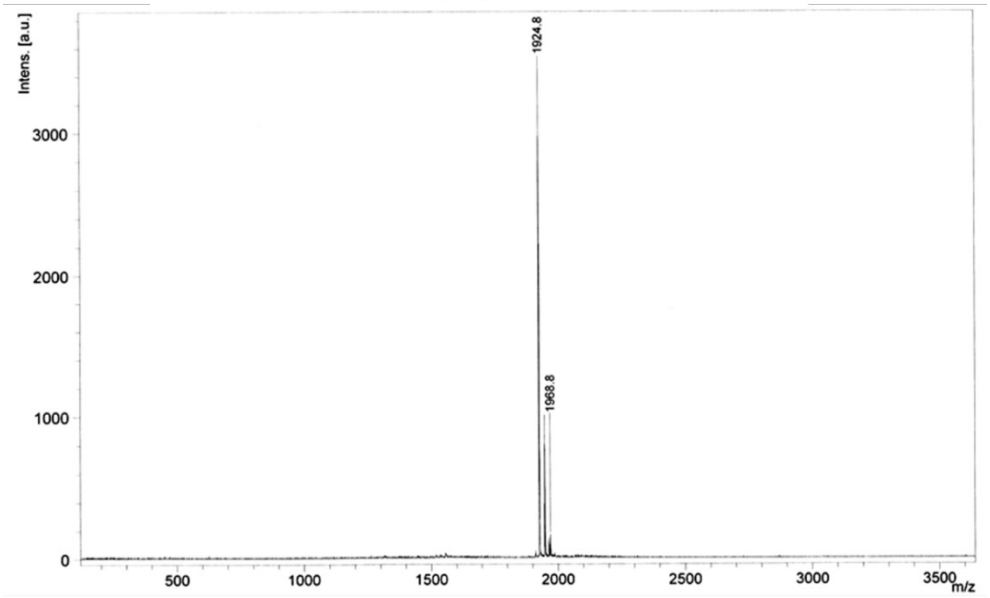
α/β/γ-**10**


a.i.
1993.3
1994.3
1995.4
1996.4
1997.6
2015.3
2016.3
2017.3
2018.4
2037.3
2038.3
2039.4
m/z

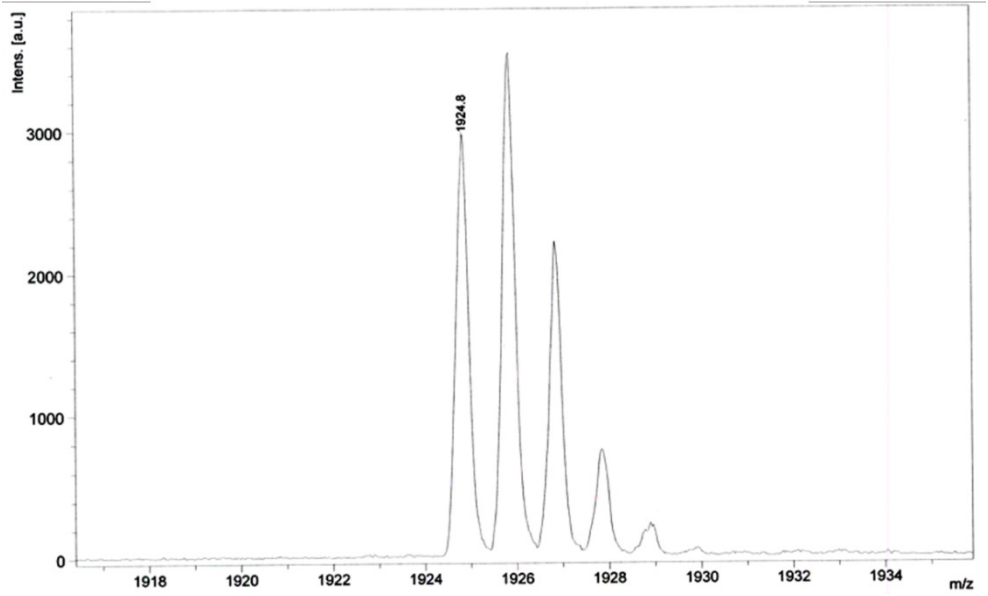
α/β/γ-**10**


a.i.
1993.3
1994.3
1995.4
1996.4
1997.6
2015.3
2016.3
2017.3
2018.4
2037.3
2038.3
2039.4
m/z

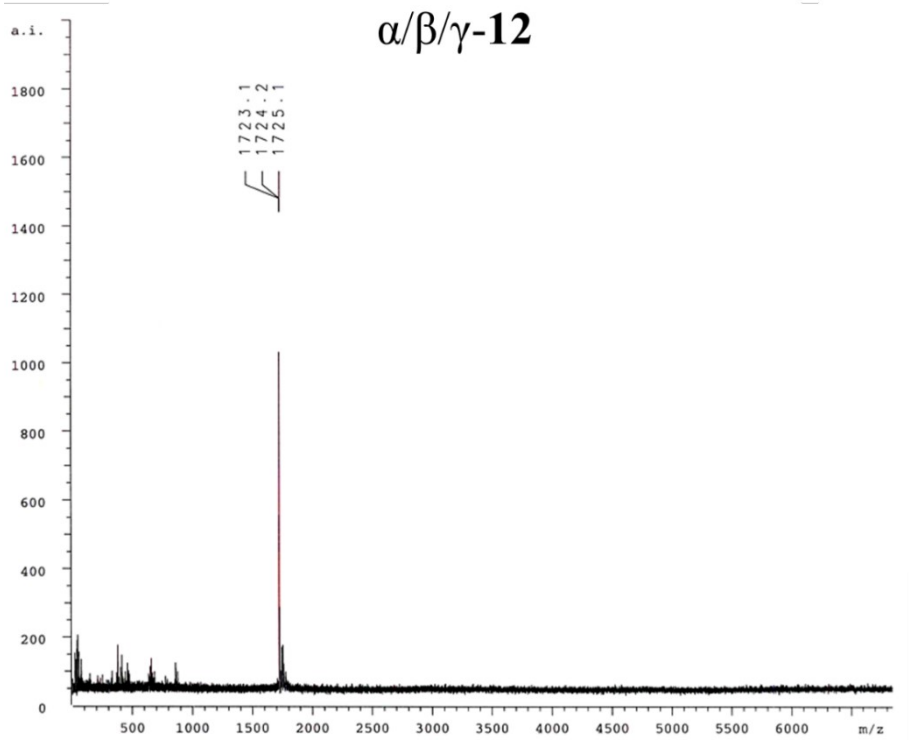
α/β/γ-**11**


Intens. [a.u.]
3000
2000
1000
0
1924.8
1968.8
500
1000
1500
2000
2500
3000
3500
m/z

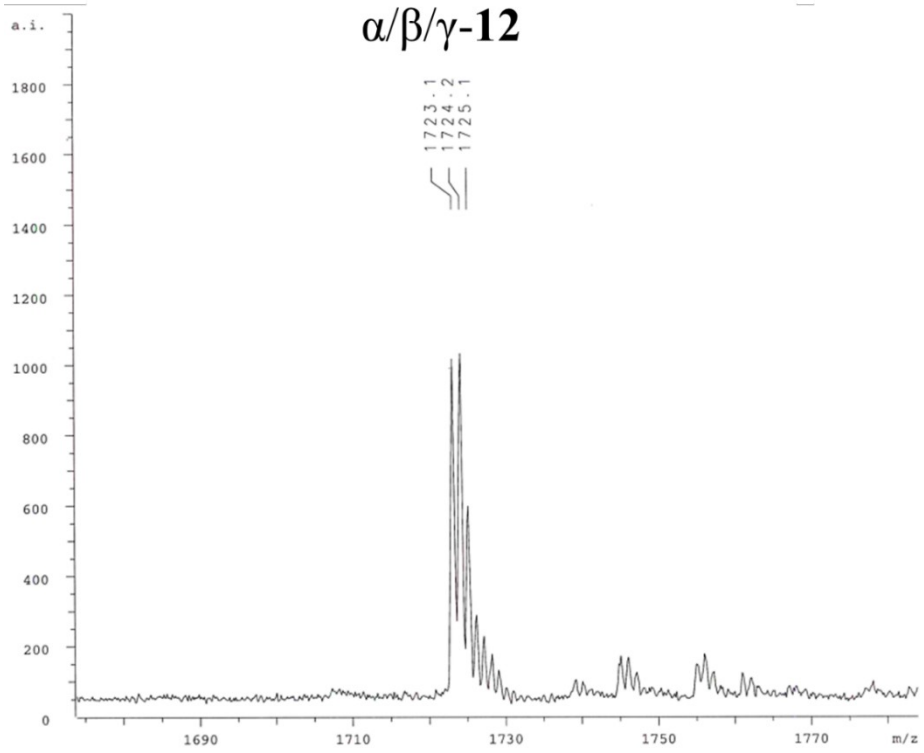
α/β/γ-**11**

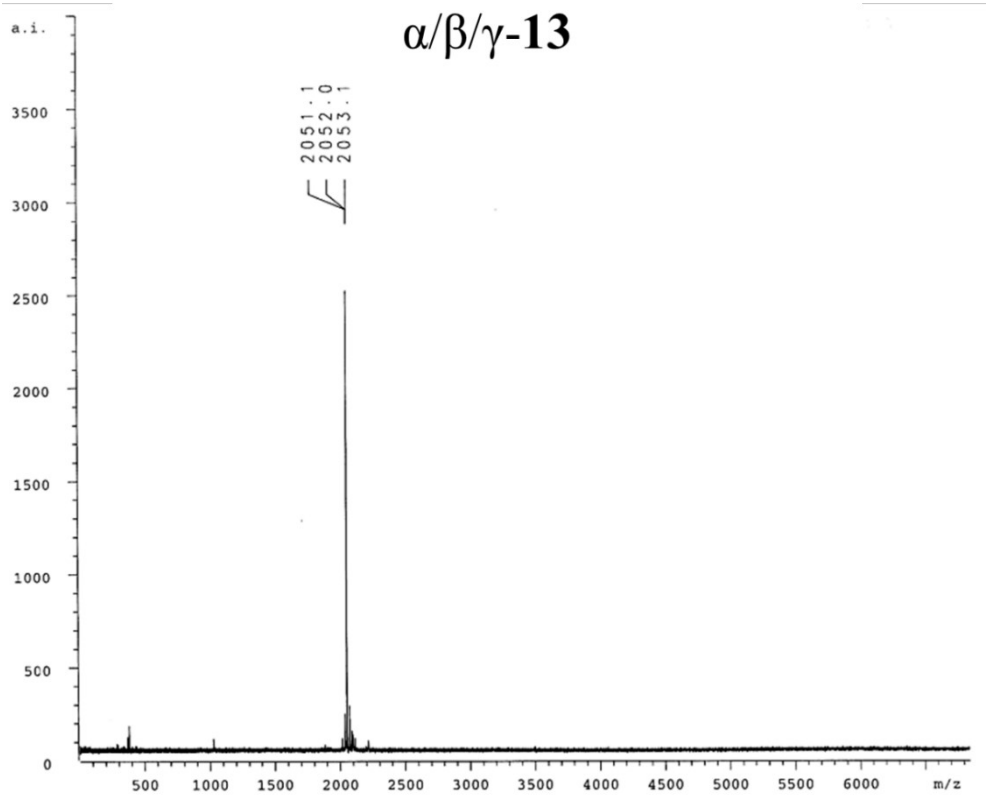

Intens. [a.u.]
3000
2000
1000
0
1924.8
1918
1920
1922
1924
1926
1928
1930
1932
1934
m/z

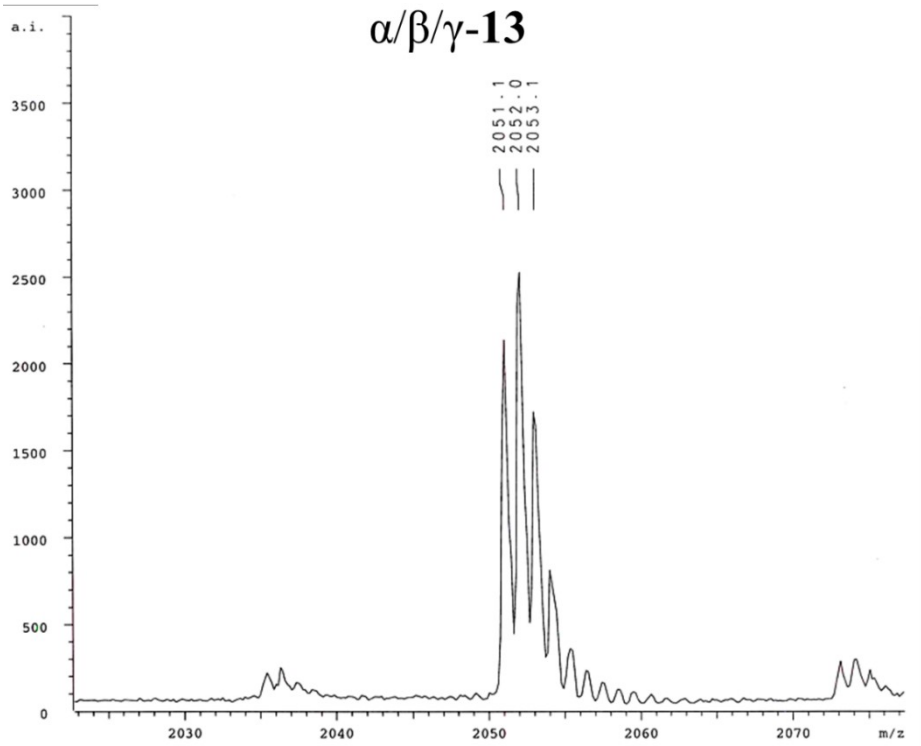
α/β/γ-**12**

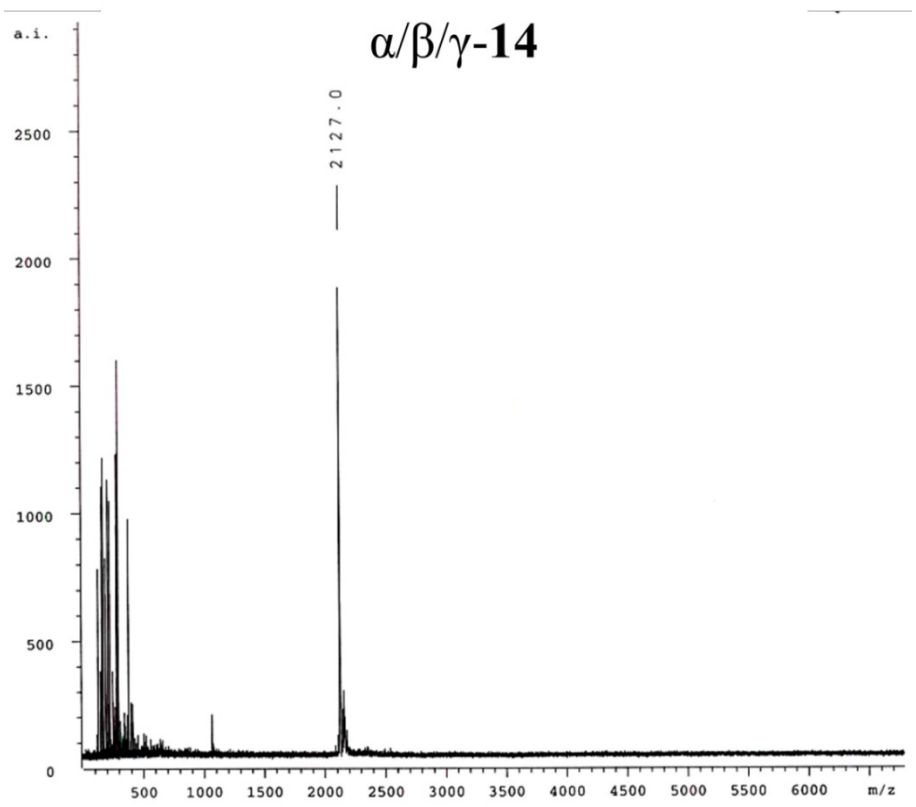


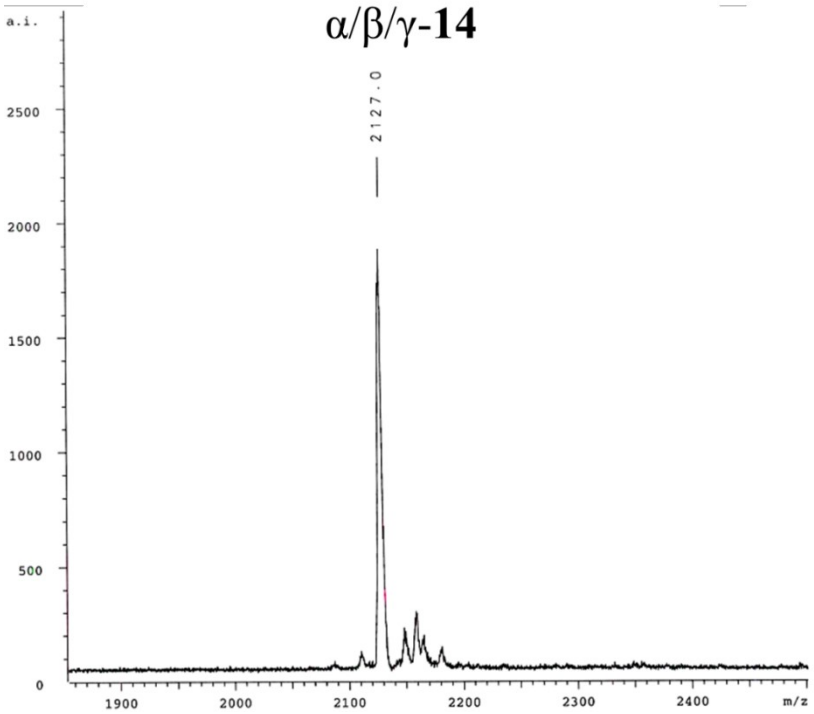
α/β/γ-**12**

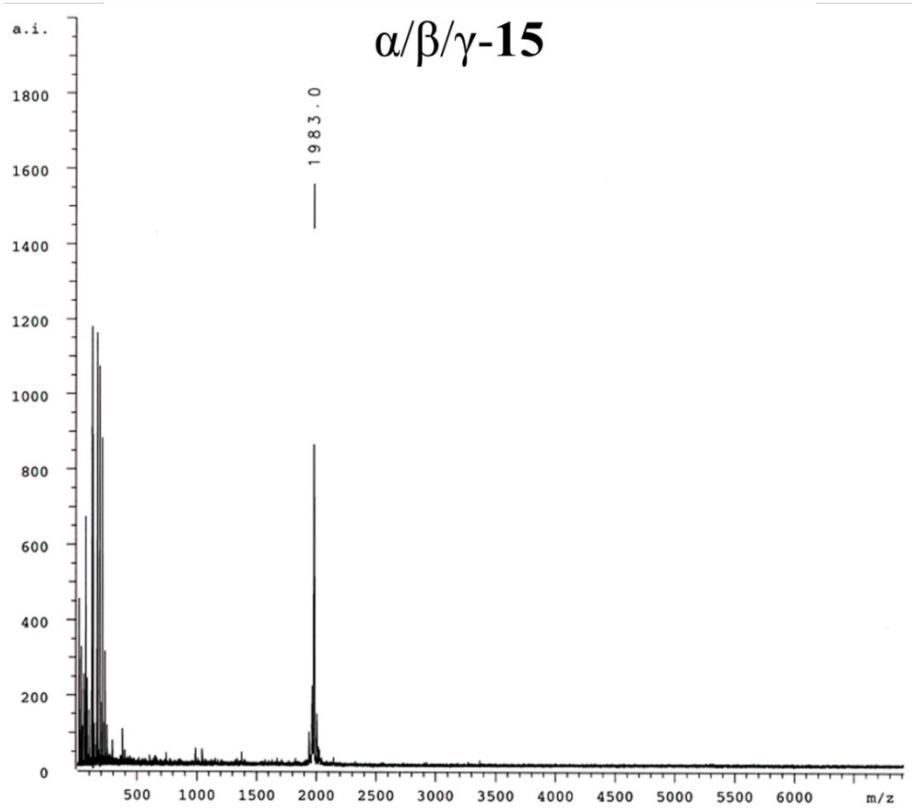


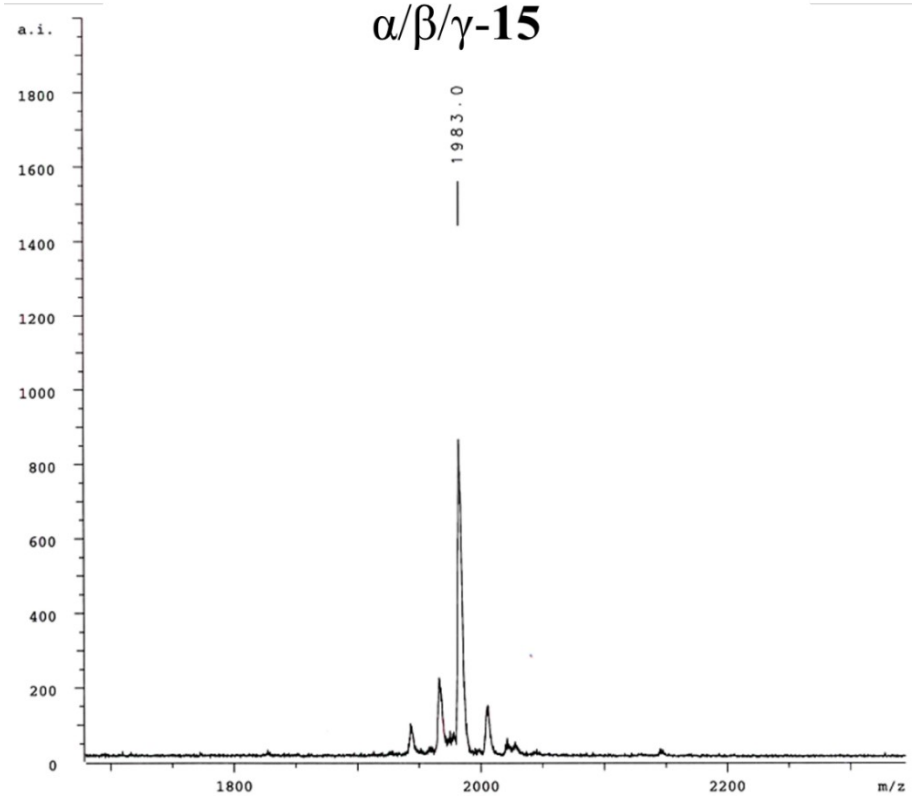

α/β/γ-13
a.i.
2051.1
2052.0
2053.1
3500
3000
2500
2000
1500
1000
500
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z


α/β/γ-13
a.i.
2051.1
2052.0
2053.1
3500
3000
2500
2000
1500
1000
500
0
2030
2040
2050
2060
2070
m/z

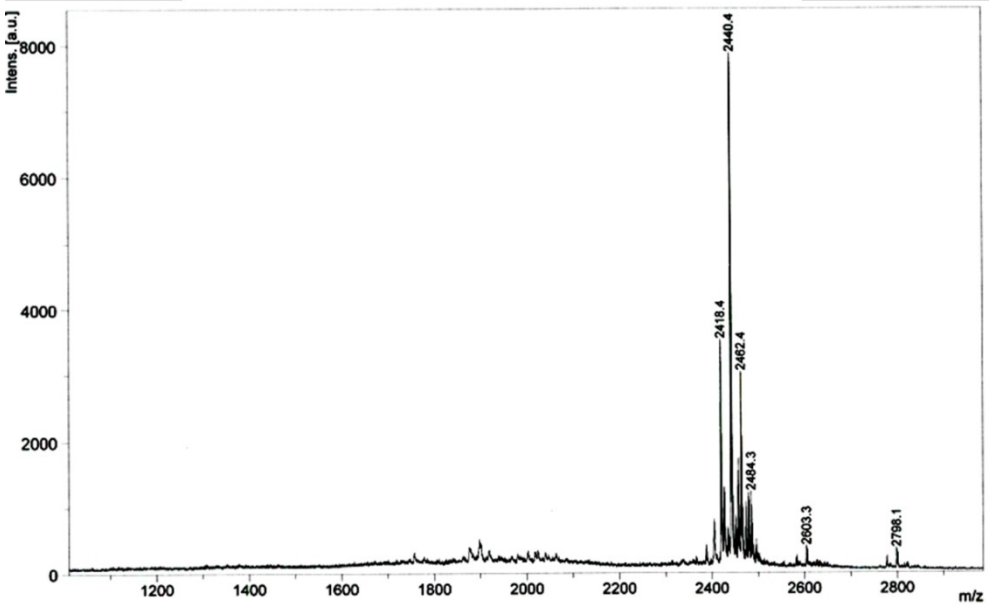

α/β/γ-14
a.i.
2127.0
2500
2000
1500
1000
500
0
500 1000 1500 2000 2500 3000 3500 4000 4500 5000 5500 6000 m/z


α/β/γ-14
a.i.
2127.0
2500
2000
1500
1000
500
0
1900 2000 2100 2200 2300 2400 m/z

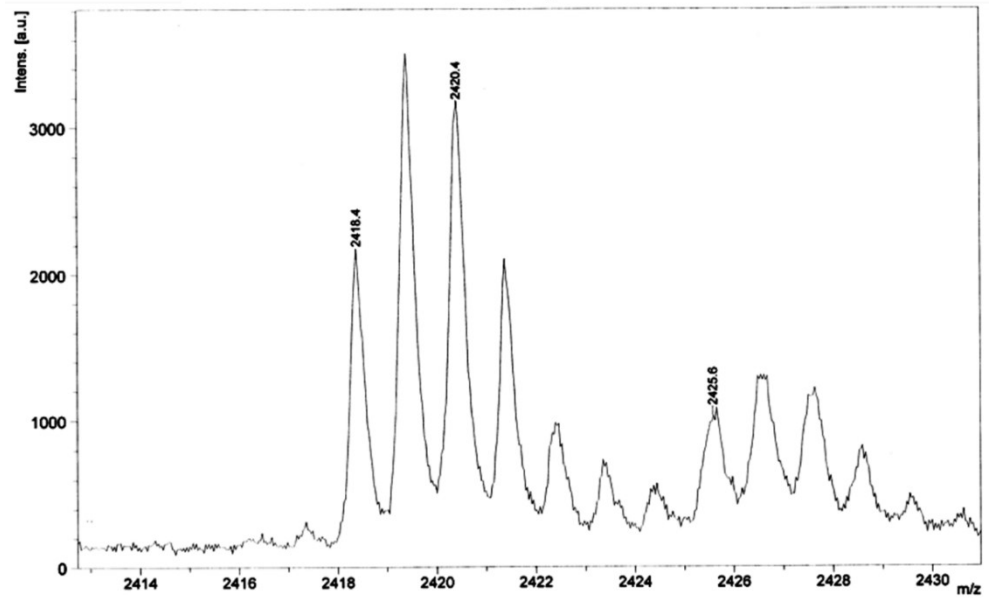

α/β/γ-15
a.i.
1983.0
1800
1600
1400
1200
1000
800
600
400
200
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
m/z


α/β/γ-15
a.i.
1983.0
1800
1600
1400
1200
1000
800
600
400
200
0
1800
2000
2200
m/z

Flu-α/β/γ 8



Flu-α/β/γ 8



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