Electronic Supporting Information

Triphenyl Phosphonium Functionalized Amphiphilic Peptides as Promising Antibacterial and Anticancer Agents

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Instrumentations:

NMR experiments: All NMR studies were carried out on Bruker DPX 400 MHz and Brüker DPX 500 MHz spectrometers at 300 K. Compounds concentrations were in the range 1–10 mM in $CDCl_3$ or DMSO-d_6.

Mass spectrometry: Mass spectra were recorded on a Q-Tof micro™ (Waters Corporation) mass spectrometer by positive mode electro spray ionisation process.

Field Emission Gun Transmission Electron Microscope (FEG-TEM) studies. FEG-TEM images were recorded on a UHR-FEG-TEM JEM-2100F at 200 kV. During FEG-TEM experiment. Hydrogels were made at minimum gelation concentration (MGC) and then 10 μL of gel was taken in a screw cap vial and diluted with 1 mL Milli-Q water. Then, a drop of dilute solution was placed on a carbon coated copper grid (300 mesh) and dried by slow evaporation. The grid was then allowed to dry in a vacuum for two days and then images were taken.

Field Emission Scanning Electron Microscopic studies (FE-SEM). FE-SEM experiments were performed by using a Jeol Scanning Microscope JSM-6700F.

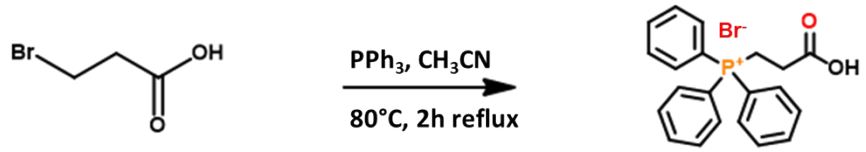
Dynamic light scattering (DLS). DLS was recorded using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK) equipped with a laser beam of He-Ne, functioning at 633 nm at a scattering angle of 173° at room temperature.

Confocal laser scanning microscopy (CLSM). Bacteria cell imaging data were recorded with a CARL-ZEISS inverted laser scanning confocal microscope (LSM880).

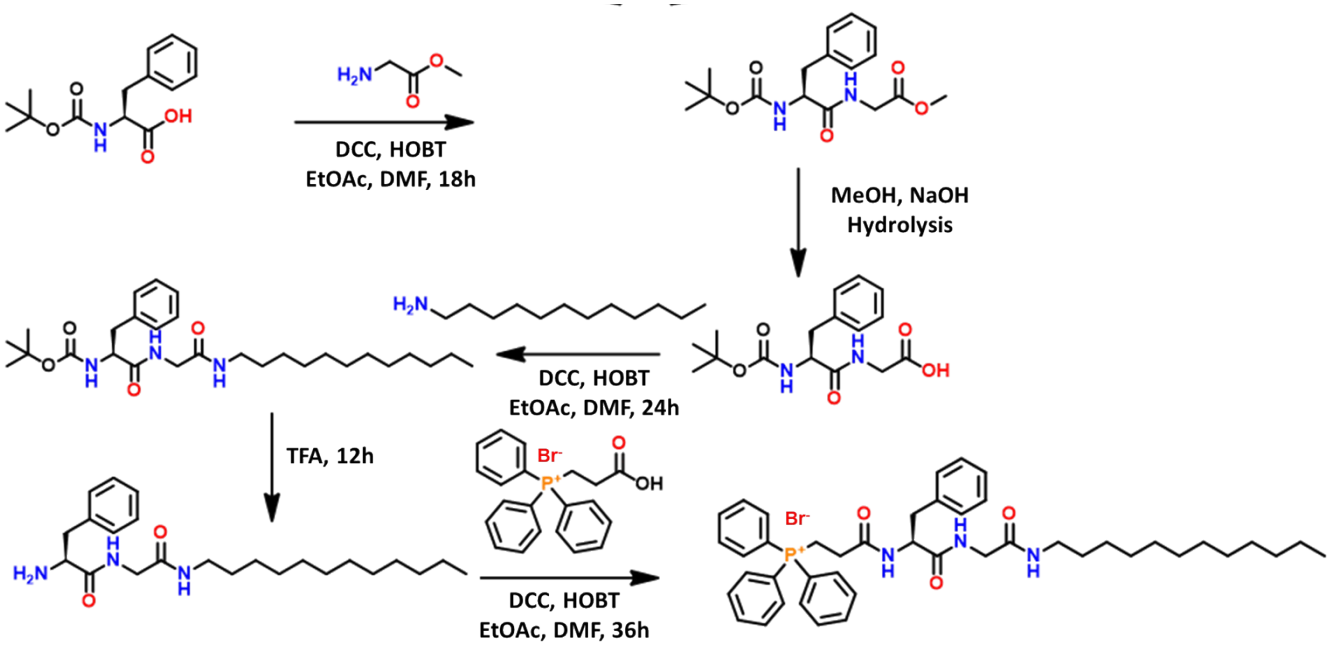
Fluorescence spectroscopy. All fluorescence spectra were recorded in a Horiba JobinYvon (Fluoromax-3, Xe-150 W, 250-900 nm) fluorescence spectrophotometer.

Synthetic Scheme:

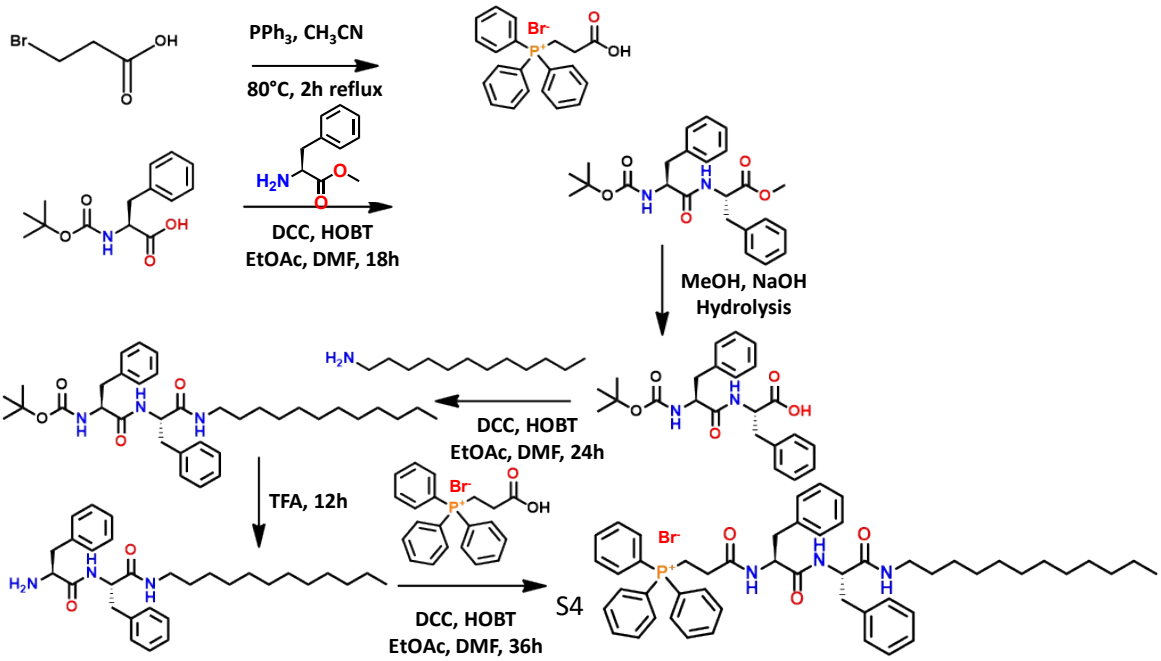
A. *Synthesis of 2-carboxyethyl-(triphenyl)-phosphanium [Ph_3P^+-C_2-COOH]:*



B. *Synthesis of Ph_3P^+-C_2-Phe-Gly-C_{12} [PHFGC_{12}]:*



C. *Synthesis of Ph_3P^+-C_2-Phe-Phe-C_{12} [PHFFC_{12}]:*



Experimental Section

Chemicals and Materials. Dodecylamine (min 98% pure), L-phenylalanine (ExiPlus, 99%), L-glycine (min 99%), DCC (99% pure), hydroxybenzotriazole (HOBt) (extrapure, min 99%), 3-Bromopropanoic acid (min 97%, Sigma-Aldrich), Triphenylphospine (min 98%, SRL), silica gel (100−200 mesh min 90%, Brockmann activity grade 2−3). All the solvents acetonitrile, chloroform, methanol, petroleum ether, ethyl acetate, THF, Trifluoroacetic acid (TFA) DMF and DMSO (99.8% pure) were all purchased from SRL. NaOH (NLT 97%) was acquired from Rankem. Agar agar, type I, Mueller Hinton (MH) broth, and Luria broth (LB) were acquired from HiMedia. Millipore Milli-Q grade water was used in all experiments. Thiazolyl blue tetrazolium bromide (extrapure AR, 98%), 2,7-dichlorofluorescein diacetate (extrapure, 97%), 8-anilino-1-naphthalenesulfonic acid (ANS) dye (extrapure, 97%) purchased from Sigma-Aldrich, SYTO-9 and Propidium Iodide (LIVE/DEAD BacLight Bacterial Viability Kit). 10% Titrion X were all purchased from SRL. All bacteria strains such as *Bacillus subtilis* (*B. subtilis*) (ATCC 23857), *Escherichia coli* (*E. coli*) (ATCC 25922), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (MTCC 1688), and *Staphylococcus aureus* (*S. aureus*) (MTCC 96), and Ovarian adenocarcinoma cell line, SKOV-3 and normal epithelial cell line, HEK 293 were grown in RPMI, 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% Penicillin-Streptomycin (Gibco). The ovarian adenocarcinoma cell line (SKOV3) and the normal human epithelial cell line (HEK 293) used in our experiments were procured from the National Centre for Cell Science (NCCS), Pune, India

Synthesis and Characterization of P1 and P2. The peptide amphiphiles P1 and P2 were synthesized via conventional solution-phase methods, employing a racemization-free fragment condensation approach. Detailed synthetic procedures are provided in the Supporting Information (Scheme S1). The final compounds were thoroughly characterized using mass spectrometry (Figure S1, S5 and S9), ^{1}H NMR spectroscopy (Figure S2, S6 and S10), ^{13}C NMR

spectroscopy (Figure S3, S7 and S11) and ^{31}P NMR spectroscopy (Figure S4, S8 and S12). NMR studies were conducted on a Bruker DPX400 MHz or Bruker DPX500 MHz spectrometer at 300 K, with concentrations ranging from 5 to 10 mM in $CDCl_3$ or DMSO-d_6. Mass spectra remained obtained using a Q-Tof micro™ mass spectrometer (Waters Corporation) with positive-mode electrospray ionization.

Self-assembly study protocol. The critical micelle concentrations (CMC) of both P1 and P2 were determined using Nile red, a hydrophobic fluorescent dye. A series of 1% (v/v) DMSO-water solution of P1 and P2 were prepared with concentrations ranging from 500 μM to 0.5 μM. Separately, 1 mM Nile red solution in THF was prepared, and a fixed volume was added to each P1 and P2 solution, ensuring a final Nile red concentration of 1μM in all cases. The solutions were left overnight in open vials to allow for equilibration with the dye. Fluorescence emission spectra were then recorded with an excitation wavelength of 550 nm (slit width 3), while emission was monitored at 636 nm (slit width 3). The emission intensity at 636 nm (I_{636}) was plotted against the logarithm of the P1 and P2 concentrations, and the CMC was determined from the intersection of two linear fits to the data.

Study for MIC determination. *B. subtilis* (ATCC 23857), *E. coli* (ATCC 25922), *P. aeruginosa* (MTCC 1688), and *S. aureus* (MTCC 96) were cultured overnight in Luria Broth (LB) at 37 °C. The minimum inhibitory concentrations (MICs) of peptides were determined using the micro broth dilution method, following the clinical and laboratory standards institute (CLSI) guidelines. Bacterial cultures were grown in LB broth to reach a final concentration of approximately 10^5 CFU/mL. Stock solutions of each peptide amphiphiles were prepared in 1% (v/v) DMSO-water at a concentration of 0.5 mM, with the pH adjusted to 7.00. Peptides were added to the wells at varying concentrations (250–3.9 μM) through serial dilutions in LB medium. Then, 20 μL of each bacterial suspension (10^5 CFU/mL) was added to 96-well plates, bringing the total volume in each well to 200 μL. The plates were incubated at 37°C under

static conditions for 18–24 hours. Following incubation, the optical density (OD) at 600 nm was measured to assess bacterial growth in a micro plate reader (Spectra Max ID5 Multi-Mode). The lowest concentration of each peptide that inhibited bacterial growth was recorded as the MIC. Positive controls (broth with bacteria) and negative controls (1% (v/v) DMSO-water) were included in each experiment to validate results. All MIC determinations were performed in triplicate to ensure accuracy and reproducibility.

Study for Disc Diffusion assay. Gram-positive bacteria S. aureus and B. subtilis, along with Gram-negative bacteria E. coli and P. aeruginosa, were cultured in Luria Broth (LB) medium and incubated at 37°C for overnight. The initial bacterial concentration was 6×10^5 CFU/mL. Sterile plastic plates (90 mm) were used to prepare a gel bed containing 1.5% (w/v) LB and 2% (w/v) agar. A 20 µL bacterial suspension was uniformly spread onto the agar plates. The antimicrobial activity of two test substances, P1 and P2, was assessed using the agar disc-diffusion method. Sterile discs were treated with minimum inhibitory concentrations (MICs) of P1 and P2 were placed onto the gel bed containing the bacterial cultures. The plates were incubated at 37°C for 18-24 hours to allow bacterial growth and interaction with the test compounds. 1% (v/v) DMSO-water solution was used as control. After incubation, the zone of inhibition (clear areas around the discs where bacterial growth was inhibited) was measured. This zone provided a visual indication of the effectiveness of P1 and P2 against each bacterial strain. The assay helped in determining the antimicrobial potential of P1 and P2 against both Gram-positive and Gram-negative bacteria.

Bacterial morphology analysis by FESEM. *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* bacteria were cultured in LB medium and incubated for 18 h at 37 °C. Afterward, P2 was treated with the bacterial solutions at MICs value and incubated for an additional 24 h. The suspensions were centrifuged at 5000 rpm for 5 minutes to collect the precipitates, which were then washed with a 0.87% NaCl solution. The bacterial precipitates were subsequently fixed in

2.5% glutaraldehyde in PBS at room temperature for 30 minutes and stored overnight at 4 °C. Following fixation, the pellets were collected by centrifugation and washed twice with Milli-Q water. The samples were then dehydrated through an ethanol gradient (30%, 50%, 70%, 90%, and 100%), with each concentration applied for 10 minutes. Once dehydrated, the samples were cast onto a glass plate for imaging. Field emission scanning electron microscopy (FESEM) was used to capture images, with the instrument set to operate at 5 kV. This method ensured that detailed observations of the bacterial morphology could be obtained following sample preparation, fixation, and dehydration.

Bacterial cell imaging by Fluorescence Microscopy. For fluorescence microscopy, *S. aureus* and *E. coli* were cultured in LB at 37 °C with shaking (150 rpm) until reaching mid-log phase. The cultures were washed twice with PBS, centrifuged for 5 minutes at 2000 rpm, and diluted to 1×10^5 CFU/mL in PBS. The bacteria were then incubated with compound P2 at its MICs for 20 hours at 37 °C. Then the bacteria were washed again and resuspended in PBS. Next, the bacterial suspension was divided into two sets (control and P2-treated). The control group consisted of untreated bacteria. Both sets were stained with SYTO 9 and PI (final concentration of 10 μM each) and incubated for 15 minutes in the dark. After staining, 10 μL of the suspension was placed onto a clean glass slide, covered with a glass coverslip, and imaged using an Olympus IX73 inverted fluorescence microscope (Tokyo, Japan). This method allowed visualization of live (green, SYTO 9-stained) and dead (red, PI-stained) bacteria for further analysis.

Membrane permeability assays. 8-anilinonapthalene-1-sulfonic acid (ANS) uptake assay was used to evaluate the outer membrane (OM) permeability of the bacterial test candidates in response to the peptides P1 and P2. *P. aeruginosa* were grown to the logarithmic phase (OD_{600} = 0.5−0.6) and harvested by centrifugation at 4000 rpm for 5 minutes. The bacterial cells were washed twice with sterile HEPES buffer and then resuspended in HEPES buffer (5 mM, pH

7.4). ANS solution (10 µL, 0.5 mM) was added to the bacterial suspension and incubated in the dark at room temperature for 30 minutes at 37 °C. Following incubation, bacterial suspensions were mixed with peptides P1 and P2 at varying concentrations such as MIC/4, MIC/3, MIC/2, MIC and 2MIC maintaining a total volume of 100 µL per sample in 96-well plates. Fluorescence emission was recorded over time at an emission wavelength of 520 nm, with excitation at 380 nm. A 10 µM Triton X solution served as the positive control and 1% (v/v) DMSO-water as negative control. Each experiment was repeated three times, and the resulting data were analyzed with standard deviation error bars incorporated to reflect variability.

The inner membrane permeability was performed by using the nucleic acid-binding red fluorescent dye PI.[52] *S. aureus and P. aeruginosa* were grown to an optical density (OD_{600}-0.5-0.6). The cells were then treated with varying concentrations such as MIC/4, MIC/3, MIC/2, MIC and 2MIC of compounds P1 and P2 for 60 minutes at 37 °C in a 96-well plate, with a total volume of 100 µL per sample. After treatment, the bacteria cells were washed twice with 5 mM HEPES buffer (pH 7.4) by centrifugation at 5000 rpm for 10 minutes. They were then resuspended in the same buffer. Following resuspension, 10 µL of a PI solution (0.5 mM) was added to each sample and incubated in the dark for 30 minutes to allow for staining. The fluorescence intensity of the samples was measured using a 96-well plate reader, with an excitation wavelength of 493 nm and an emission wavelength of 636 nm. Triton X (10 µM) was used as a positive control and 1% (v/v) DMSO-water as negative control for cell membrane disruption. Biological triplicates were performed for each condition to ensure accuracy and reproducibility of the results. This assay aimed to assess cell membrane integrity following treatment with P1 and P2 by measuring PI uptake, which indicates compromised cell membranes.

Cell culture. Ovarian adenocarcinoma cells (SKOV3) and normal epithelial cells (HEK 293) were cultured at 37 °C in a humidified incubator with 5% CO_2. SKOV3 cells were maintained in RPMI media, and HEK 293 cells were grown in DMEM media, both supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Gibco) antibiotic. When the cells reached 75–80% confluency, they were harvested using 0.025% trypsin and 0.52 mM EDTA. Afterward, the cells were seeded at the desired density to allow for growth prior to the designed experiments.

Cell viability assay. 5×10^3 SKOV3 cells were seeded in a 96-well flat-bottom transparent plate and allowed to grow overnight in a humified chamber. The SKOV3 and HEK 293 cells were subjected to the treatment of the novel drugs P1 and P2 on the following day, for duration of 24 h at different drug concentrations 1 - 100 μM. 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) tetrazolium dissolved in PBS was added to each well and incubated for 3 h at 37°C followed by the addition of 150 μL DMSO in each well and reading was observed in a microplate plate reader at 570 nm. To determine the effect of the 0-500 μM P1 or P2 on the normal epithelial cells, cell viability assay on HEK 293 cells was also performed.

Morphological analysis. SKOV3 cells were seeded at a count of 5×10^5 cells on a 22 mm glass coverslip kept in a 35mm petri dish. The cells were treated with P2 for 24 h and after completion of the treatment the cells were fixed in 2% paraformaldehyde followed by staining with 4′,6′-Diamidino-2 phenylindole (DAPI) for 2 min in the dark at room temperature. DPX mounting was performed and analyzed under fluorescence microscopy.

Assessment of Reactive Oxygen Species production. To determine the generation of Reactive Oxygen Species (ROS), 2×10^7 cells were seeded and allowed to grow overnight and then treated with P2 for a period of 24 h. After the treatment, the cells were incubated with 10

mM DCF-DA followed by incubation at 37°C for 15 min, and analyzed with a BD LSRFortessa.

Determination of apoptotic, mitochondrial proteins and percentage of apoptotic cells. For detecting the anti-apoptotic and pro-apoptotic proteins in the P2 treated (for 24h) SKOV3 cells, primary antibodies of pro-apoptotic marker (Bax), anti-apoptotic marker (Bcl2) and mitochondrial protein (Cyt c) were added to the cells (1:500 dilution) and incubated at 4°C for 2 h. Following the incubation, the cells were tagged with secondary antibodies (FITC and APC tagged) at a dilution of 1:1000 and incubated for 1hr. The level of expression of the markers was then analyzed using a BD LSR flow cytometer. The untreated and treated cells were incubated with Annexin V for a duration of 20 min in the dark at room temperature and flow cytometric analysis was performed.

Analysis of Caspase proteins. The cell culture supernatant collected from the control cells and P2 treated cells was used for determining the secretory levels of caspase 3, caspase 8, and caspase 9. The analysis was performed using three distinct ELISA kits Human Active Caspase-3 Immunoassay (RnD Systems, Catalog Number KM300), Human CASP 9 (Caspase 9) ELISA Kit (Assay Genie, Catalog Number HUES01822), and Human Caspase 8 ELISA Kit (Invitrogen, Catalog Number BMS2024) as per manufacturer's protocol.

Analysis of the expressional status of APAF-1. The cells were seeded in coverslip and allowed to adhere overnight. After the treatment with P2 for 24 h at 37°C, the cells were incubated with primary antibody APAF-1 rabbit polyclonal (1:500) (Catalog Number A0751) overnight at 4°C. On the following day, Rhodamine (TRITC) conjugated Goat anti-Rabbit IgG (H+L) (Catalog Number AS040) was added for 2 h and mounting was done with DPX for imaging under confocal microscope (LSM 980, Zeiss Germany).

Statistical Analysis. The graphs were prepared using GraphPad Prism software (version 5) by unpaired t-test wherever applicable. Image analysis was carried out using Fiji-ImageJ software (https://imagej.net/Fiji). Flow cytometry analysis was performed using FlowJo software. P-value < 0.001 and<0.05 and above were considered statistically significant.

Measurement of mitochondrial membrane potential: For JC1 staining, SKOV3 and HEK 293 cells were seeded in 35 mm culture petri dish. After the treatment with P1 and P2, fresh media was added into the petri dish followed by the addition of (1 µL in 1 mL concentration form 10 mg/mL stock solution) JC1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide) and incubation for 30 mins. The samples were acquired in flow cytometer, BDLSRFortessa after washing with PBS. The analysis was performed in FACSDiva software. In JC-1 staining assays, the green fluorescence indicates mitochondria with low membrane potential that is associated with unhealthy or damaged mitochondria. On the contrary, healthy mitochondria with high membrane potential exhibit red fluorescence due to JC-1 aggregating in the mitochondria. The JC1 staining in normal cells showed almost 99% of cells with JC1 aggregates in control, P1 and P2 with no significant difference in them. Furthermore, cell percentages of JC1 aggregates in SKOV3 cells, showed a significant difference between control and P2 and no significant difference with P1. Therefore, the data showed P2 effecting the SKOV3 cells via mitochondria mediated pathway without affecting the mitochondria of normal cells.

Synthetic Procedure:

Synthesis of 2-carboxyethyl-(triphenyl)-phosphanium [Ph_3P^+-C_2-COOH]: 1-bromo propanoic acid (1.52 g, 10 mM) and triphenyl phosphine (3.9 g, 15 mM) were taken in a 250 mL round bottom flask and 100 mL acetonitrile (CH_3CN) was added to dissolve it. After that, the mixture was put in an oil bath at 80°C temperature and reflux for 2 h. After 2 h CH_3CN was evaporated in vacuum from the reaction mixture and white solid was obtained. Purification was done using a silica gel column (100–200 mesh) using chloroform and methanol as eluents. The compound was coming at chloroform: methanol (95:5) mixture.

Yield: 3.1 g (9.22 mM, 89%).

HRMS (m/z) calculated for $C_{20}H_{21}O_2P^+$ (M): Exact mass: 355.1195, **Mass obtained**: 335.0970, 336.1019 $(M+H)^+$.

^{1}H NMR (400 MHz, DMSO): δ 12.72 (s, 1H, -COOH), 7.88 (s, 15H, aromatic), 3.82-3.72 (m, 2H), 2.70-2.46 (s, 2H).

^{13}C NMR (101 MHz, DMSO): δ 171.5, 162.2, 135.0, 133.7, 130.3, 118.5, 117.6, 35.7, 30.7, 26.8.

^{31}P NMR (162 MHz, DMSO): δ 26.48.

Synthesis of Boc-Phe-Gly-OMe [B-F-G-OMe]: Boc-Phe-OH (2.65 g, 10 mM) was taken in a 250 mL round bottom flask and 10 mL (dimethyl formamide) DMF was added to dissolve it. After that the mixture was cooled in an ice-water bath having temperature 0°C–10°C. H_2N-Gly-COOMe was isolated from the corresponding methyl ester hydrochloride (1 g, 12 mM) by neutralization, subsequent extraction with ethyl acetate and concentration to 50 mL. Then it was added to the reaction mixture, followed immediately by DCC (2.472 g, 12 mM) and HOBt (1.52 g, 12 mM). The reaction mixture was stirred for 36 h. The reaction mixture was filtered through a sintered glass crucible and the DCU (dicyclohexyl urea) was filtered off. The organic

layer was washed with brine (2×50 mL) and then dried over anhydrous sodium sulphate and evaporated in vacuum to yield peptide as a white solid. Purification was done using a silica gel column (100–200 mesh) using chloroform and ethyl acetate as eluents.

Yield: 3.1 g (9.22 mM, 92.20%).

HRMS (m/z) calculated for $C_{17}H_{24}N_2O_5$ (M): Exact mass: 336.1685, **Mass obtained**: 359.1845 $(M+Na)^+$.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38 – 7.24 (m, 5H, aromatic), 6.62 (s, 1H, NH), 5.14 (s, 1H, NH), 4.48 (s, 1H, α-H of phe), 4.14 – 3.95 (m, 2H, α-H of gly), 3.79 (s, 3H, -OCH_3), 3.25 – 3.06 (m, 2H, β-H of phe), 1.45 (s, 9H, Boc).

^{13}C NMR (126 MHz, $CDCl_3$): δ 171.7, 170.0, 155.5, 136.7, 129.4, 128.7, 127.0, 80.3, 55.7, 52.4, 41.2, 38.4, 34.0, 28.3, 25.7, 25.0.

Synthesis of Boc-Phe-Gly-COOH [B-F-G-OH]: Boc-Phe-Gly-OMe (3.0g, 8.92 mM) was taken in a 250 mL round bottom flask. The solid was dissolved in MeOH (40 mL) and 1N NaOH (15 mL) was added to the mixture. The reaction mixture was stirred for 24 h and the progress of saponification was monitored by thin layer chromatography (TLC). After 24 h methanol was removed under vacuum, the residue was taken in 50 mL of water, washed with diethyl ether (2×50 mL). Then the pH of the aqueous layer was adjusted to 2 using 1(N) HCl and it was extracted with ethyl acetate (3×50 mL). The extracts were dried over anhydrous sodium sulphate, and evaporated in vacuum to yield as a white solid product. Purification was done using a silica gel column (100–200 mesh) using chloroform and methanol as eluents.

Yield: 2.7g (8.36 mM, 90 %).

HRMS (m/z) calculated for $C_{16}H_{22}N_2O_5$ (M): Exact mass: 322.1529, **Mass obtained**: 323.1403 $(M+H)^+$, 345.1228 $(M+Na)^+$.

^{1}H NMR (400 MHz, DMSO): δ 13.27 (s, 1H,-COOH), 8.21 (s, 1H, NH), 8.01 – 7.29 (m, 6H, aromatic), 6.90 (s, 1H, NH), 4.39 – 4.03 (m, 1H, α-H of phe), 3.93 – 3.66 (m, 2H, α-H of gly), 3.02 (d, J = 17.9 Hz, 1H, β-H of phe), 2.75 (d, J = 10.5 Hz, 1H, β-H of phe), 1.34 (s, 9H, Boc).

^{13}C NMR (101 MHz, DMSO): δ 172.5, 171.6, 155.69, 138.7, 129.6, 128.4, 128.2, 127.8, 126.5, 124.9, 119.6, 110.0, 78.4, 55.9, 41.1, 37.9, 28.6.

Synthesis of Boc-Phe-Gly-C_{12} [B-F-G-C_{12}]: Boc-Phe-Gly-OH (4.8g, 15 mM) was dissolved in 10 mL DMF and 10 mL of EtOAc in a 250 mL round bottom flask. The mixture was cooled to 0°C in an ice water bath. 2.7g (19 mM) HOBt was added to it. Then H_2N-C_{12} (2.7 g, 15 mM dissolved in 15 mL ethyl acetate) followed by DCC (4g, 19 mM) was added to the reaction mixture. The reaction mixture was allowed to come to room temperature and stirred for 24 h. The reaction mixture was filtered to separate N, N-dicyclohexyl urea (DCU). The organic layer was washed with 1 (N) HCl (3 × 30 mL), brine (1 × 30 mL) and brine (2 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated in vacuum. A yellowish white material was obtained, purified using silica gel in pet ether and ethyl acetate (85:15) as eluent. After purification white colour powder was obtained.

Yield: 4g (8 mM, 83%)

HRMS (m/z) calculated for $C_{28}H_{47}N_3O_2$ (M): Exact mass: 489.3567, **Mass obtained**: 512.3171 $(M+Na)^+$.

Synthesis of H_2N-Phe-Gly-C_{12} [NH_2-F-G-C_{12}]: 3.3g (8 mM) of Boc-Phe-Gly-C_{12}, 5 mL of formic acid was added and the removal of the Boc group was monitored by TLC. After 24 h, formic acid was removed under vacuum. The residue was taken in water (10 mL) and pH was maintained by using saturated sodium carbonate solution. The resulting aqueous layer was extracted with ethyl acetate (3 × 40 mL).The ethyl acetate extract was dried over anhydrous sodium sulphate and evaporated in vacuum to obtain the white colourless sticky product. A

white material was obtained after purification in basic alumina in chloroform and methanol (9:1) as eluent.

Yield: 3g (9 mM, 90%)

HRMS (m/z) calculated for $C_{23}H_{39}N_3O_2$ (M): Exact mass: 389.3042, **Mass obtained**: 390.3068 $(M+H)^+$.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.27 – 7.13 (m, 5H, aromatic), 6.61 (s, 1H, NH), 6.42 (s, 1H, NH), 5.01 (d, J = 6.8 Hz, 1H, NH), 4.25 (q, J = 6.9 Hz, 1H, α-H of phe), 3.91 – 3.72 (m, 2H, α-H of gly), 3.19 – 2.97 (m, 4H, β-H of phe and α-H of long chain), 1.44 (t, J = 7.2 Hz, 2H, β-H of long chain), 1.36 (s, 9H, Boc), 1.22 (d, J = 5.8 Hz, 18H, 10-CH_2 of fatty acyl chain), 0.84 (t, J = 6.7 Hz, 3H, -CH_3 of long chain).

^{13}C NMR (101 MHz, $CDCl_3$): δ 171.8, 168.4, 155.7, 136.3, 129.1, 128.8, 127.1, 80.6, 56.4, 43.2, 39.6, 37.9, 31.9, 29.6, 29.6, 29.6, 29.5, 29.4, 29.3, 28.2, 26.9, 22.7, 14.1.

Synthesis of Ph_3P^+-C_2-Phe-Gly-C_{12} [PHFGC_{12}]: NH_2-F-G-C_{12} (2.0 g, 5.0 mM) was taken in a 250 mL round bottom flask and 10 mL DMF and EtOAc were added to dissolve it. After that $Ph3P^+$-C_2-COOH (1.68 g, 5.0 mM) was added to this mixture and cooled in an ice-water bath having temperature 0°C–10°C. Then DCC (0.84 g, 4.0 mM) and HOBt (0.61 g, 4.5 mM) were added to this reaction mixture and stirred for 48 h. The reaction mixture was filtered through a sintered glass crucible and the DCU was filtered off. The organic layer was washed with brine (2×50 mL) and then dried over anhydrous sodium sulphate and evaporated in vacuum to yield peptide as a white solid. Purification was done using a silica gel column (100–200 mesh) using chloroform and methanol as eluents.

Yield: 1.47g (2.5 mM, 83%).

HRMS (m/z) calculated for $C_{44}H_{57}N_3O_3P^+$ (M): Exact mass: 706.4132, **Mass obtained**: 706.4280, 707.4349 $(M+H)^+$.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.91 (d, J = 7.8 Hz, 1H, NH), 8.15 (t, J = 6.2 Hz, 1H), 7.75 – 7.66 (m, 4H, aromatic), 7.60 (tdd, J = 8.3, 6.6, 2.9 Hz, 12H, aromatic), 7.39 – 7.22 (m, 4H, aromatic), 6.98 (br, 1H, NH), 5.18 (s, 1H), 4.61 (br, 3H, α-H of phe and gly), 3.94 (dd, J = 16.8, 6.4 Hz, 1H, βH of phe), 3.79 (dd, J = 16.8, 5.5 Hz, 1H, βH of of phe), 3.73 – 3.60 (m, 1H, βH of), 3.46 (dtd, J = 15.8, 11.7, 4.5 Hz, 1H), 3.30 (dd, J = 14.2, 4.3 Hz, 1H), 3.11 (td, J = 9.2, 4.6 Hz, 2H), 3.06 – 2.95 (m, 2H), 2.60 (dtd, J = 15.5, 11.2, 4.6 Hz, 1H), 1.42 (p, J = 7.3 Hz, 2H, βH of long chain), 1.31 – 1.15 (m, 18H, 9-CH_2 of long fatty acyl chain), 0.86 (t, J = 6.8 Hz, 3H, -CH_3).

^{13}C NMR (101 MHz, $CDCl_3$): δ 172.0, 170.3, 170.1, 169.6, 142.0, 137.7, 135.4, 135.3, 133.5, 133.4, 130.6, 130.5, 129.5, 128.3, 126.5, 126.2, 125.1, 118.1, 117.9, 117.1, 110.9, 77.3, 56.6, 43.4, 39.7, 37.6, 32.0, 29.8, 29.4, 28.6, 27.0, 22.7, 19.2, 14.2.

^{31}P NMR (162 MHz, $CDCl_3$): δ 24.63 (d, J = 15.6 Hz)

Synthesis of Boc-Phe-Phe-OMe [B-F-F-OMe]: Boc-Phe-OH (2.65 g, 10 mM) was taken in a 250 mL round bottom flask and 10 mL (dimethyl formamide) DMF was added to dissolve it. After that the mixture was cooled in an ice-water bath having temperature 0°C–10°C. H_2N-Phe-COOMe was isolated from the corresponding methyl ester hydrochloride (2.1 g, 12 mM) by neutralization, subsequent extraction with ethyl acetate and concentration to 50 mL. Then it was added to the reaction mixture, followed immediately by DCC (2.472 g, 12 mM) and HOBt (1.52 g, 12 mM). The reaction mixture was stirred for 36 h. The reaction mixture was filtered through a sintered glass crucible and the DCU (dicyclohexyl urea) was filtered off. The organic layer was washed with brine (2×50 mL) and then dried over anhydrous sodium sulphate

and evaporated in vacuum to yield peptide as a white solid. Purification was done using a silica gel column (100–200 mesh) using chloroform and ethyl acetate as eluents.

Yield: 3.9 g (9.15 mM, 91.50%).

HRMS (m/z) calculated for $C_{24}H_{30}N_2O_5$ (M): Exact mass: 426.2155, **Mass obtained**: 427.2073 $(M+H)^+$.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31–7.23 (m, 8H, aromatic), 7.03 (d, J = 7.6 Hz, 2H, aromatic), 6.34 (s, 1H, NH), 5.00 (s, 1H, NH), 4.83 (d, J = 7.5 Hz, 1H, α-H of Phe), 4.39 (s, 1H, α-H of Phe), 3.72 (s, 3H, -OCH_3), 3.16 – 3.02 (m, 4H, β-H of Phe), 1.45 (s, 9H, Boc).

^{13}C NMR (101 MHz, $CDCl_3$): δ 171.3, 170.7, 155.2, 136.5, 135.6, 129.3, 129.2, 128.6, 128.5, 127.1, 126.9, 80.2, 55.7, 53.2, 52.2, 38.2, 37.9, 28.2.

Synthesis of Boc-Phe-Phe-COOH [B-F-F-OH]: Boc-Phe-Phe-OMe (3.9g, 9.1 mM) was taken in a 250 mL round bottom flask. The solid was dissolved in MeOH (40 mL) and 1N NaOH (15 mL) was added to the mixture. The reaction mixture was stirred for 24 h and the progress of saponification was monitored by thin layer chromatography (TLC). After 24 h methanol was removed under vacuum, the residue was taken in 50 mL of water, washed with diethyl ether (2×50 mL). Then the pH of the aqueous layer was adjusted to 2 using 1(N) HCl and it was extracted with ethyl acetate (3×50 mL). The extracts were dried over anhydrous sodium sulphate, and evaporated in vacuum to yield as a white solid product. Purification was done using a silica gel column (100–200 mesh) using chloroform and methanol as eluents.

Yield: 3.5 g (8.9 mM, 97 %).

HRMS (m/z) calculated for $C_{23}H_{28}N_2O_5$ (M): Exact mass: 412.1998, **Mass obtained**: 413.1935 $(M+H)^+$.

^{1}H NMR (400 MHz, DMSO): δ 12.01 (s, 1H, -COOH), 8.10 (s, 1H, NH), 7.31 – 7.19 (m, 10H, aromatic), 6.90 (s, 1H, NH), 4.51 – 4.41 (m, 1H, α-H of Phe), 4.25 – 4.08 (m, 1H, α-H of Phe), 3.09 (d, J = 13.8 Hz, 2H, β-H of Phe), 2.96 – 2.81 (m, 2H, β-H of Phe), 1.28 (s, 9H, Boc).

^{13}C NMR (101 MHz, DMSO): δ 173.3, 171.8, 155.5, 138.5, 129.6, 128.6, 128.6, 78.5, 56.1, 53.8, 37.3, 33.8, 28.5.

Synthesis of Boc-Phe-Phe-C_{12} [B-F-F-C_{12}]: Boc-Phe-Phe-OH (6.1 g, 15 mM) was dissolved in 10 mL DMF and 10 mL of EtOAc in a 250 mL round bottom flask. The mixture was cooled to 0°C in an ice water bath. 2.7g (19 mM) HOBt was added to it. Then H_2N-C_{12} (2.7 g, 15 mM dissolved in 15 mL ethyl acetate) followed by DCC (4g, 19 mM) was added to the reaction mixture. The reaction mixture was allowed to come to room temperature and stirred for 24 h. The reaction mixture was filtered to separate N, N-dicyclohexyl urea (DCU). The organic layer was washed with 1 (N) HCl (3 × 30 mL), brine (1 × 30 mL) and brine (2 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated in vacuum. A yellowish white material was obtained, purified using silica gel in pet ether and ethyl acetate (85:15) as eluent. After purification white colour powder was obtained.

Yield: 5g (8.6 mM, 82 %)

HRMS (m/z) calculated for $C_{35}H_{53}N_3O_4$ (M): Exact mass: 579.4036, **Mass obtained**: 602.4372 $(M+Na)^+$.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.30 – 7.16 (m, 10H, aromatic), 6.95 (s, 1H, NH), 6.34 (d, J = 6.4 Hz, 1H, NH), 4.71 (d, J = 25.2 Hz, 1H, NH), 4.47 (q, J = 6.5 Hz, 1H, α-H of Phe), 4.17 (dt, J = 7.8, 5.8 Hz, 1H, α-H of Phe), 3.26 – 3.10 (m, 2H, α-H of long chain), 2.99 – 2.89 (m, 4H, β-H of Phe), 1.40 (d, J = 7.3 Hz, 2H, β-H of long chain), 1.32 (s, 9H, Boc), 1.25 (s, 15H, -CH_2 of long chain), 0.88 (t, J = 6.8 Hz, 3H, -CH_3).

^{13}C NMR (101 MHz, $CDCl_3$): δ 171.2, 170.2, 162.83, 155.5, 136.6, 136.5, 136.2, 129.5, 129.5, 129.4, 129.3, 128.9, 128.8, 128.7, 128.56, 127.3, 127.3, 127.1, 127.0, 80.8, 80.6, 57.1, 54.0, 53.6, 50.9, 39.8, 37.6, 36.6, 33.9, 32.0, 31.6, 29.7, 29.7, 29.7, 29.6, 29.4, 29.3, 29.3, 29.3, 28.3, 28.2, 26.9, 25.6, 25.0, 22.8, 14.2.

Synthesis of Ph_3P^+-C_2-Phe-Phe-C_{12} [PHFFC_{12}]: NH_2-F-F-C_{12} (2.3 g, 5.0 mM) was taken in a 250 mL round bottom flask and 10 mL DMF and EtOAc were added to dissolve it. After that Ph_3P^+-C_2-COOH (1.68 g, 5.0 mM) was added to this mixture and cooled in an ice-water bath having temperature 0°C–10°C. Then DCC (0.84 g, 4.0 mM) and HOBt (0.61 g, 4.5 mM) were added to this reaction mixture and stirred for 48 h. The reaction mixture was filtered through a sintered glass crucible and the DCU was filtered off. The organic layer was washed with brine (2×50 mL) and then dried over anhydrous sodium sulphate and evaporated in vacuum to yield peptide as a white solid. Purification was done using a silica gel column (100–200 mesh) using chloroform and methanol as eluents.

Yield: 1.5 g (1.9 mM, 76 %).

HRMS (m/z) calculated for $C_{51}H_{63}N_3O_3P^+$ (M): Exact mass: 796.4602, Mass obtained: 796.3876, 797.3931 $(M+H)^+$.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.95 (s, 1H, NH), 7.86 (s, 1H, NH), 7.73 – 7.56 (m, 15H, aromatic), 7.16 – 7.00 (m, 10H, aromatic), 6.48 (s, 1H, NH), 4.68 – 4.55 (m, 1H, α-H of Phe), 4.48 – 4.35 (m, 1H, α-H of Phe), 3.76 – 3.58 (m, 4H, α-H and β-H of phosphonium chain), 3.53 – 3.31 (m, 2H, α-H of long chain), 3.21 – 3.02 (m, 4H, β-H of Phe), 2.89 – 2.53 (m, 2H, β-H of long chain), 1.33– 1.19 (m, 18H, 9 -CH_2 of long chain), 0.89 – 0.85 (t, 3H, -CH_3 of long chain).

^{13}C NMR (126 MHz, $CDCl_3$): δ 171.7, 171.0, 170.5, 141.8, 137.3, 135.2, 133.5, 130.4, 129.32, 128.2, 126.0, 125.1, 117.8, 111.0, 57.3, 55.0, 39.6, 37.1, 31.9, 30.9, 29.6, 29.3, 26.9, 22.7, 14.1.

^{31}P NMR (202 MHz, $CDCl_3$): δ 24.40.

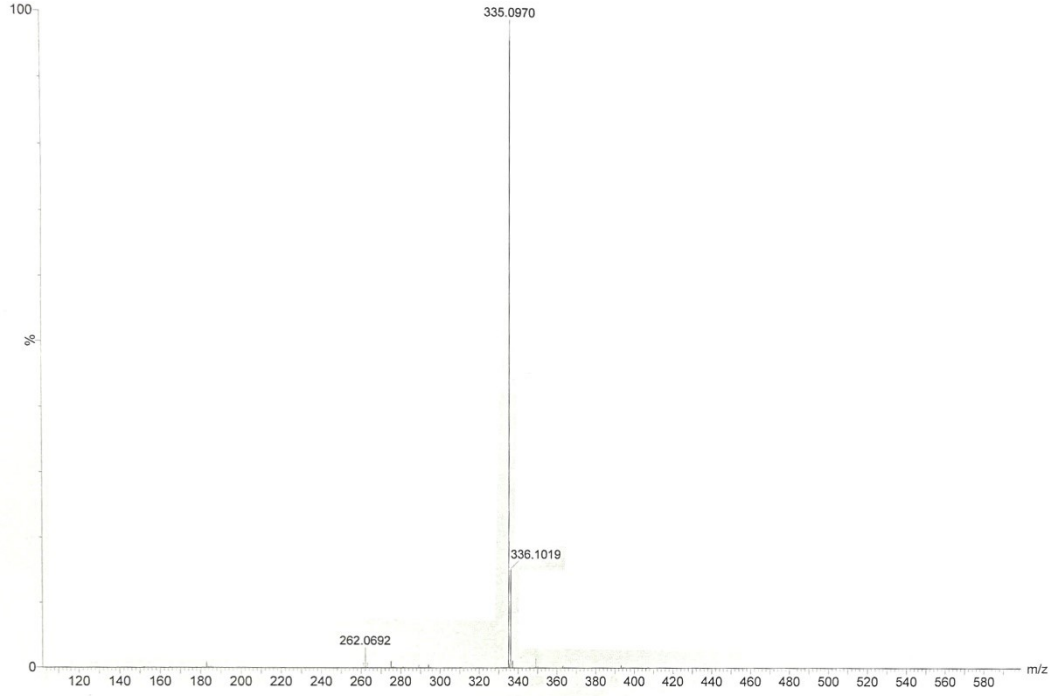


Fig. S1 HR-MS spectra of 2-carboxyethyl-(triphenyl)-phosphanium [Ph_3P^+-C_2-COOH]

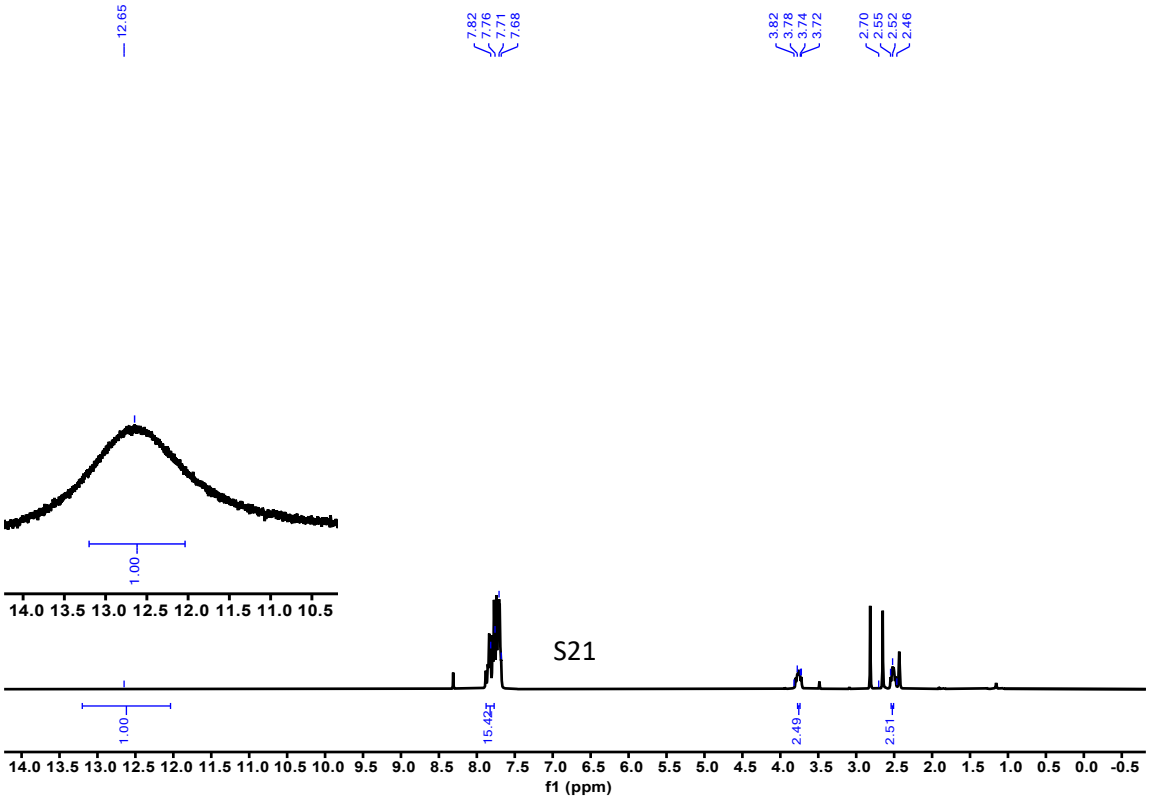


Fig. S2 ^{1}H NMR spectra of 2-carboxyethyl-(triphenyl)-phosphanium [Ph_3P^+-C_2-COOH]

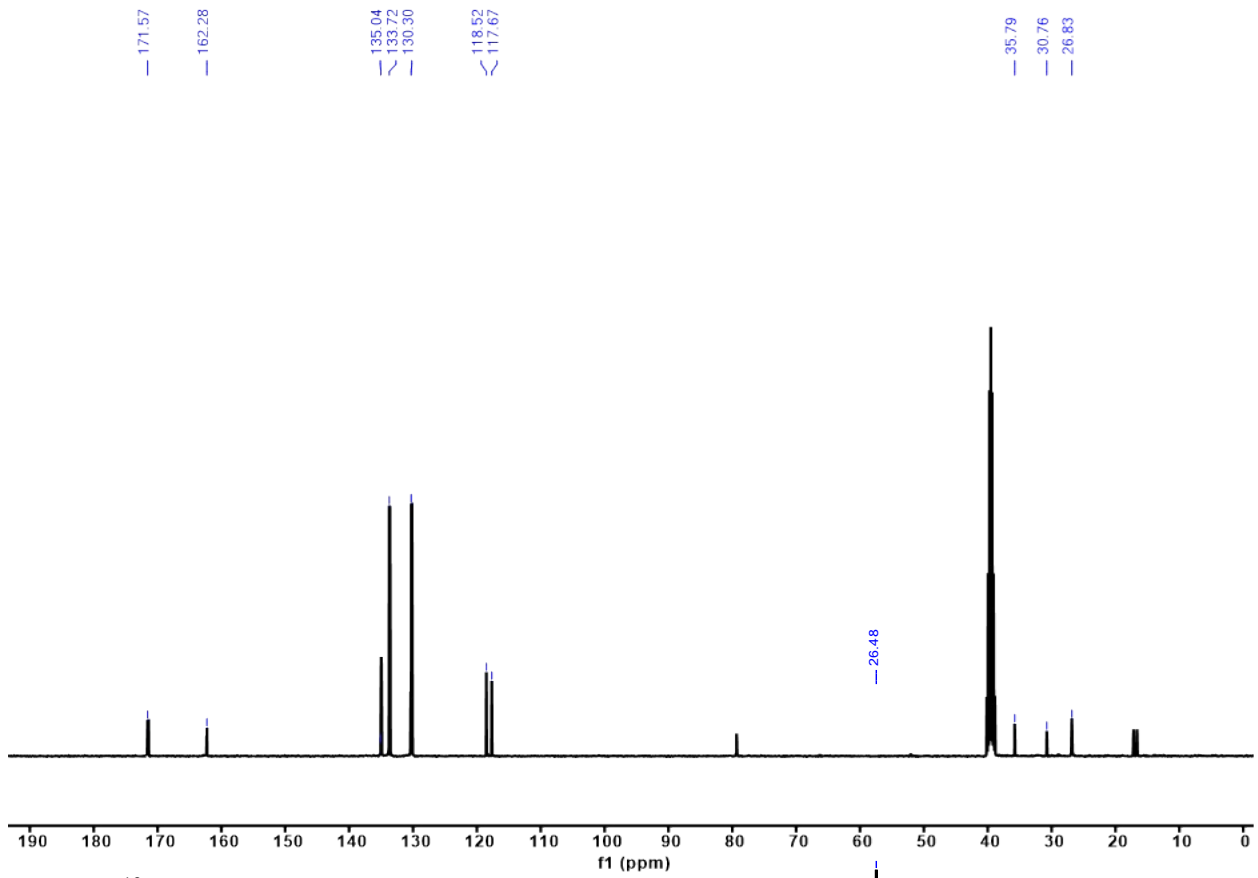


Fig. S3 ^{13}C NMR spectra of 2-carboxyethyl-(triphenyl)-phosphanium [Ph_3P^+-C_2-COOH]

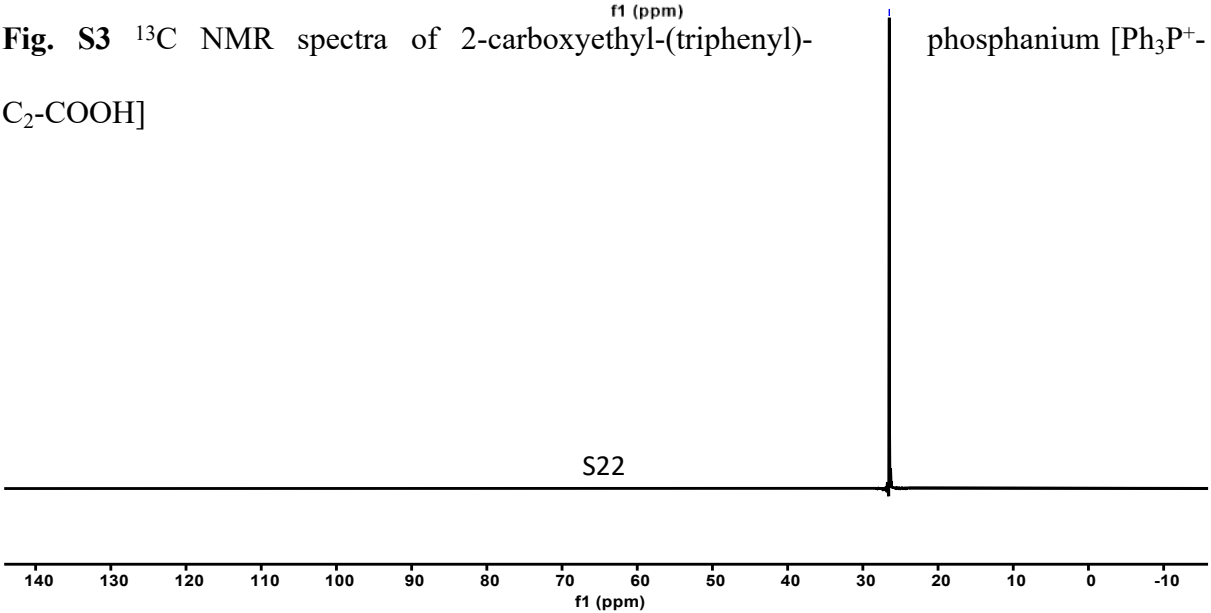


Fig. S4 ^{31}P NMR spectra of 2-carboxyethyl-(triphenyl)-phosphanium [Ph_3P^+-C_2-COOH]

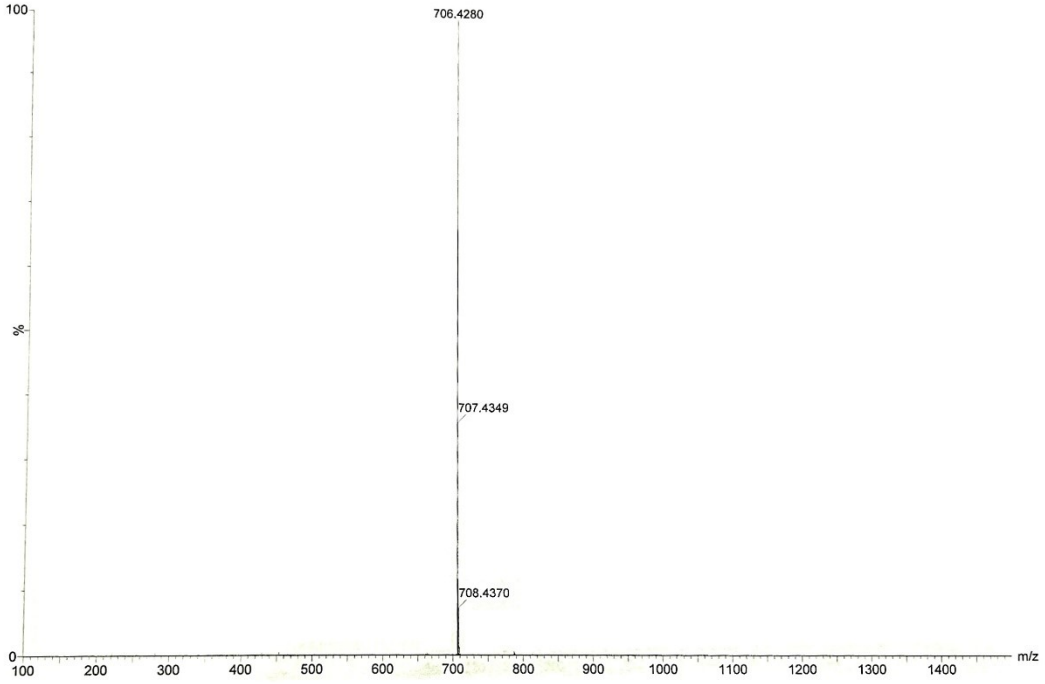


Fig. S5 HR-MS spectra of **Ph_3P^+-C_2-Phe-Gly-C_{12} [PHFGC_{12}]**

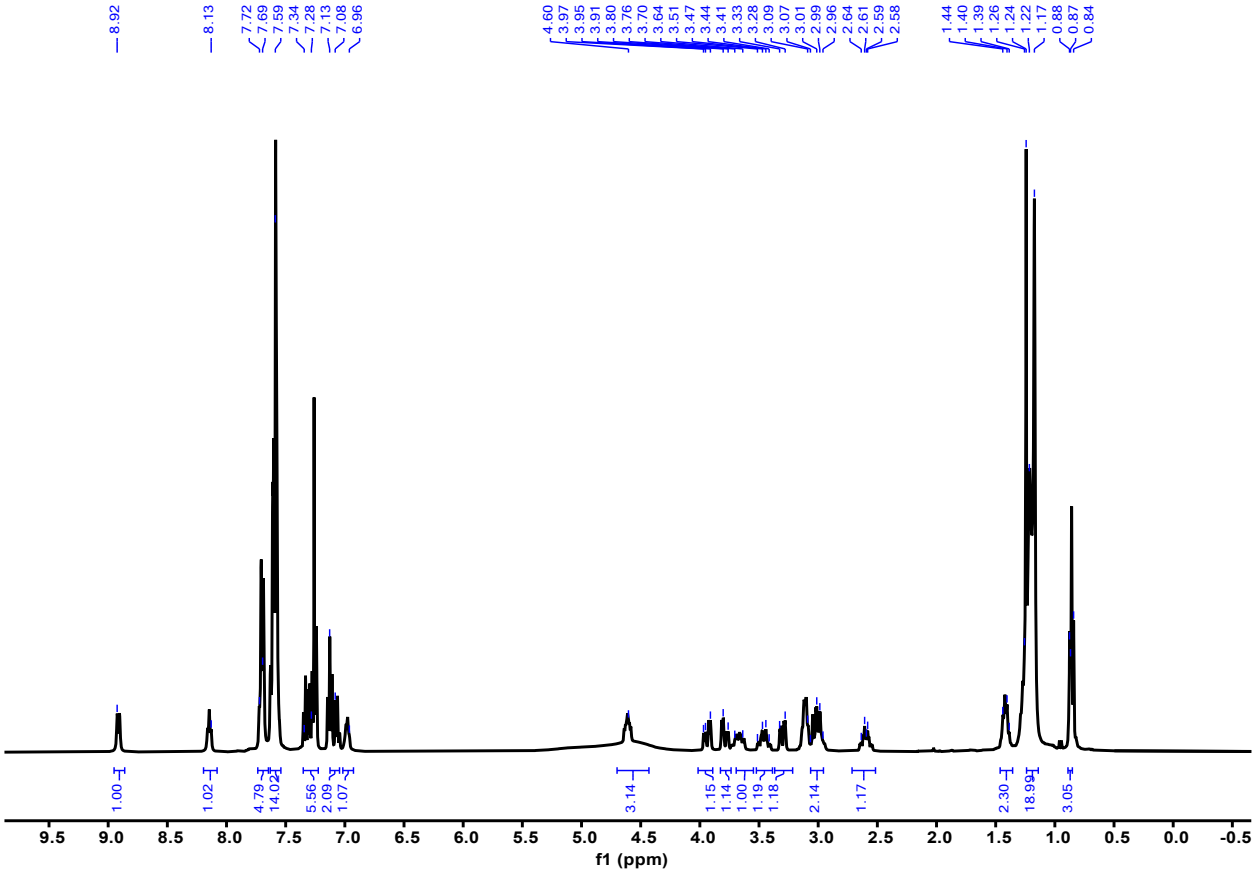


Fig. S6 ^{1}H NMR spectra of **Ph_3P^+-C_2-Phe-Gly-C_{12} [PHFGC$_{12}$]**

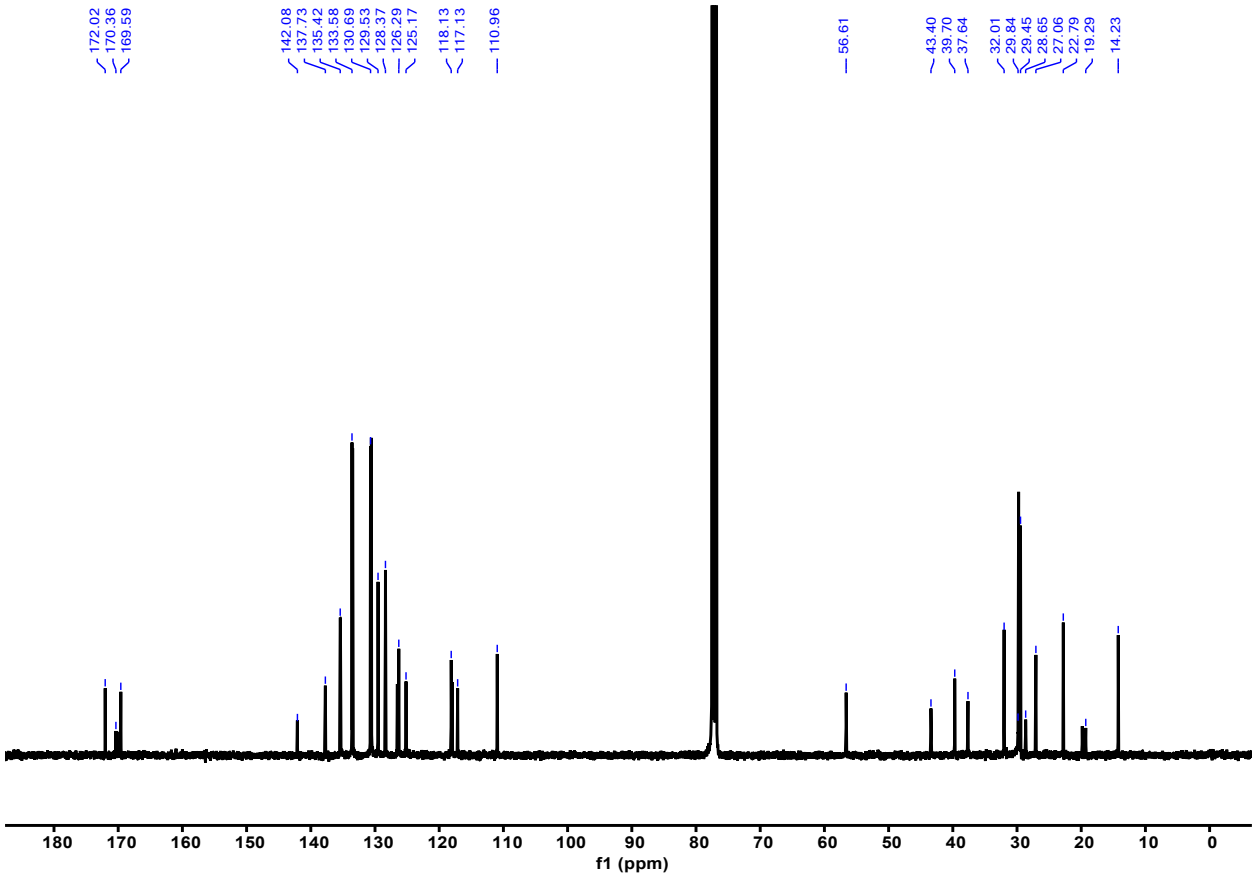


Fig. S7 ^{13}C NMR spectra of **Ph_3P^+-C_2-Phe-Gly-C_{12} [PHFGC$_{12}$]**

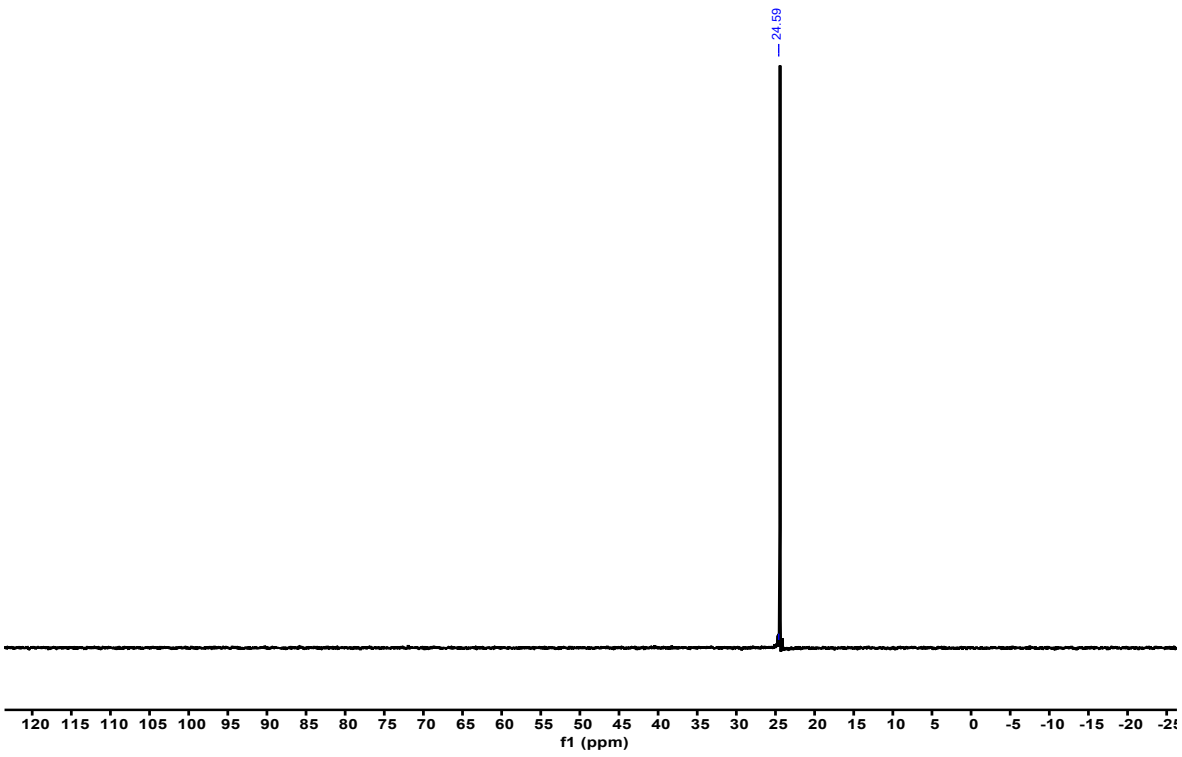


Fig. S8 ^{31}P NMR spectra of **Ph_3P^+-C_2-Phe-Gly-C_{12} [PHFGC_{12}]**

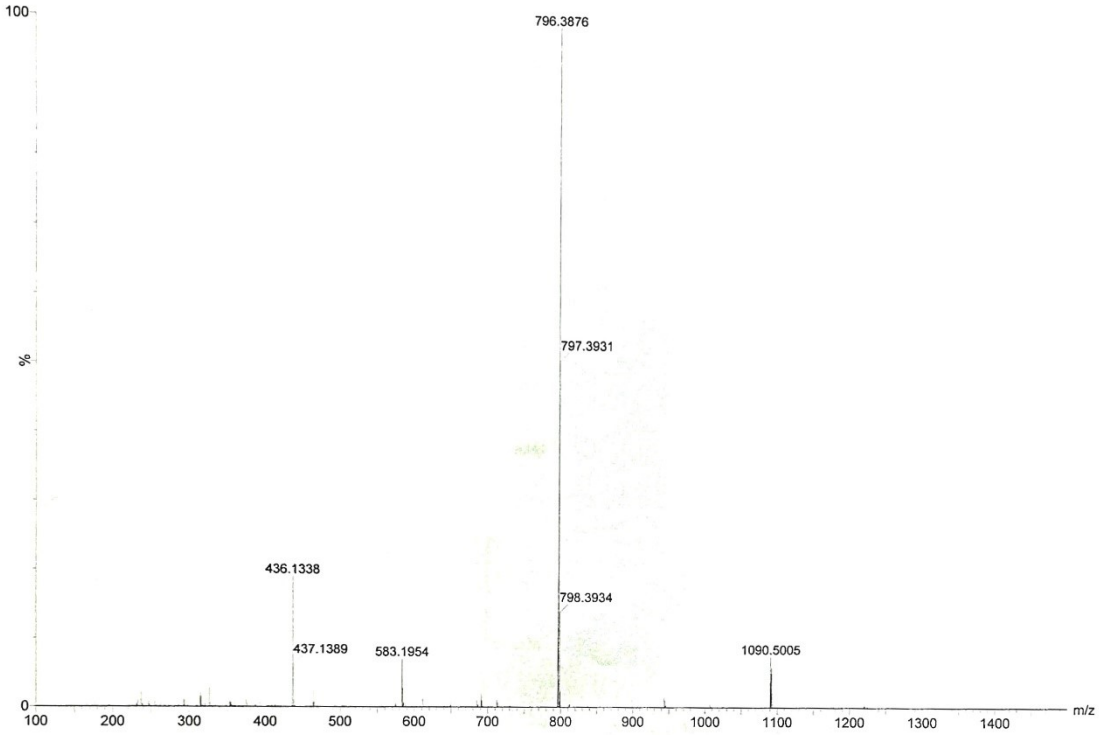


Fig. S9 HR-MS spectra of **Ph_3P^+-C_2-Phe-Phe-C_{12} [PHFFC_{12}]**

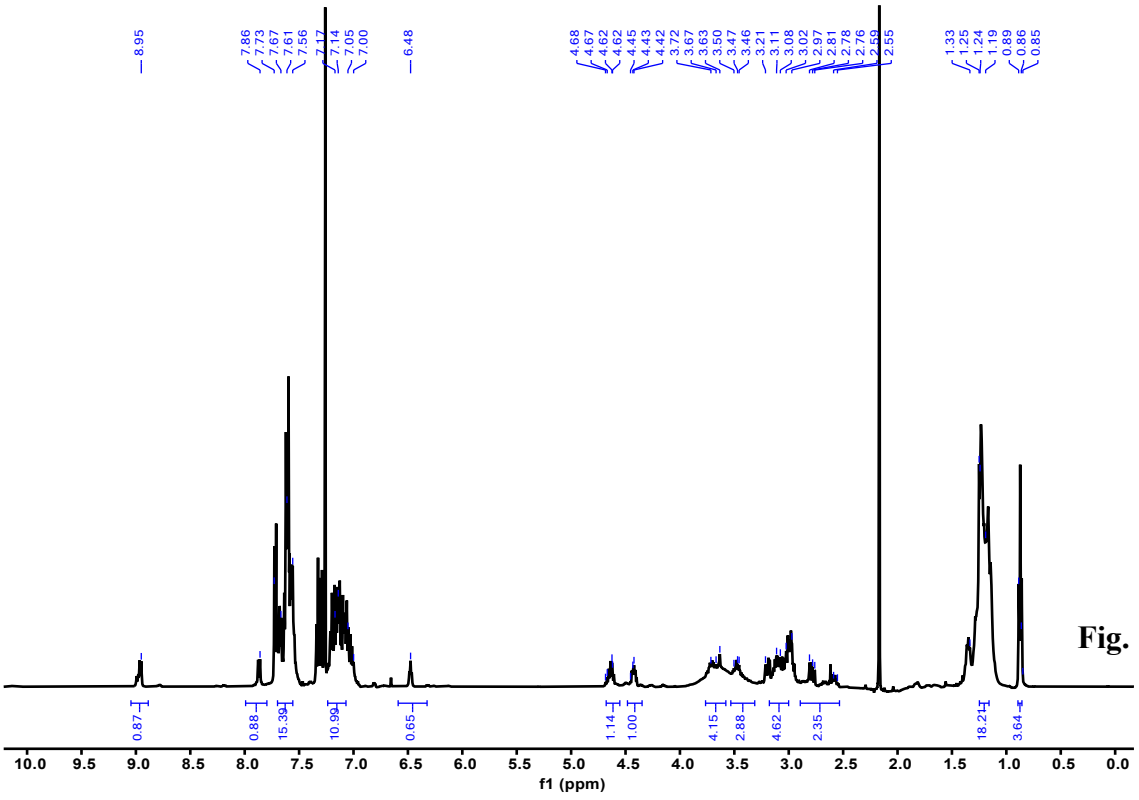


Fig. S10

^{1}H NMR spectra of **Ph_3P^+-C_2-Phe-Phe-C_{12} [PHFFC_{12}]**

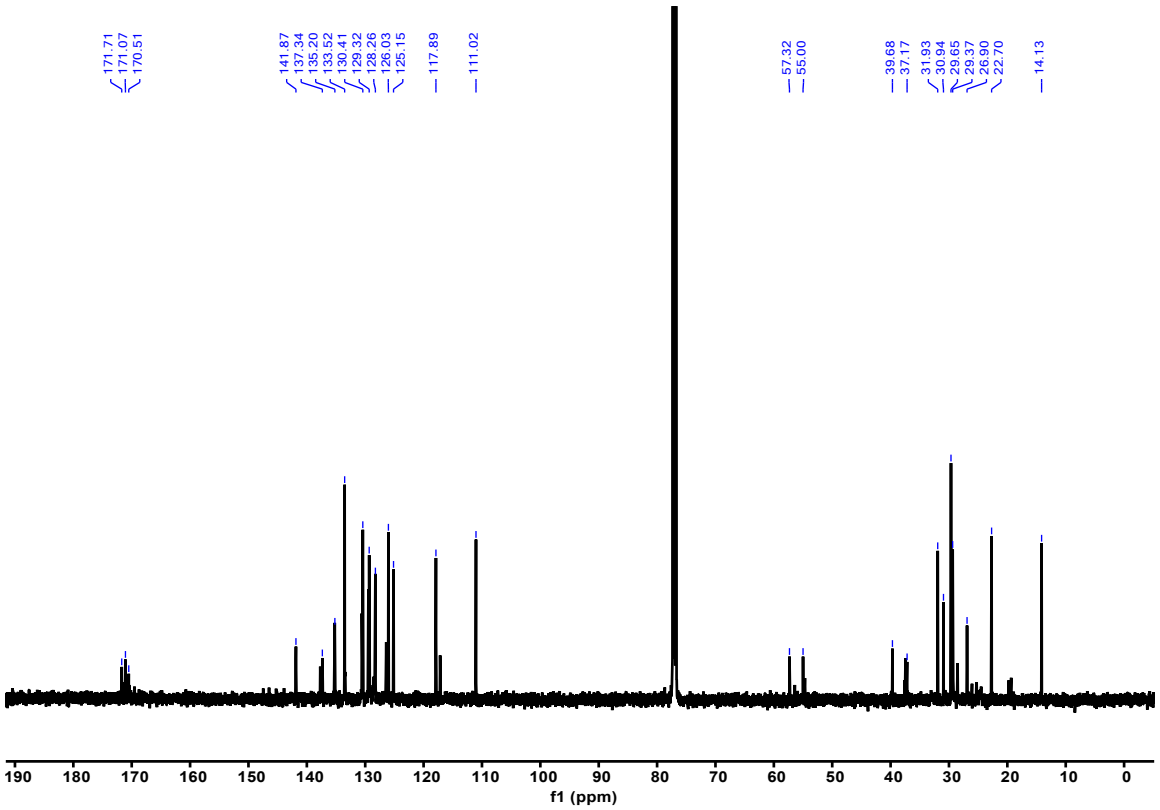


Fig. S11 ^{13}C NMR spectra of **Ph_3P^+-C_2-Phe-Phe-C_{12} [PHFFC_{12}]**

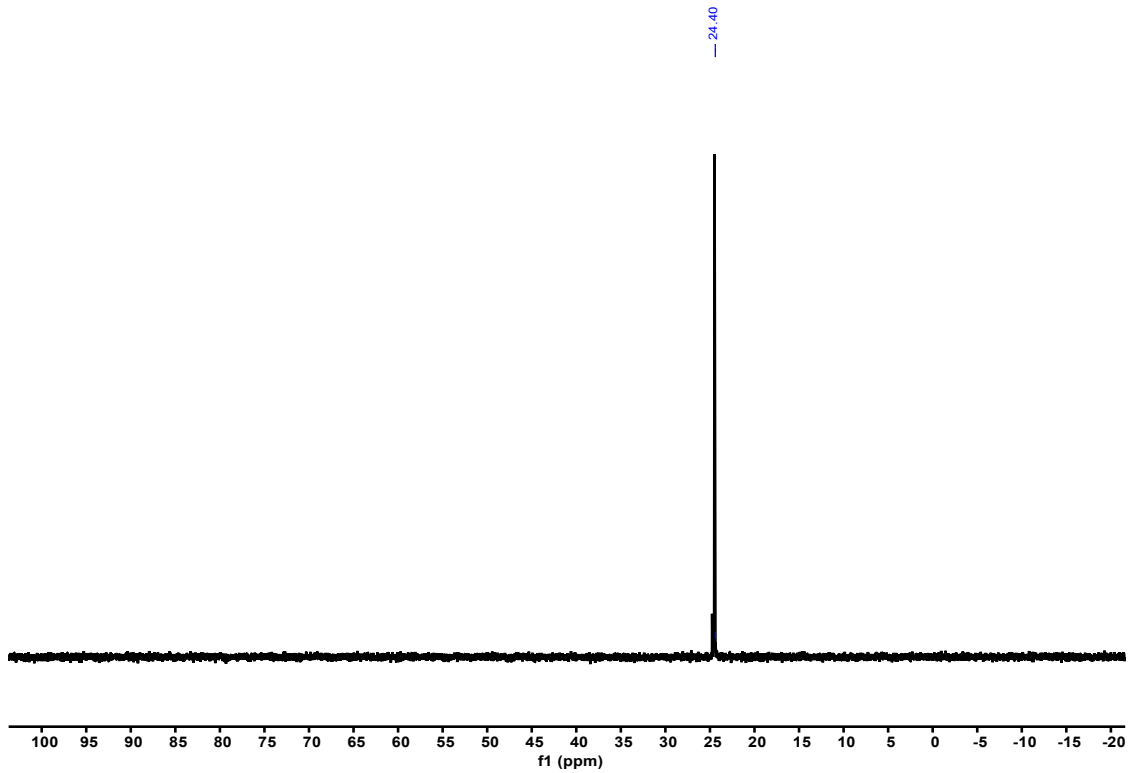


Fig. S12 ^{31}P NMR spectra of **Ph_3P^+-C_2-Phe-Phe-C_{12} [PHFFC_{12}]**

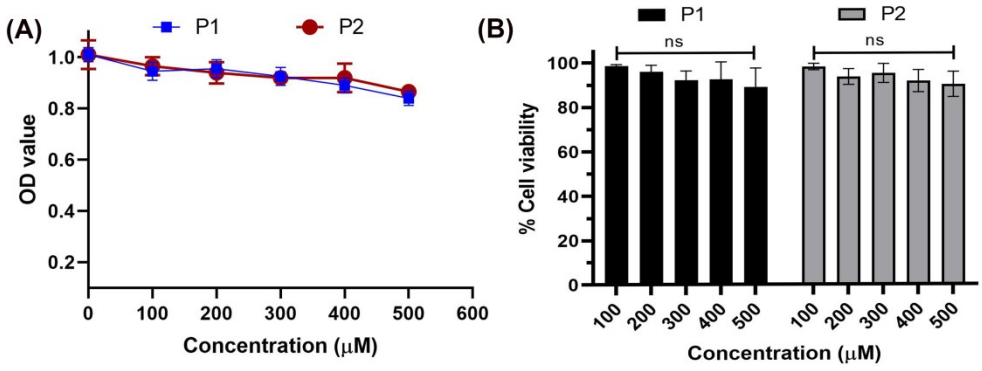


Fig. S13 (A) MTT analysis in normal human epithelial cells HEK 293. (B) Cell viability representation under the effect of the compounds P1 and P2 at different concentrations. (*P<0.5, **P<0.01, ***P<0.001, ****P<0.0001); ns denotes non-significant.

Table S1. Bacterial zone of inhibition (ZOI) diameters (mm) determined by the disc diffusion method and the minimum inhibitory concentrations (MIC) of P1 and P2 peptide amphiphiles for Gram-negative and Gram-positive bacteria.

Bacteria	**P1**		**P2**	
	ZOI (mm)	**MIC (μM)**	**ZOI (mm)**	**MIC (μM)**
P. aeruginosa	18	63 ± 3	18	40 ± 2
E. coli	18	62 ± 2	18	50 ± 3
B. subtilis	18	60 ± 4	18	45 ± 2
S. aureus	20	52 ± 2	20	48 ± 2

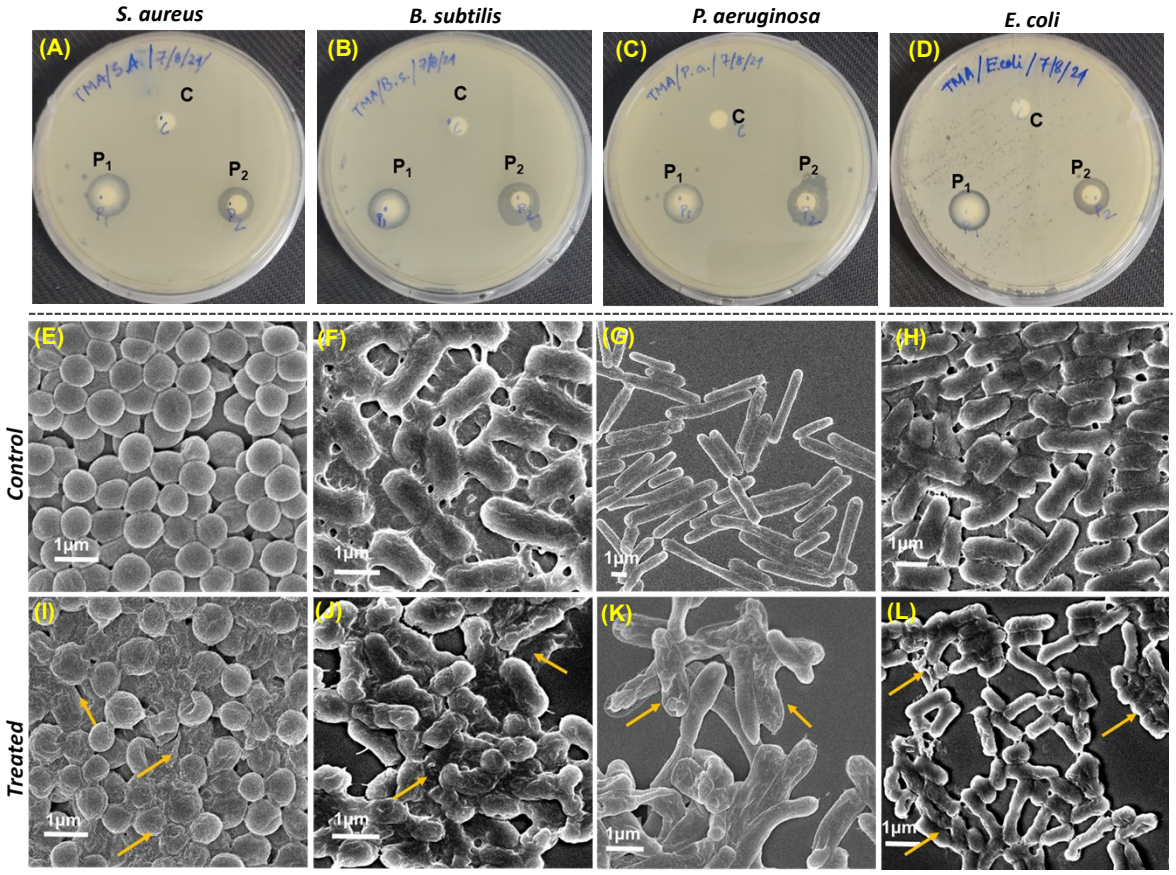


Fig. S14 Measurements of zone of inhibition of (A) *S. aureus*, (B) *B. subtilis* (C) *P. aeruginosa*, and (D) *E. coli*. FESEM images of (E) *S. aureus*, (F) *B. subtilis* (G) *P. aeruginosa*, and (h) *E. coli* without treated with P2 (control). FESEM images of (E) *S. aureus*, (F) *B. subtilis* (G) *P. aeruginosa*, and (h) *E.coli* treated with P2 (treated).

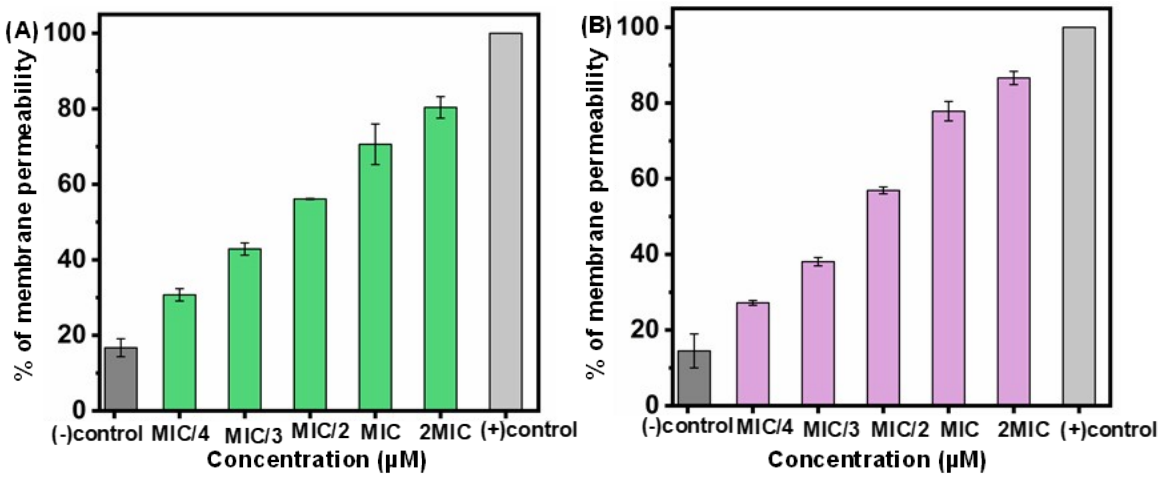


Fig. S15 Outer membrane permeability of *P. aeruginosa* treated with (A) P1 and (B) P2

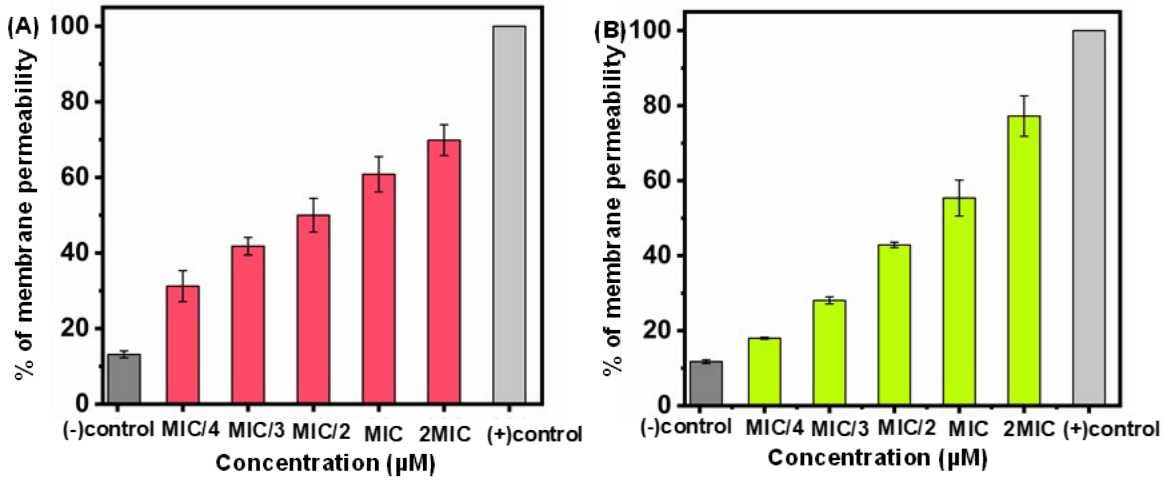


Fig. S16 Inner membrane permeability of *P. aeruginosa* treated with (A) P1 and (B) P2

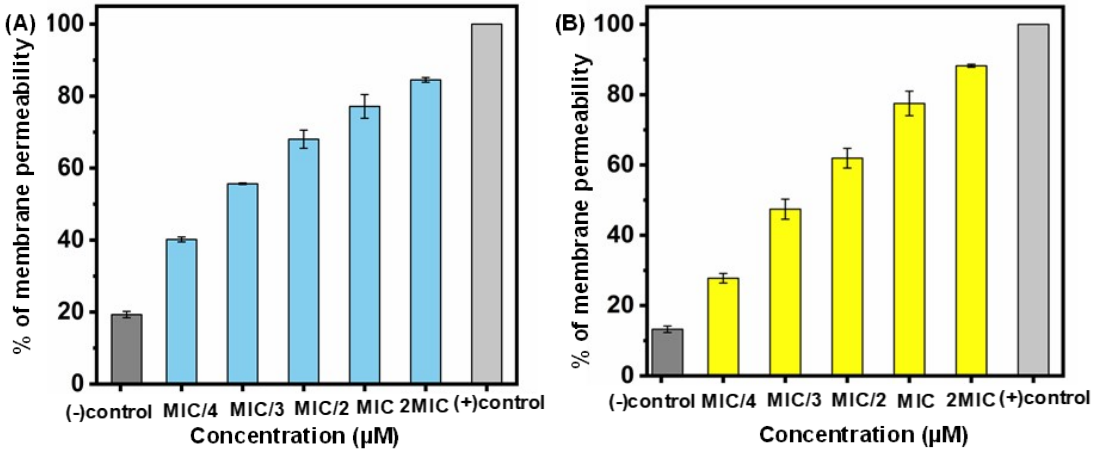


Fig. S17 Inner membrane permeability of *S. aureus* treated with (A) P1 and (B) P2

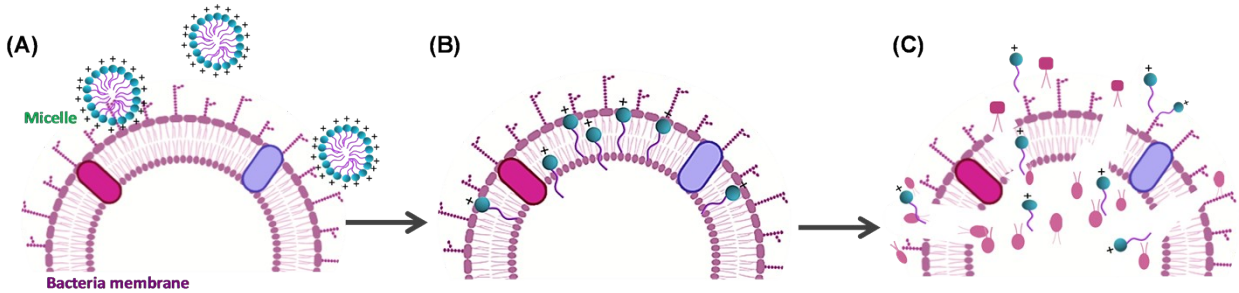


Fig. S18 Proposed model illustrating the probable mechanism disrupt the bacterial cell membrane leading to antibacterial activity. (A) Electrostatic interaction between negatively charged phospholipid membrane and positively charged micelles. (B) Cell membrane permeation by the nanostructures. (C) Disruption of phospholipid membrane and cell lysis.

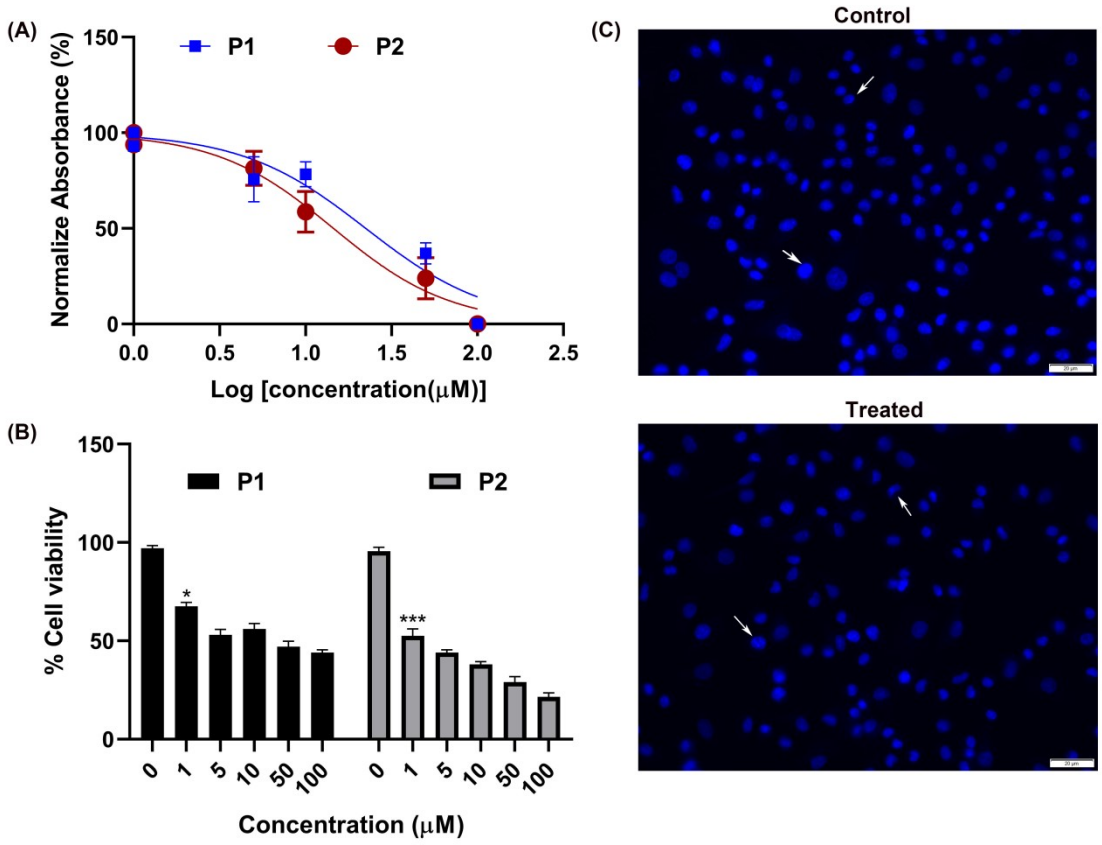
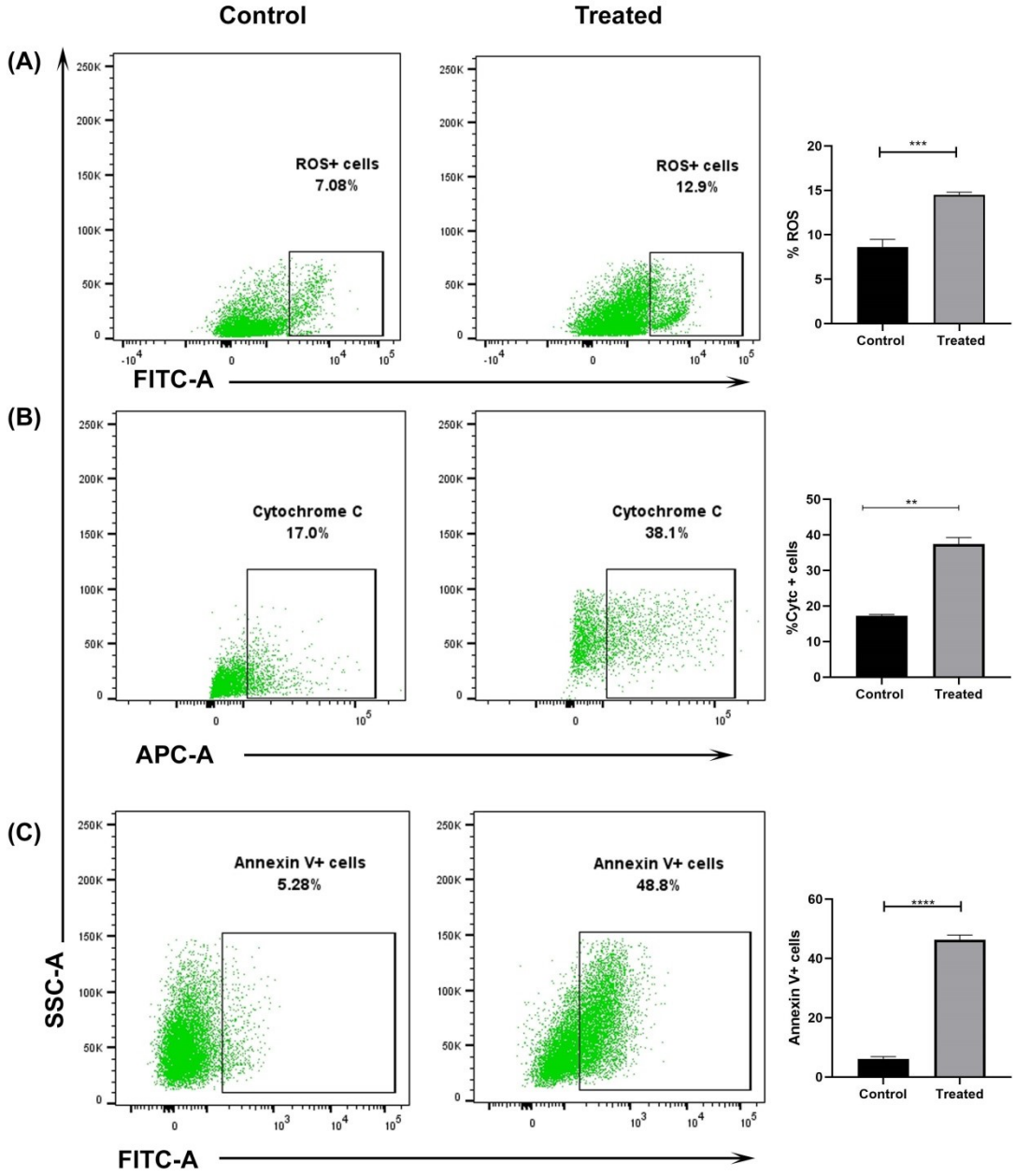


Fig. S19 (A) MTT analysis: impact of P1 and P2 on the viability of ovarian adenocarcinoma cells (SKOV3). (B) Percentage of cell viability representation under the effect of the



compounds at different concentrations. (C) Nuclear fragmentation of SKOV3 cells in control and P2 treated cells (arrows indicate the fragmentation). (*P<0.5, **P<0.01, ***P<0.001, ****P<0.0001).

Fig. S20 Flow cytometric analysis of ROS generation, Cyt c release and annexinV+ cells. (A) Flow cytometric analysis generation of ROS in control and treated cells. (B) Flow cytometric

analysis of Cyt c+ in control and treated cells. (C) Flow cytometric analysis of annexin V+ in control and treated cells (*P<0.5, **P<0.01, ***P<0.001, ****P<0.0001).

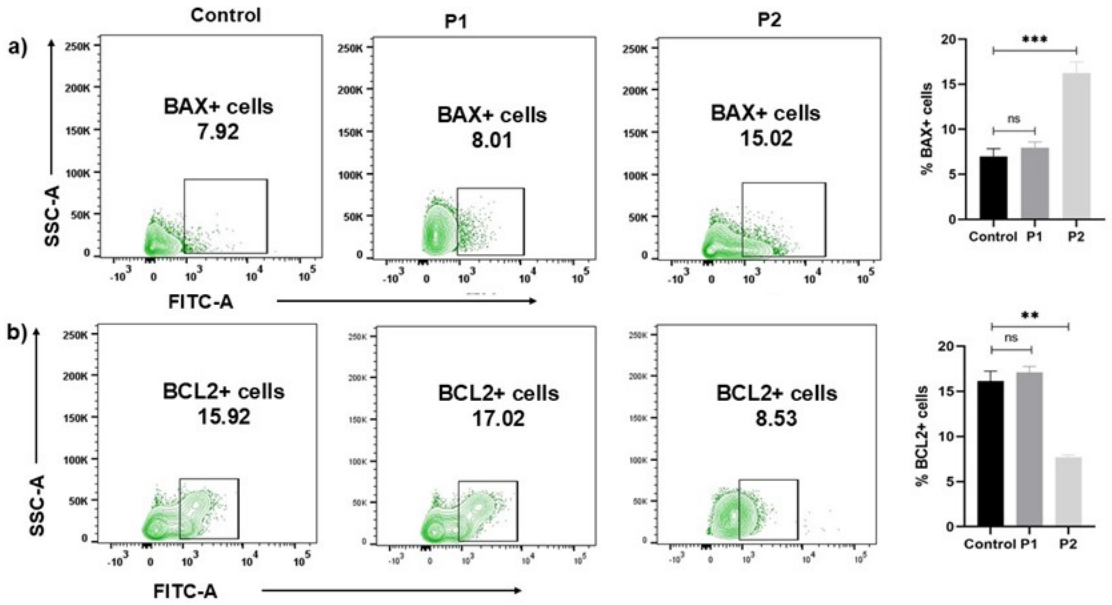


Fig. 21 Analysis of functional activity of P1 and P2 in SKOV3 cells. Flow cytometry analysis showed a) the expression of BAX in control, P1 and P2, b) the expression of BCL2 in control, P1 and P2. (Comparative data presented with the respective analysis) (Data represented as Mean±SD, all the significant values showed as *P<0.1, **P<0.01, ***P<0.001, ****P<0.0001)

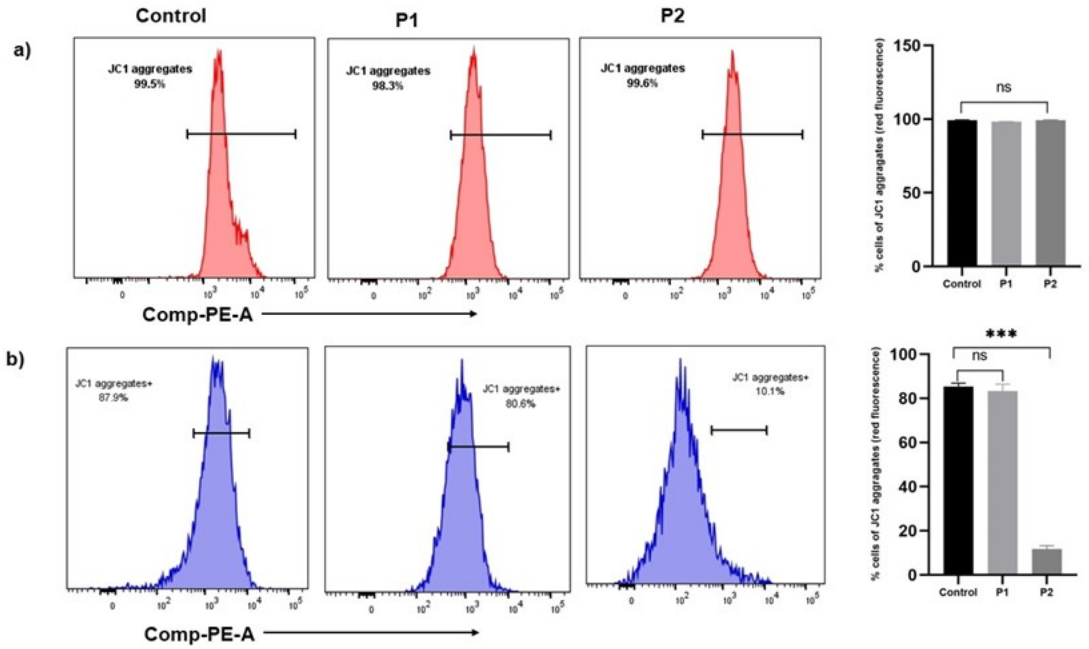


Fig. S22 Analysis of JC1 staining assay. Flow cytometry analysis showed a) expression of JC-1 aggregates in normal cells (HEK 293) with control, P1 and P2, b) expression of JC-1 aggregates in SKOV3 with control, P1 and P2. (Comparative data presented with the respective analysis). (Data represented as Mean±SD, all the significant values showed as *P<0.1, **P<0.01, ***P<0.001, ****P<0.0001)