Supporting Information

Systemic co-delivery of a homo-serine derived ceramide analog and curcumin to tumor vasculature inhibits mouse tumor growth

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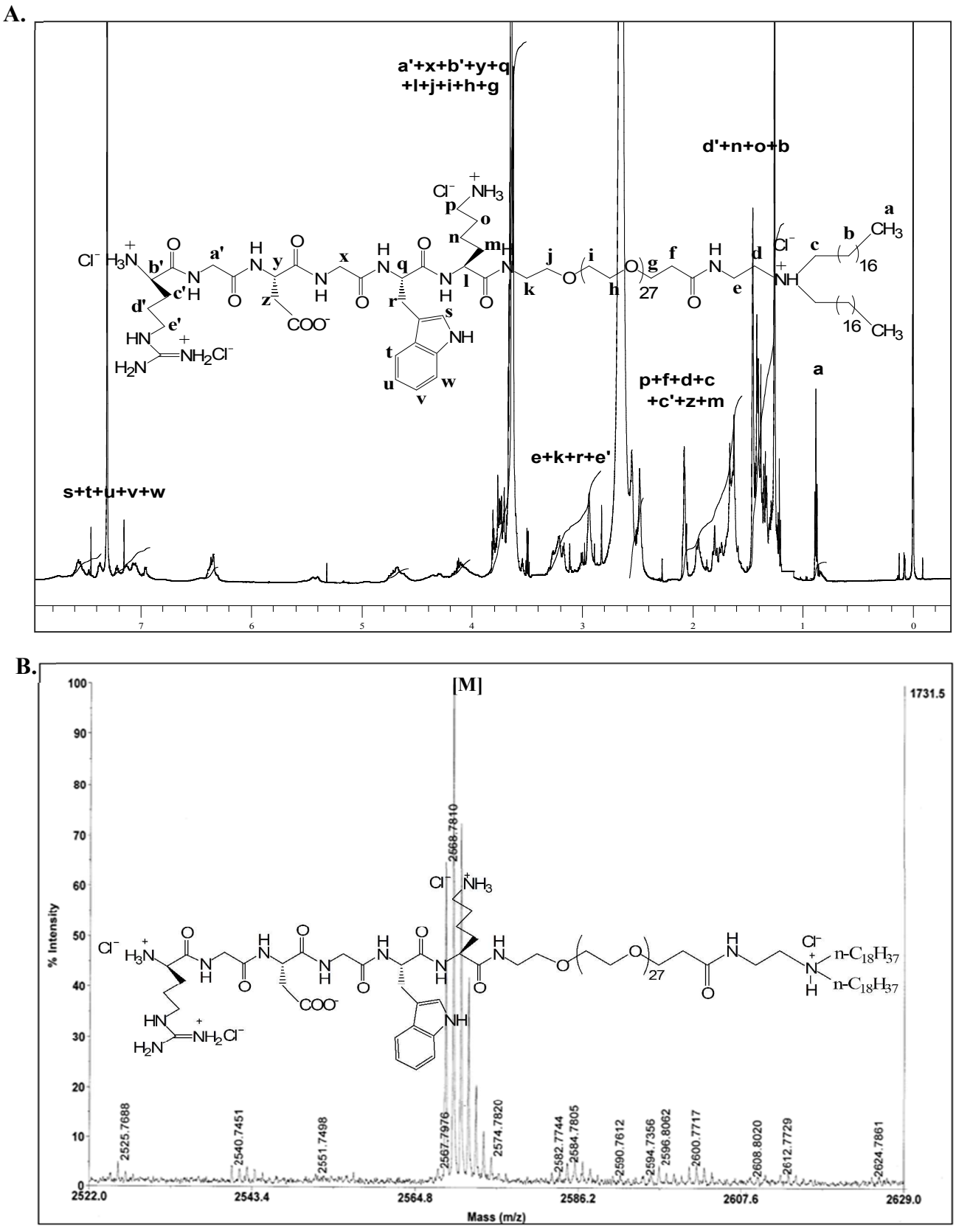


Figure S1. ^{1}H NMR (**A**) and MALDI-mass (**B**) of pegylated RGDGWK-lipopeptide **1**.

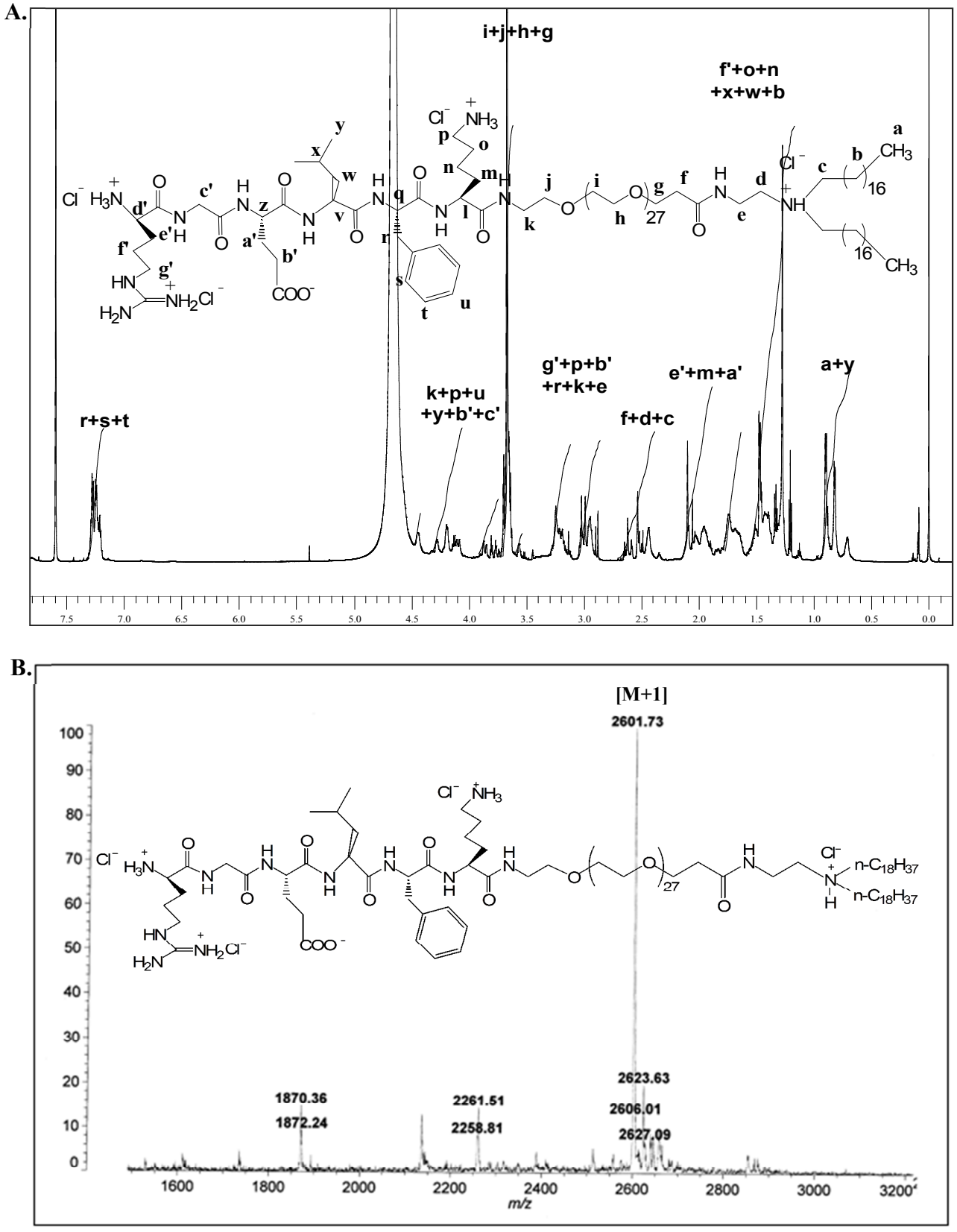
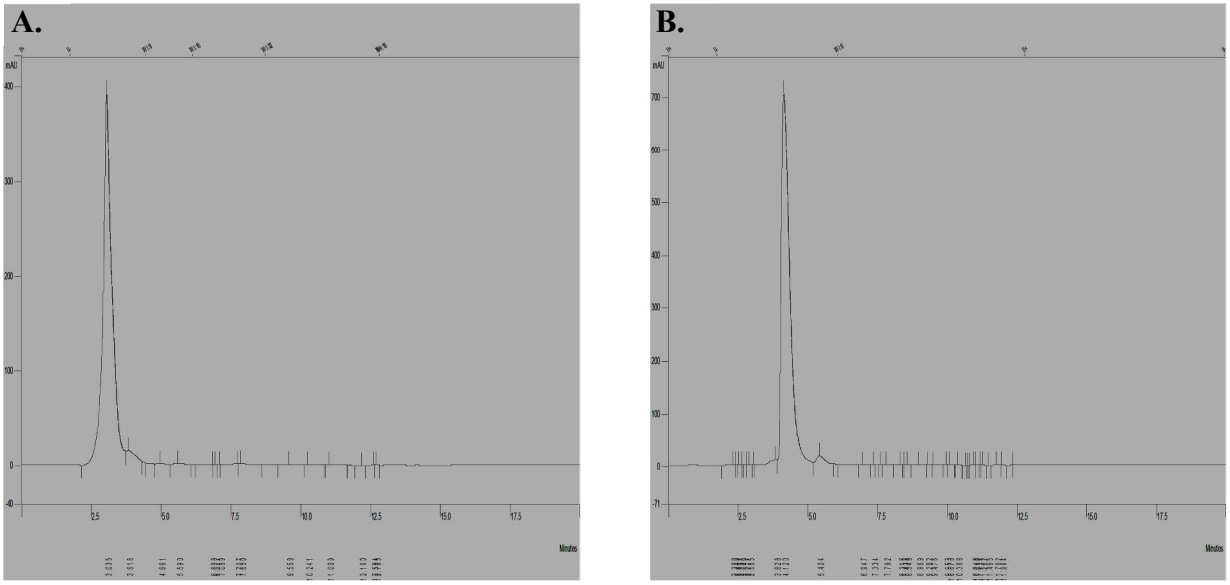


Figure S2. ^{1}H NMR (**A**) and MALDI-mass (**B**) of pegylated RGELFK-lipopeptide **2**.

HPLC Chromatogram of RGDGWK-lipopeptide **1**.



HPLC Chromatogram of RGELFK-lipopeptide **2**.

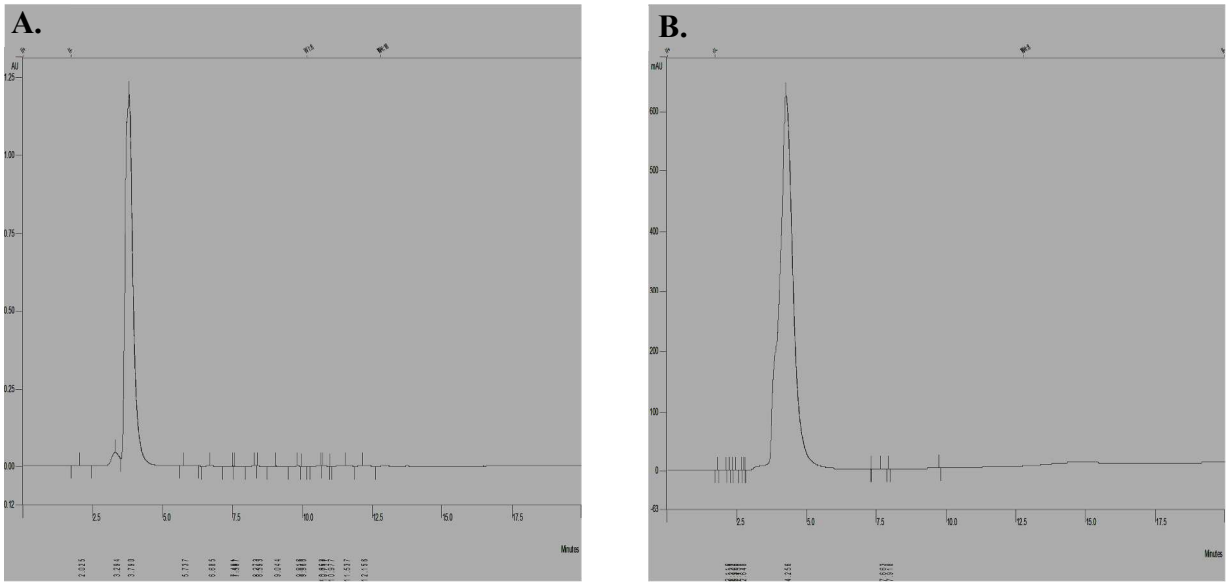


Figure S3. HPLC Chromatograms of pegylated RGDGWK-lipopeptide **1** & pegylated RGELFK-lipopeptide **2.** Mobile Phase: Methanol (**A**); Methanol:Water, 95:5, v/v, (**B**).

HPLC Conditions: System: Varian 1100 series, Column: Lichrospher® 100, RP-18e (5 µm), Flow Rate: 1.0 mL/min (0-20 min), Typical Column Pressure: 60-65 Bars, Detection: UV at 210

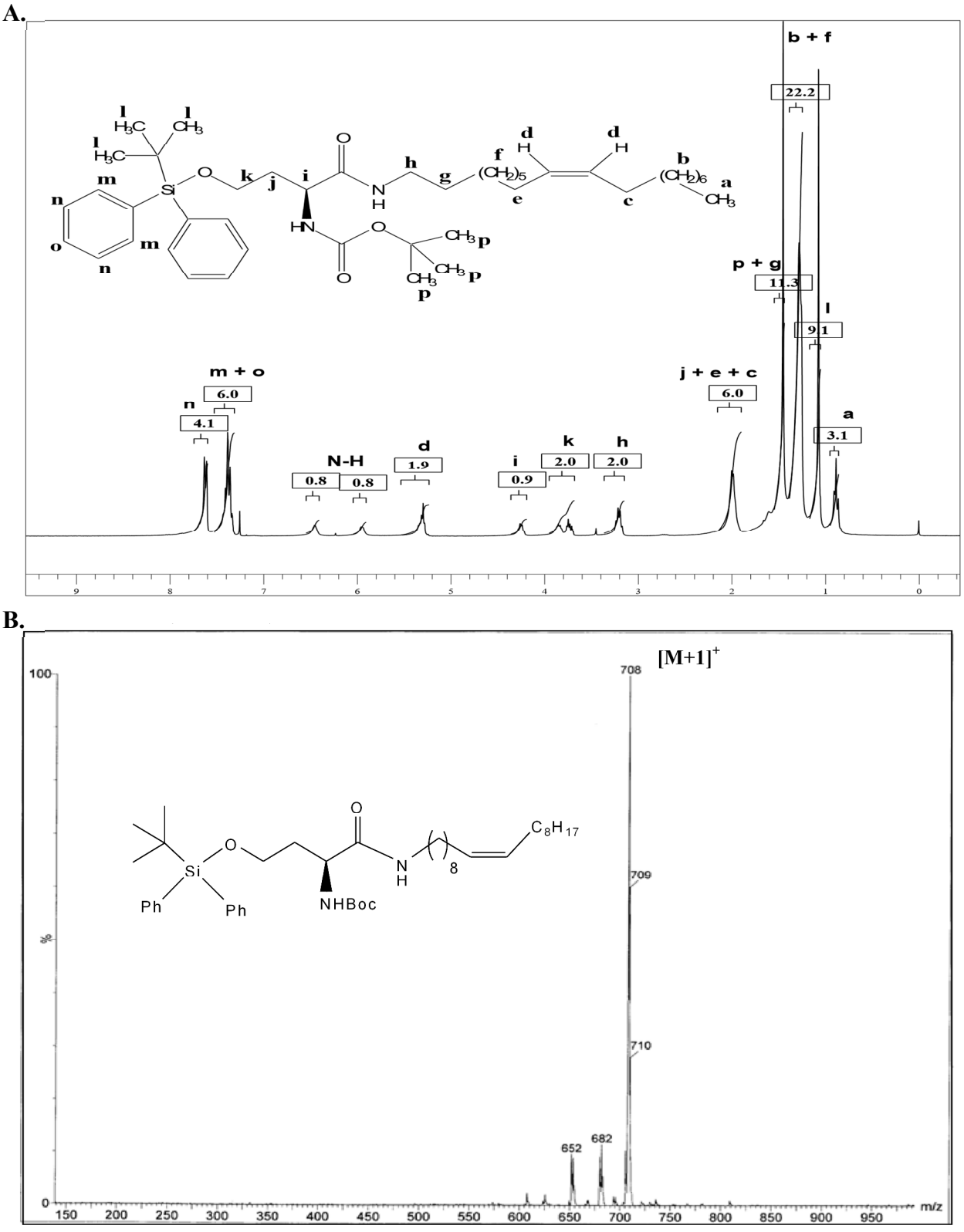


Figure S4. ^{1}H NMR (**A**) and ESI-MS (**B**) of IIIb (Scheme 3).

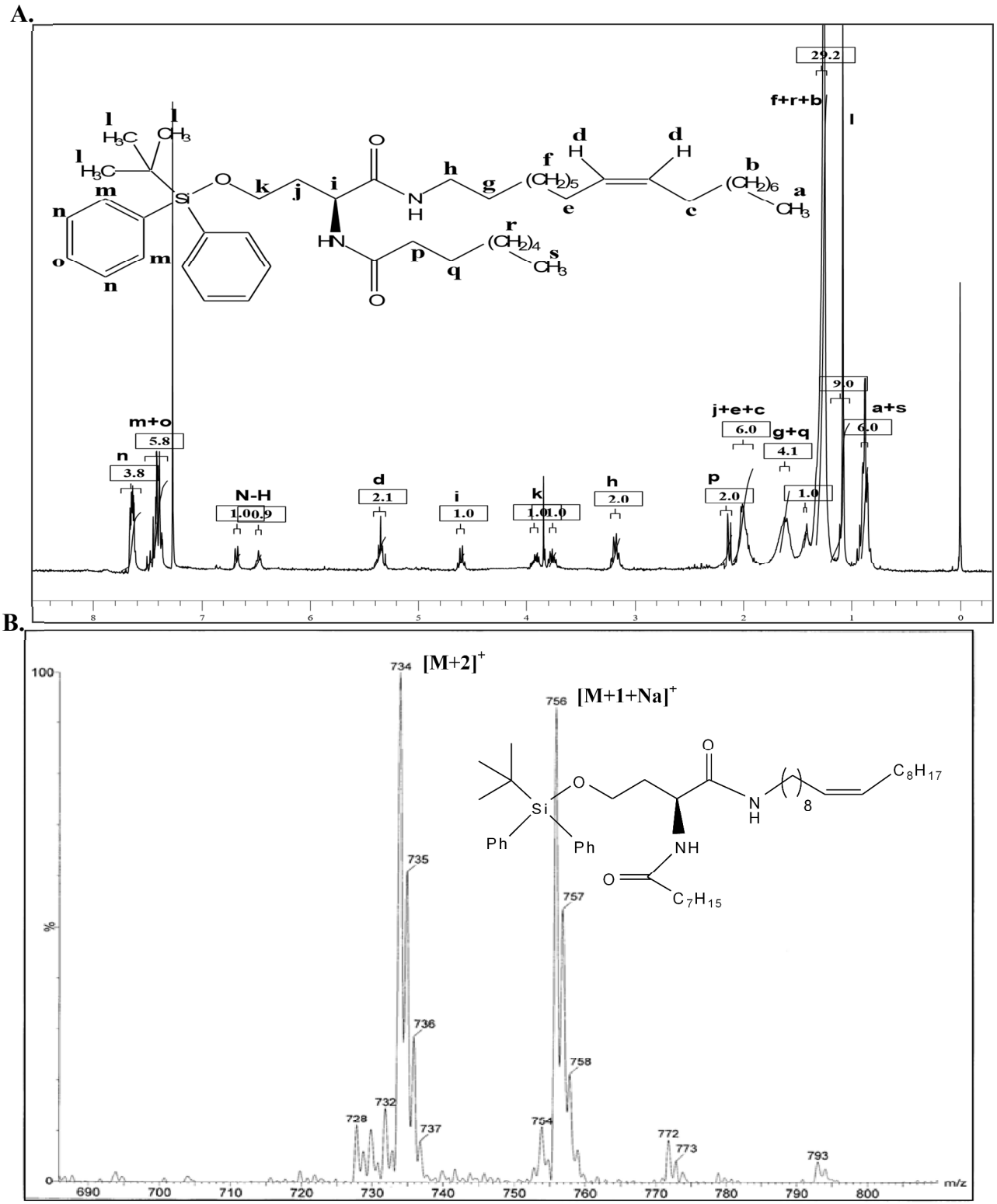


Figure S5. ^{1}H NMR (**A**) and ESI-MS (**B**) of IVb (Scheme 3).

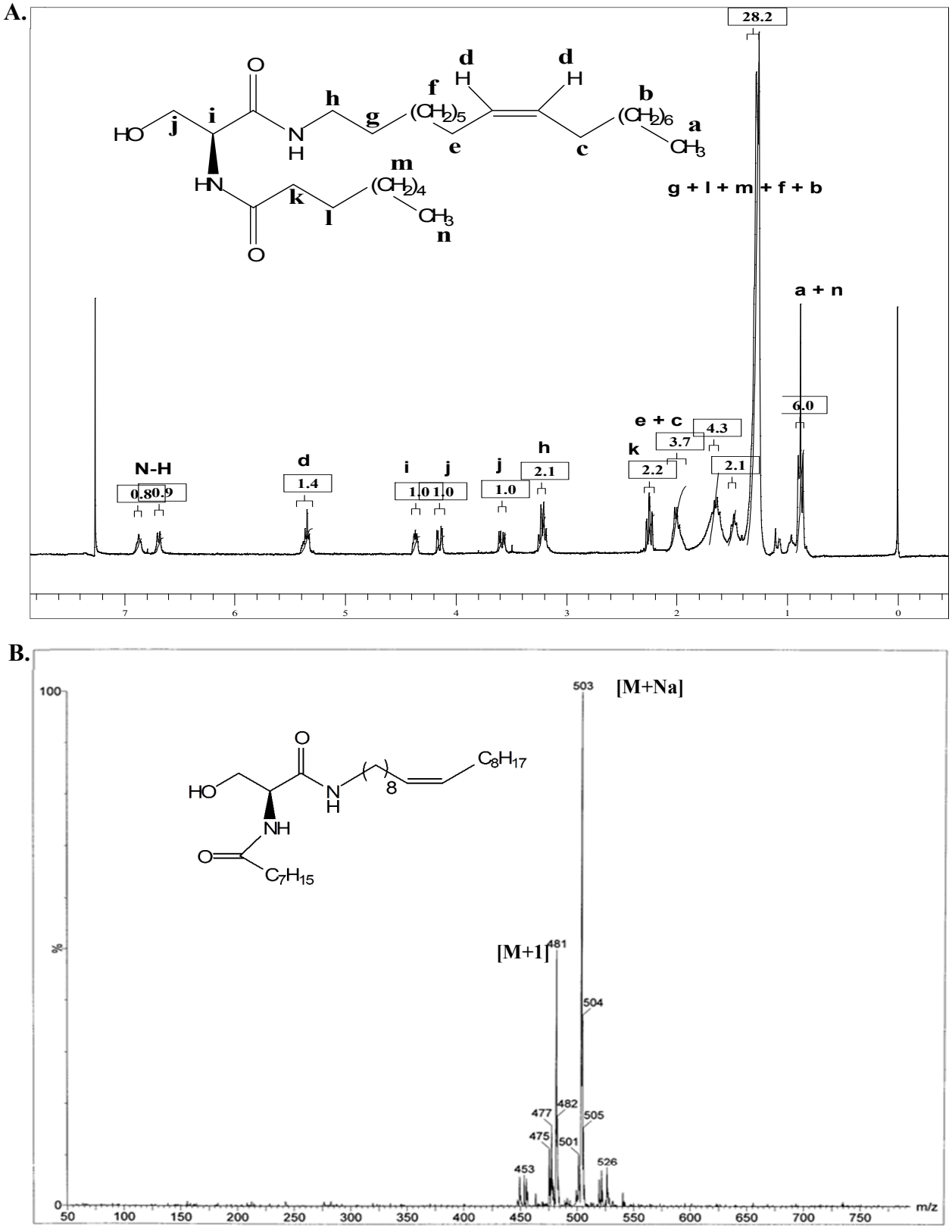


Figure S6. ^{1}H NMR (**A**) and ESI-MS (**B**) of C18-oleyl.

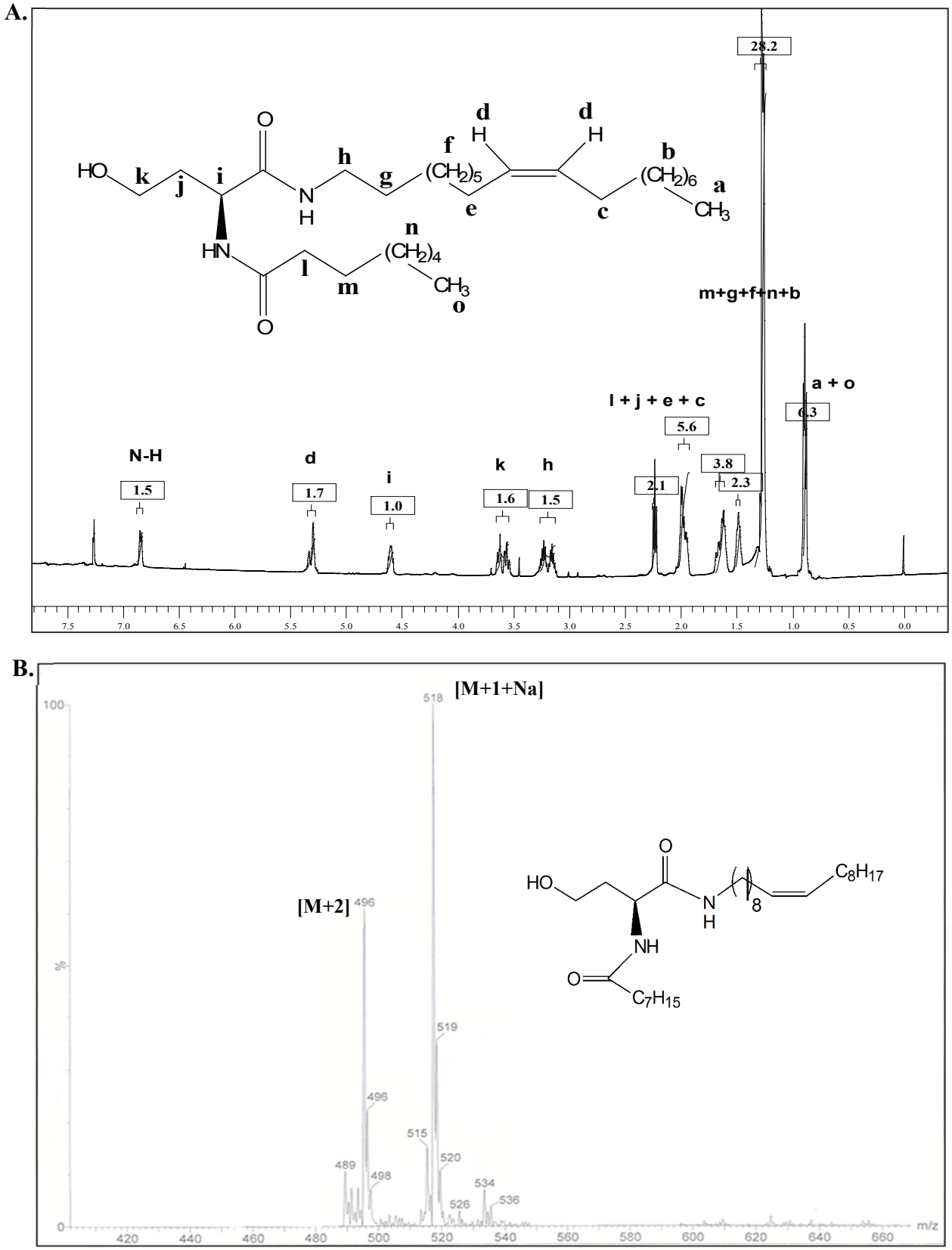


Figure S7. ^{1}H NMR (**A**) and ESIMS (**B**) of HC18-oleyl.

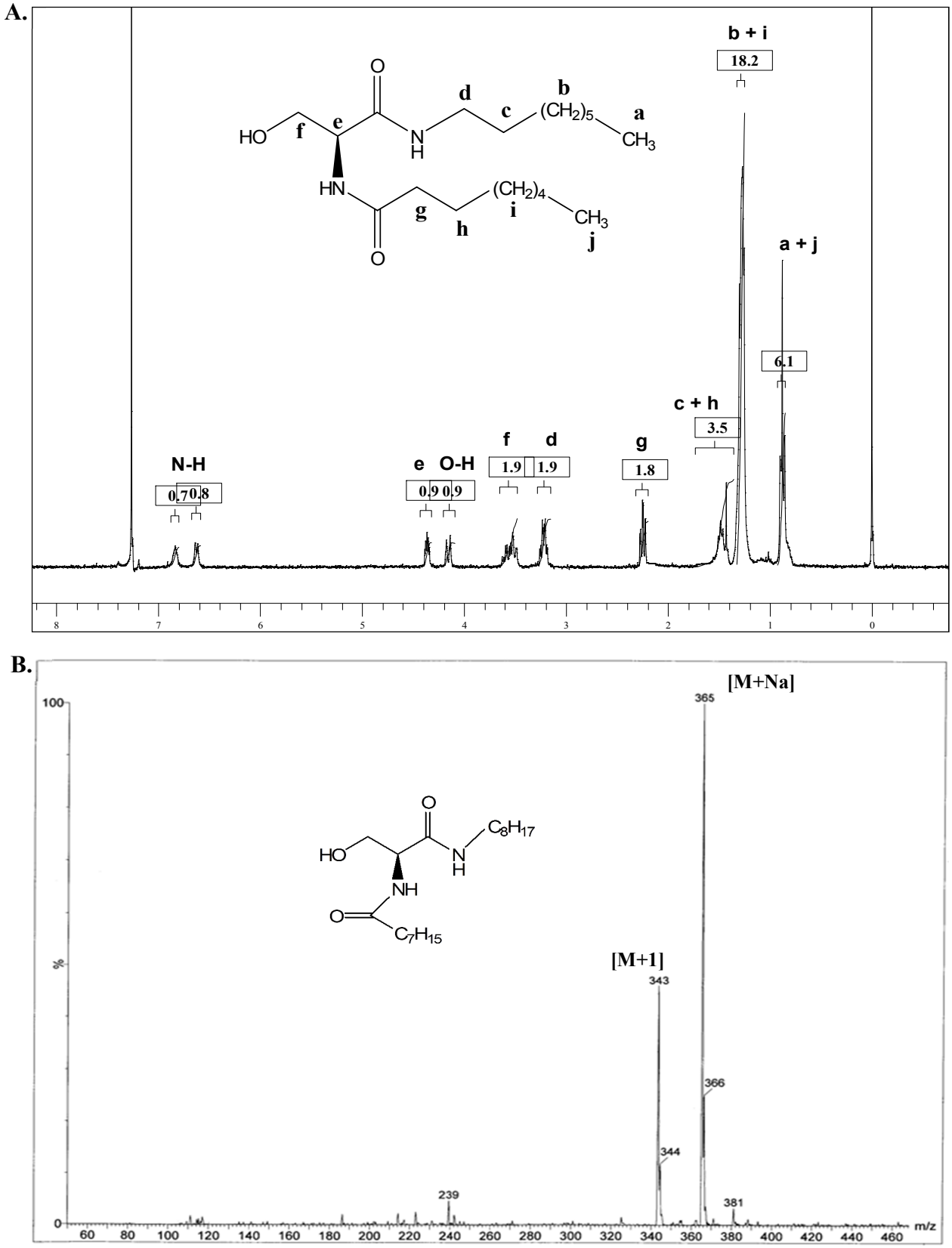
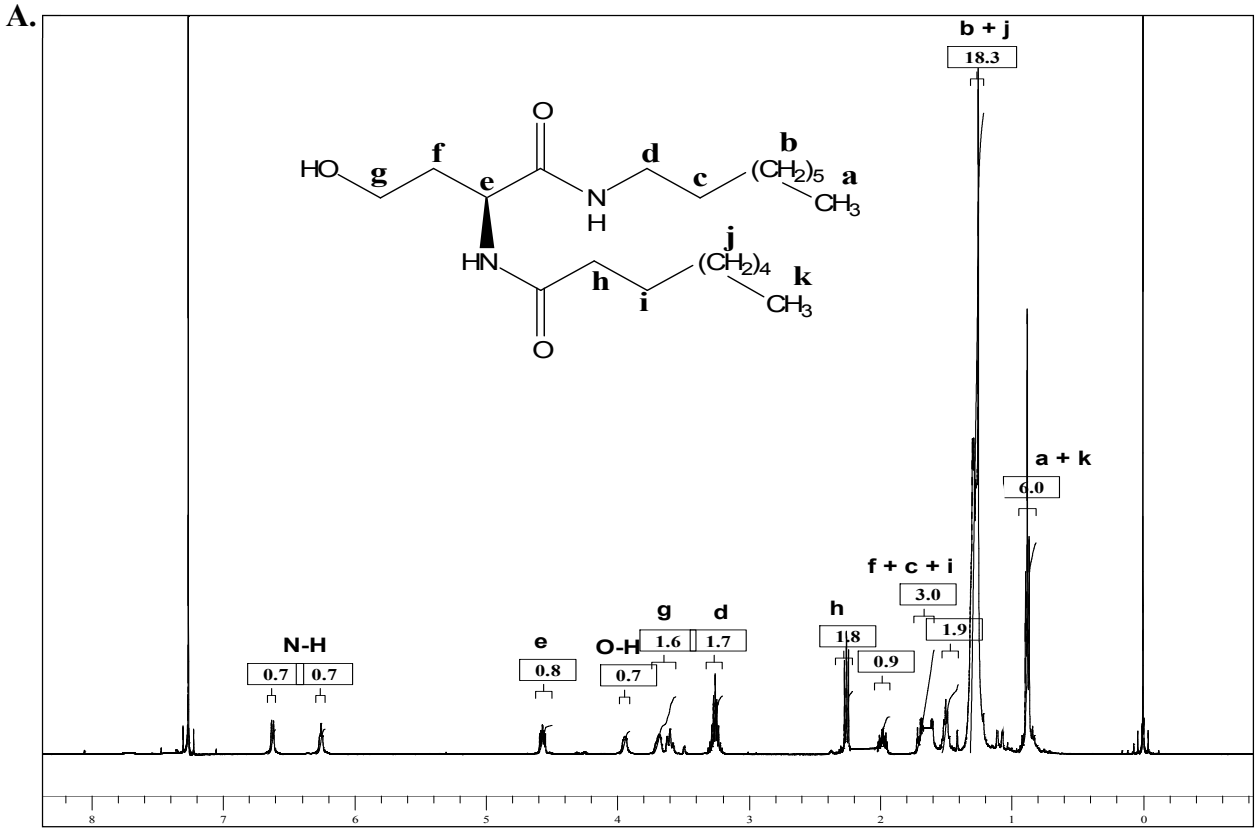


Figure S8. ^{1}H NMR (**A**) and ESIMS (**B**) of C8.



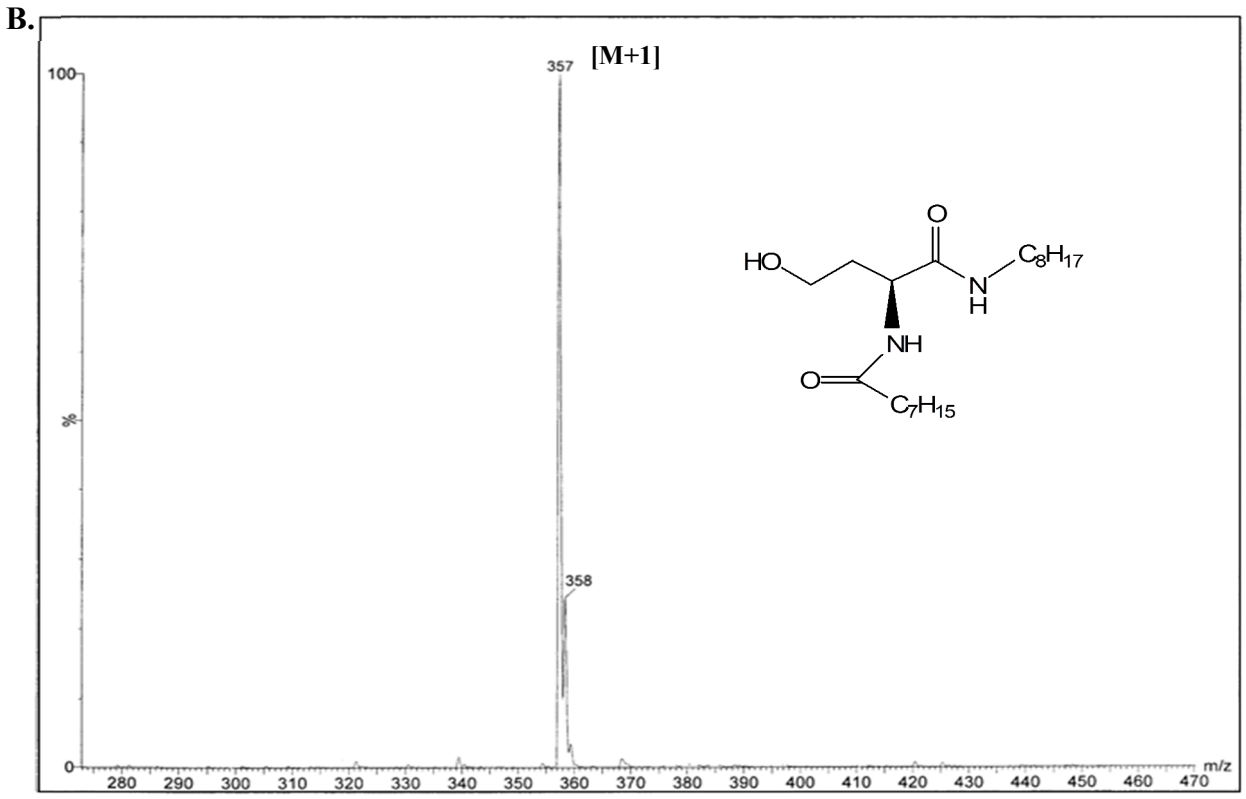


Figure S9. ^{1}H NMR (**A**) and ESIMS (**B**) of HC8.

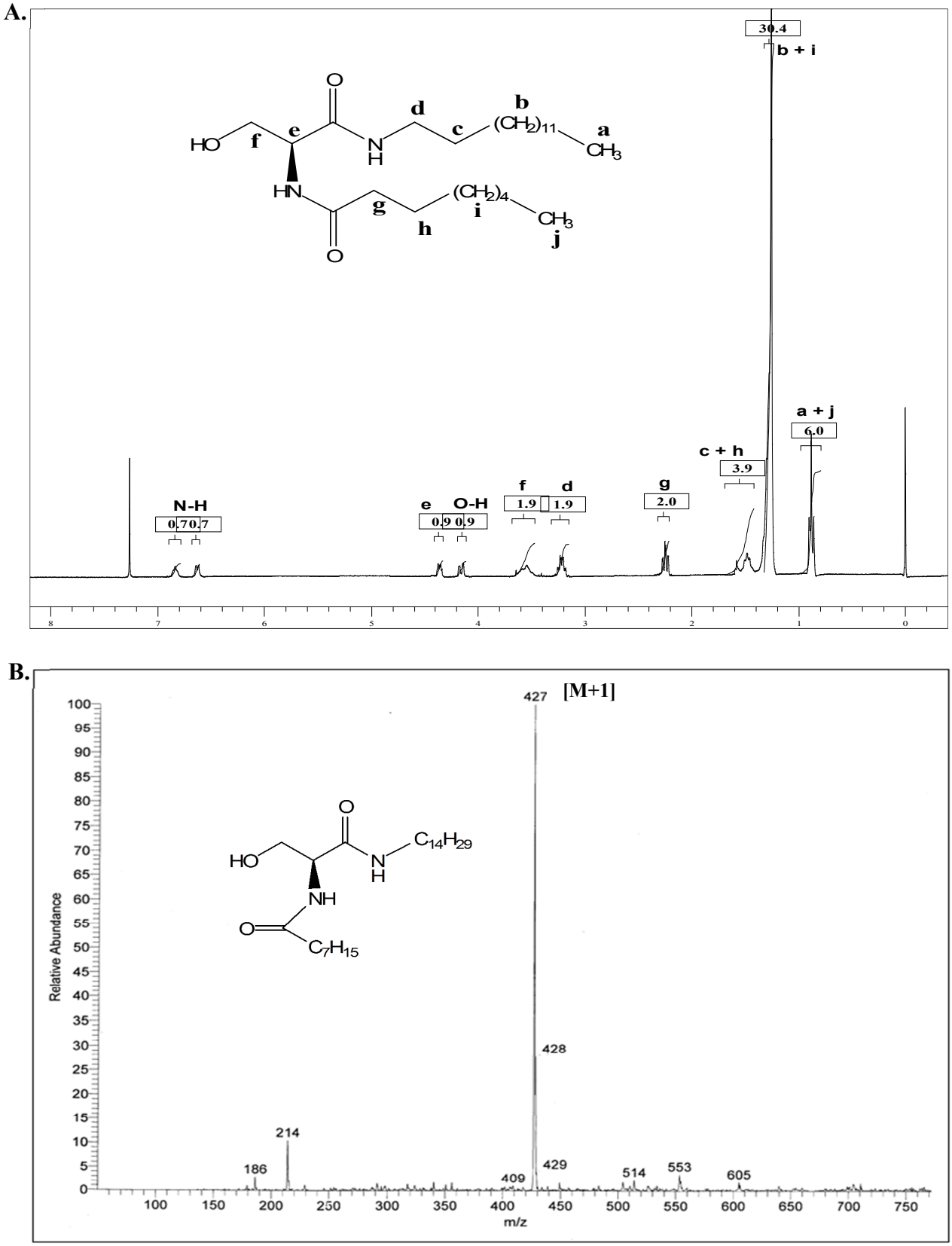


Figure S10. ^{1}H NMR (**A**) and ESIMS (**B**) of C14.

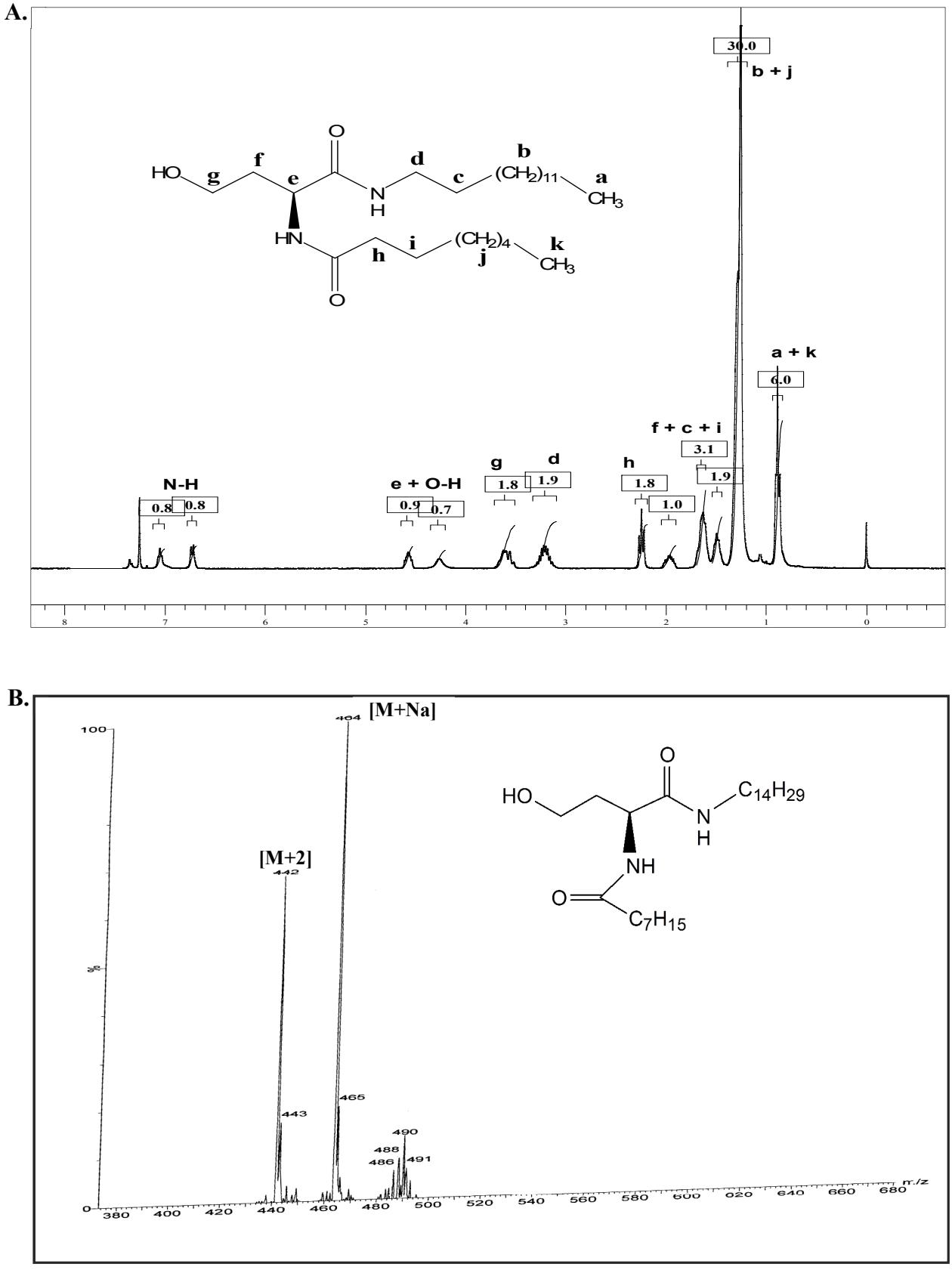


Figure S11. ^{1}H NMR (**A**) and ESIMS (**B**) of HC14.

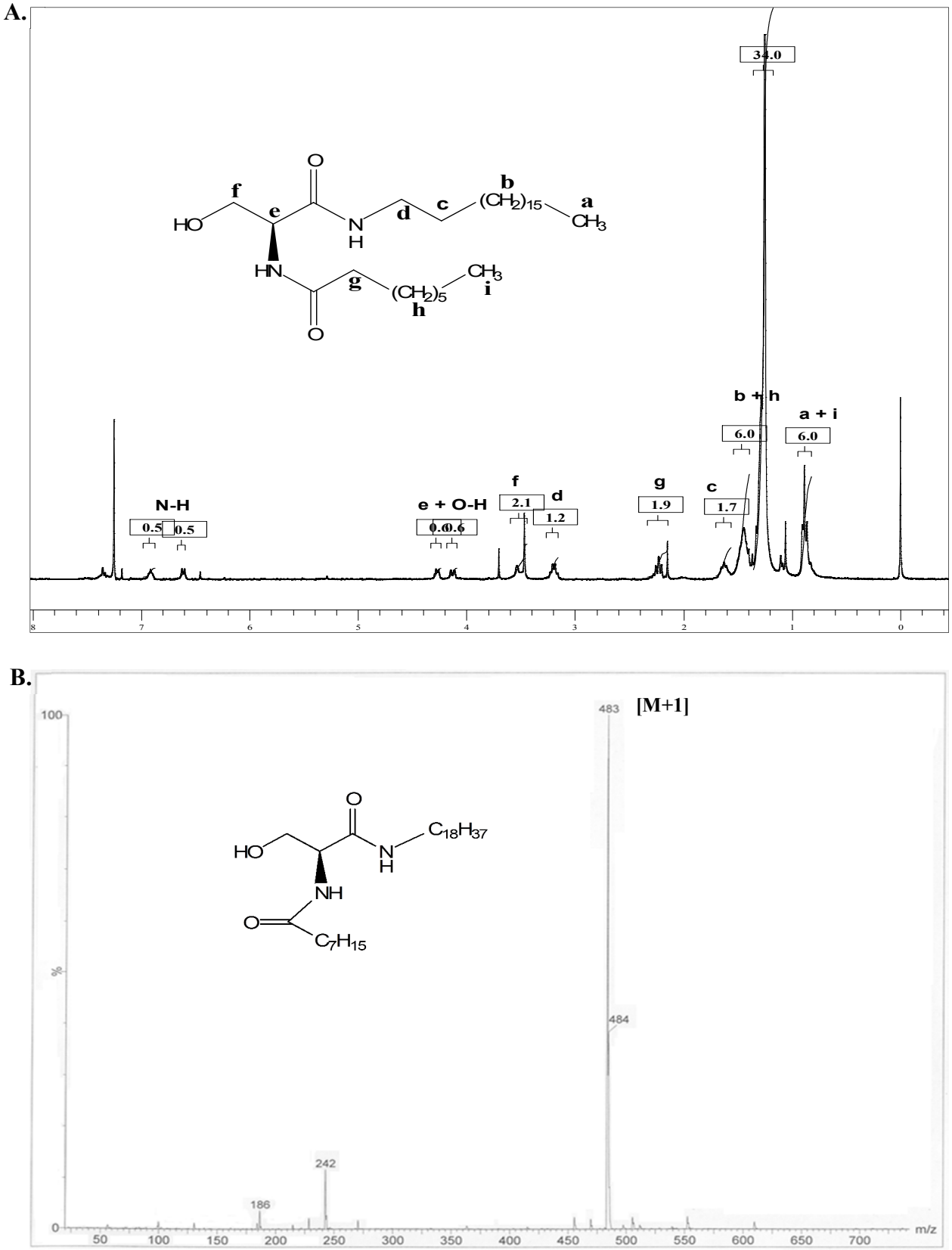


Figure S12. ^{1}H NMR (**A**) and ESIMS (**B**) of C18.

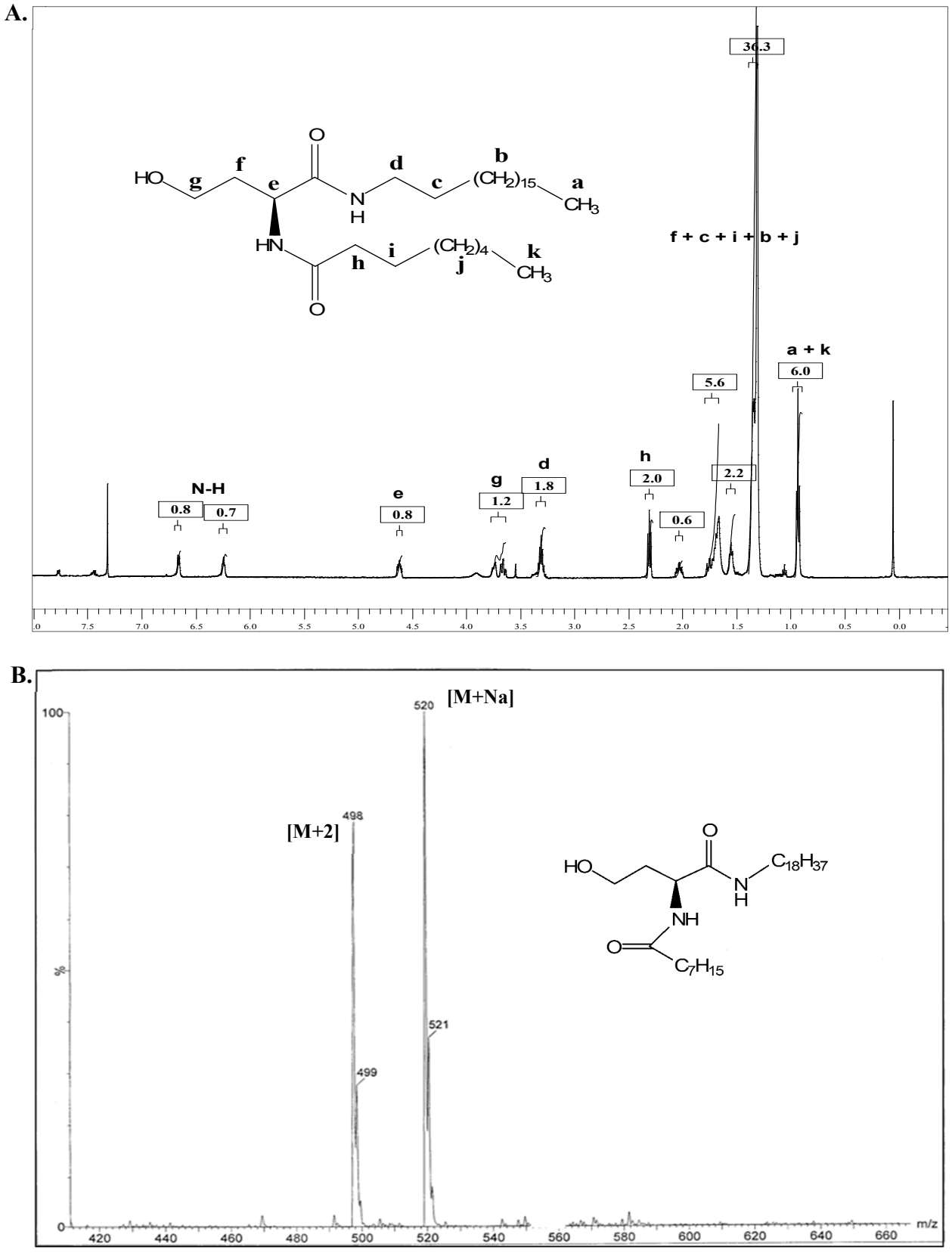
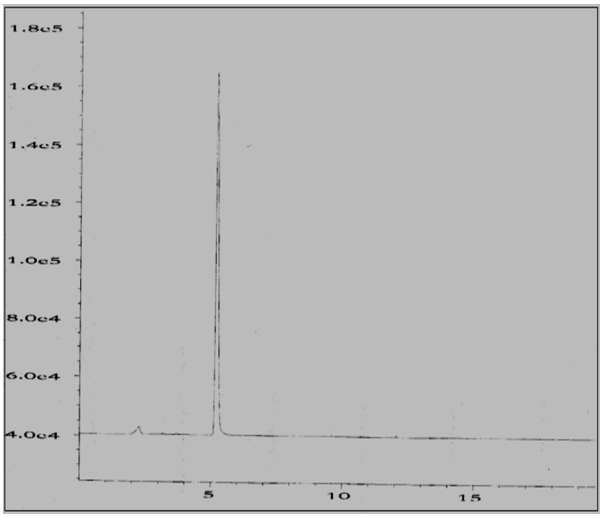


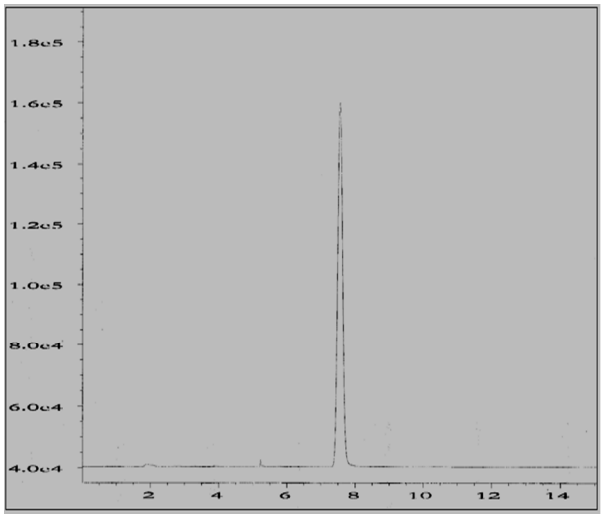
Figure S13. ^{1}H NMR (**A**) and ESIMS (**B**) of HC18.

HPLC Chromatogram of **C18-oleyl**

A.

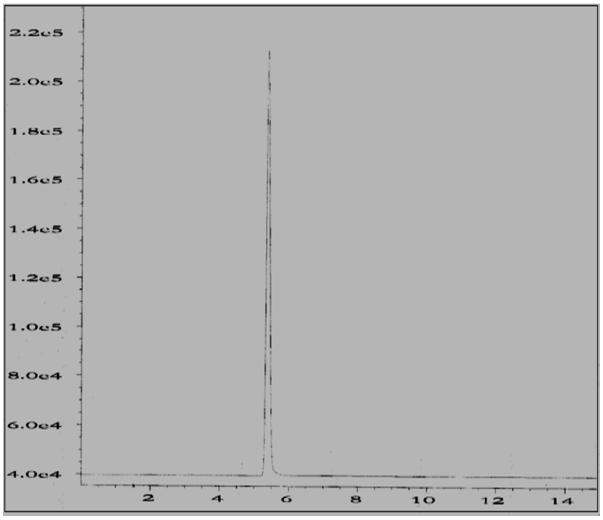


B.



HPLC Chromatogram of **HC18-oleyl**

A.



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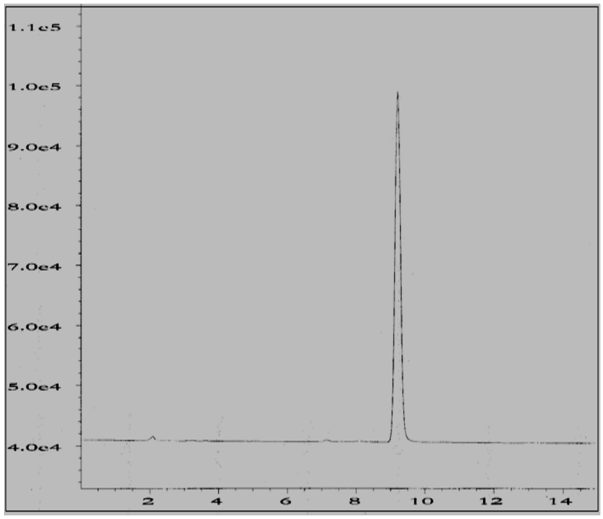
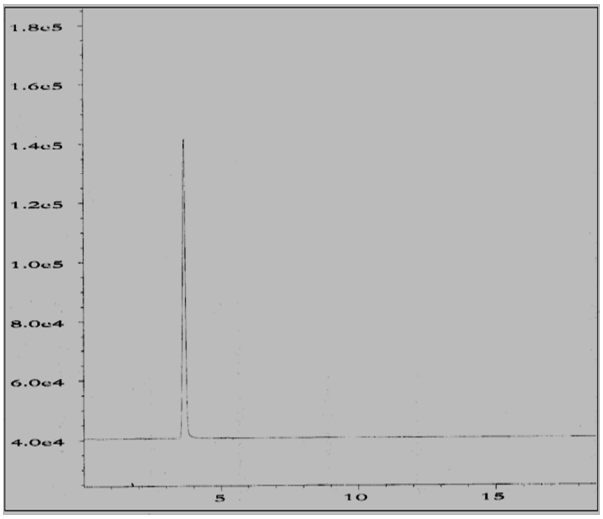


HPLC Chromatogram of **C8**

A.



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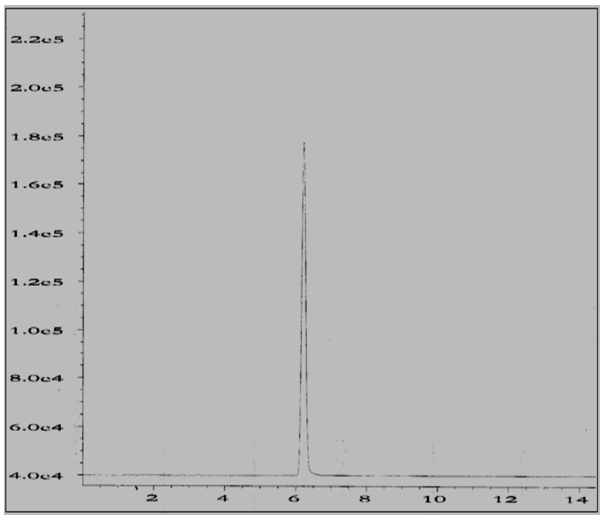
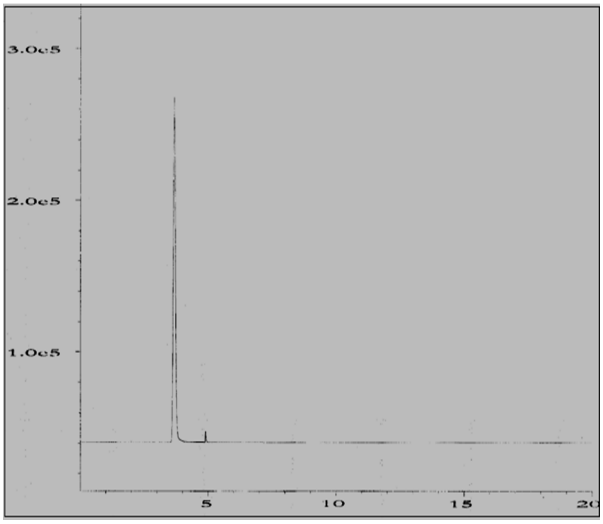


HPLC Chromatogram of **HC8**

A.



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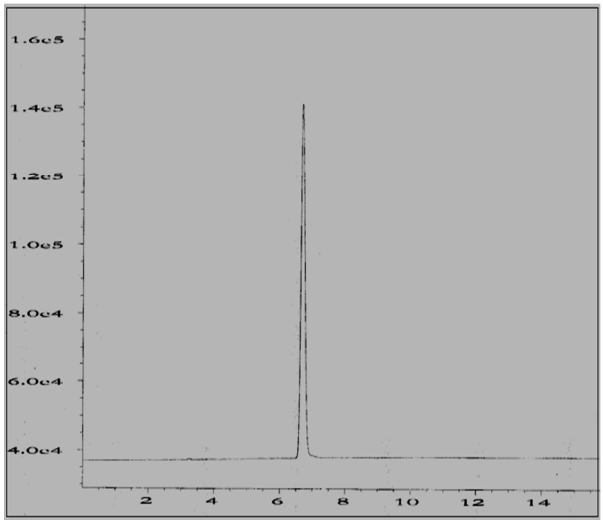
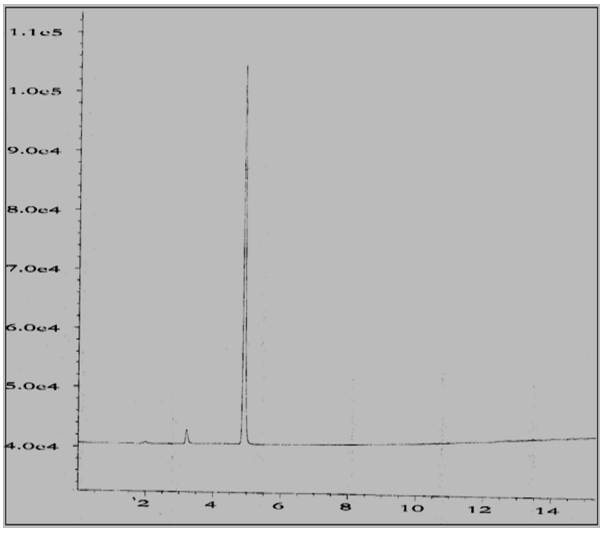


HPLC Chromatogram of **C14**

A.



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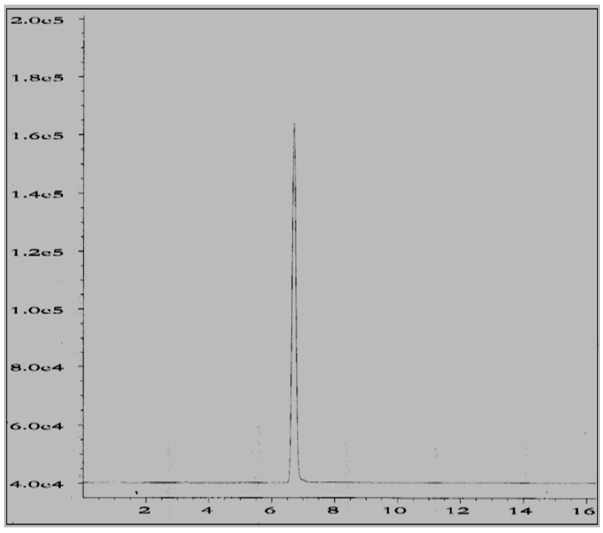
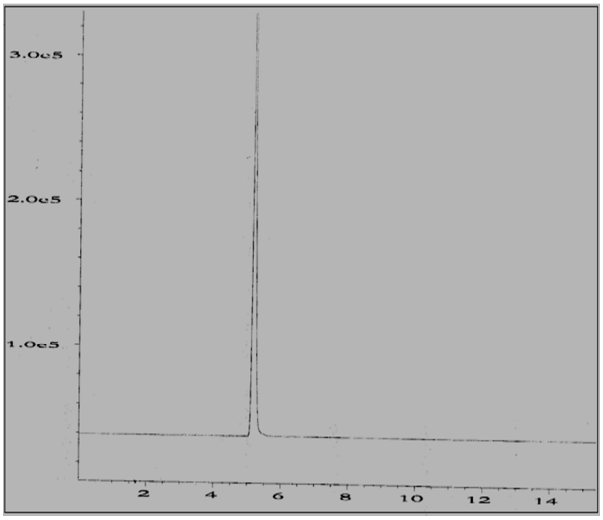


HPLC Chromatogram of **HC14**

A.



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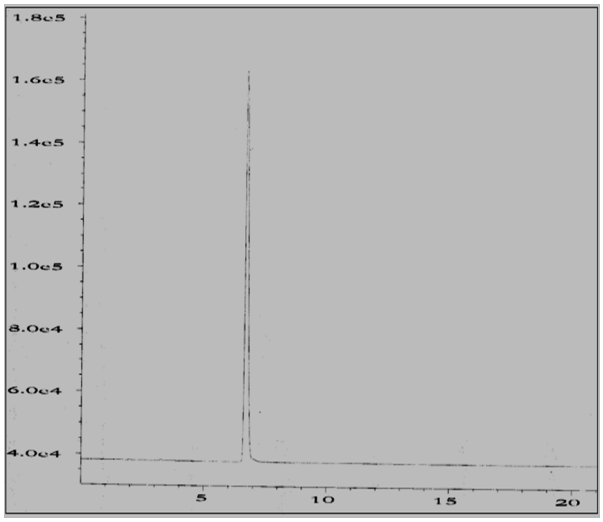
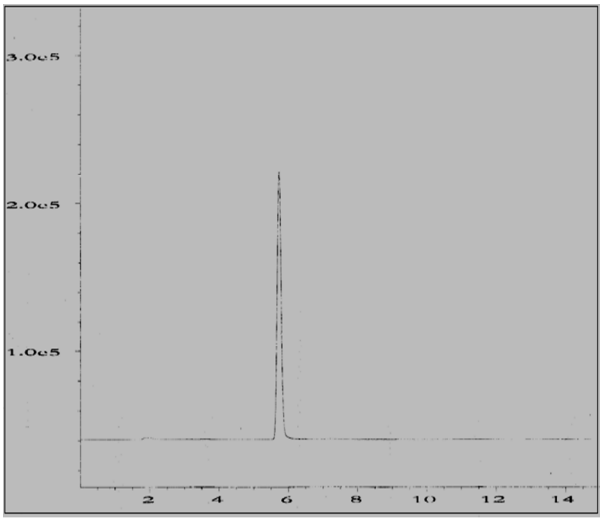


HPLC Chromatogram of **C18**

A.



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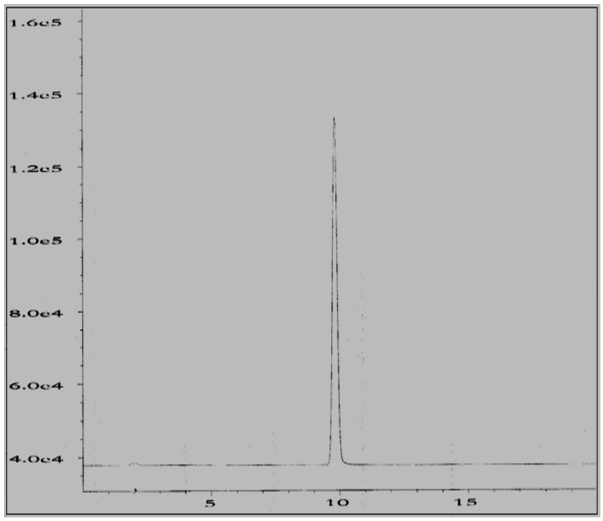
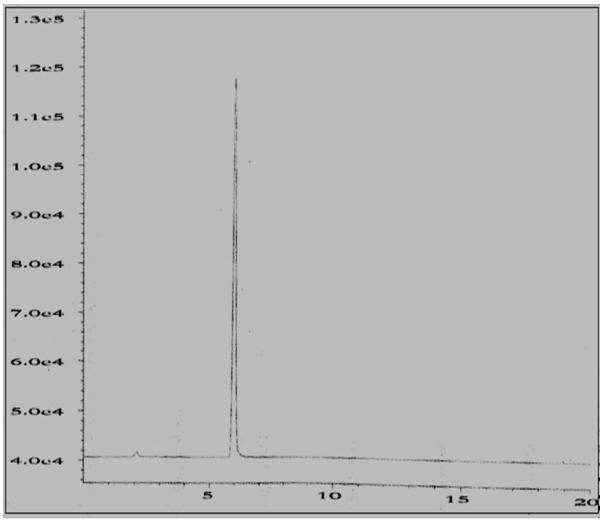


HPLC Chromatogram of **HC18**

A.



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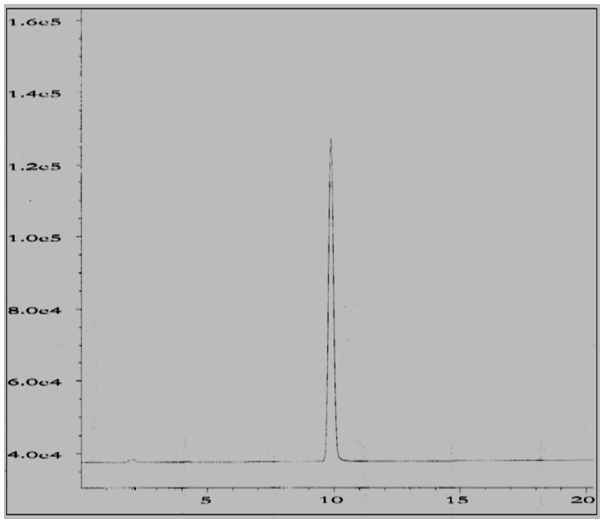
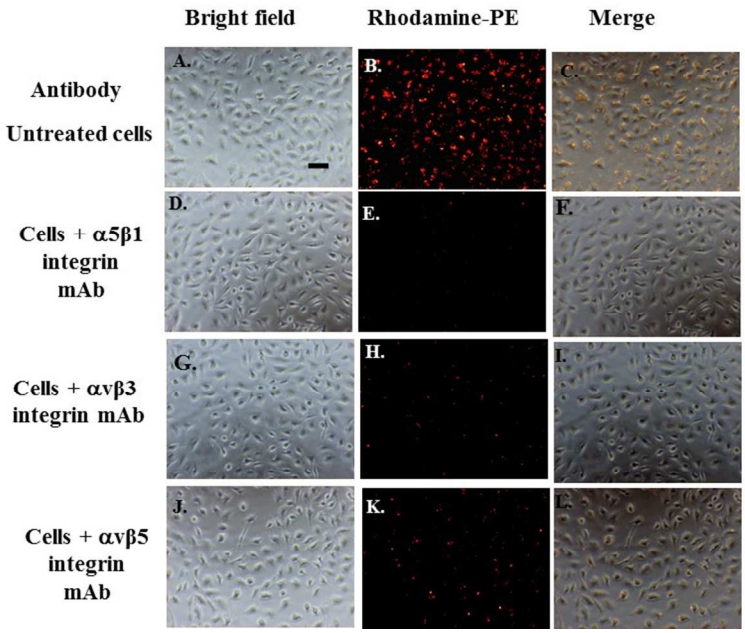


Figure S14. HPLC Chromatograms of synthesized ceramide analogs. Mobile Phase: Methanol (**A**); Methanol:Water, 95:5, v/v, (**B**). HPLC Conditions: System: Varian 1100 series, Column: Lichrospher® 100, RP-18e (5 µm), Flow Rate: 1.0 mL/min (0-20 min), Typical Column Pressure: 60-65 Bars, Detection: UV at 210 nm.

Table S1. Sizes and Zeta potentials (ξ) of Liposomes containing drugs

Drug entrapped in Liposome containing pegylated RGDGWK-lipopeptide 1	**Hydrodynamic diameter(nm)**	**Zeta Potentials(mV)**
Curcumin	190±5	3.8±1.7
HC18-oleyl	196±4	3.9±2.1
Curcumin & HC18-oleyl	208±3	4.2±1.6
C18-oleyl	194±6	3.7±2.3
Curcumin & C18-oleyl	212±5	4.3±1.7
C8	189±3	3.2±1.2
Curcumin & C8	199±5	3.8±1.5
HC8	191±4	3.5±1.4
Curcumin & HC8	201±6	4.2±2.1
C14	196±5	3.7±1.7
Curcumin & C14	215±4	3.9±1.3
HC14	198±5	3.5±1.4
Curcumin & HC14	219±5	4.1±1.7
C18	197±6	3.3±1.1
Curcumin &C18	211±6	3.9±1.7
HC18	195±3	3.5±1.2
Curcumin & HC18	209±5	4.1±1.9
C8-cer	206±5	3.4±3.1
curcumin & C8-cer	218±2	3.9±1.8

I.



II.

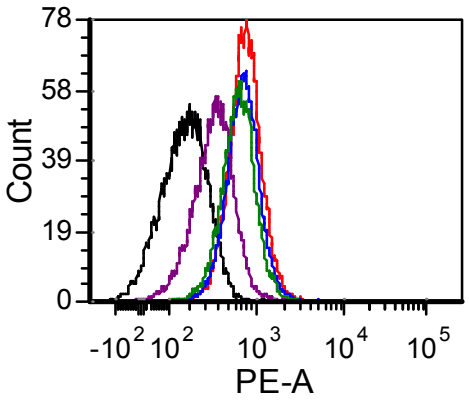


Figure S15. Liposomes of pegylated RGDGWK-lipopeptide **1** enter endothelial cells mainly via α5β1 integrin receptor. B16F10 cells were pre-saturated with monoclonal antibodies against α5β1, αvβ3 or αvβ5 integrins and then treated with Rh-PE (red) labeled liposomes of pegylated RGDGWK-lipopeptide **1** for 3 h. **I.** Epifluorescence microscopic images (Scale bar equals to 50 μm) were taken for cells without antibody treatment (**A**-**C**) as well as for cells pre-treated with mAbs against α5β1 integrins (**D**-**F**), αvβ3 integrins (**G**-**I**) and αvβ5 integrins (**J**-**L**). **II.** Flow

cytometric uptake analysis of Rh-PE labelled liposome in absence of any antibody (red) and in presence of monoclonal antibodies against α5β1 (violet), αvβ3 (green) or αvβ5 (blue) integrins compared to untreated cells (black). Data shown here are representative of two separate experiments.

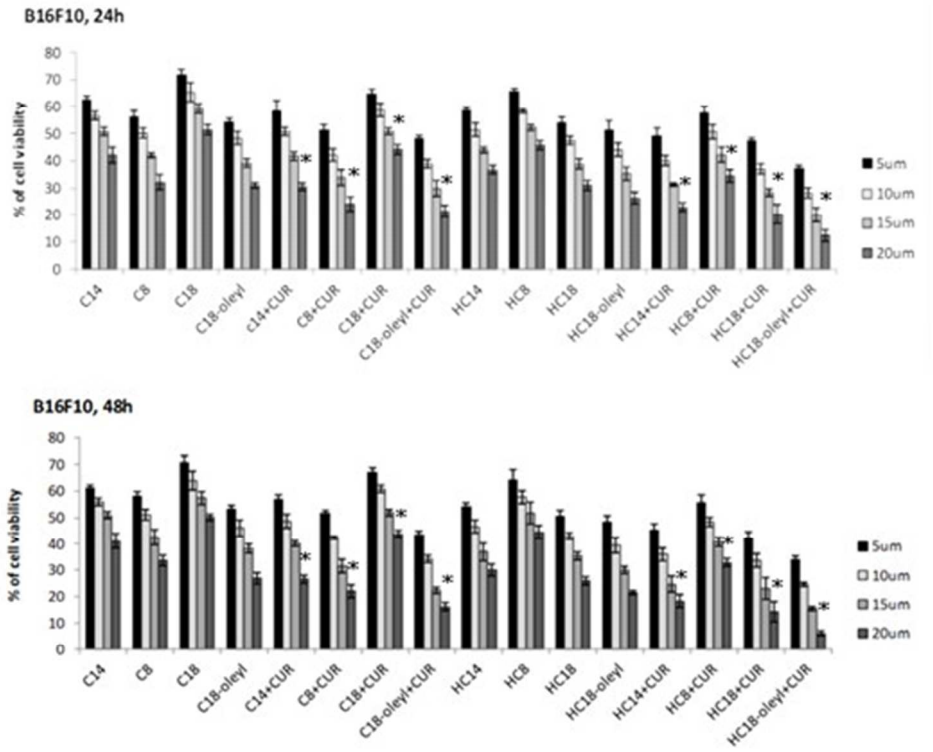
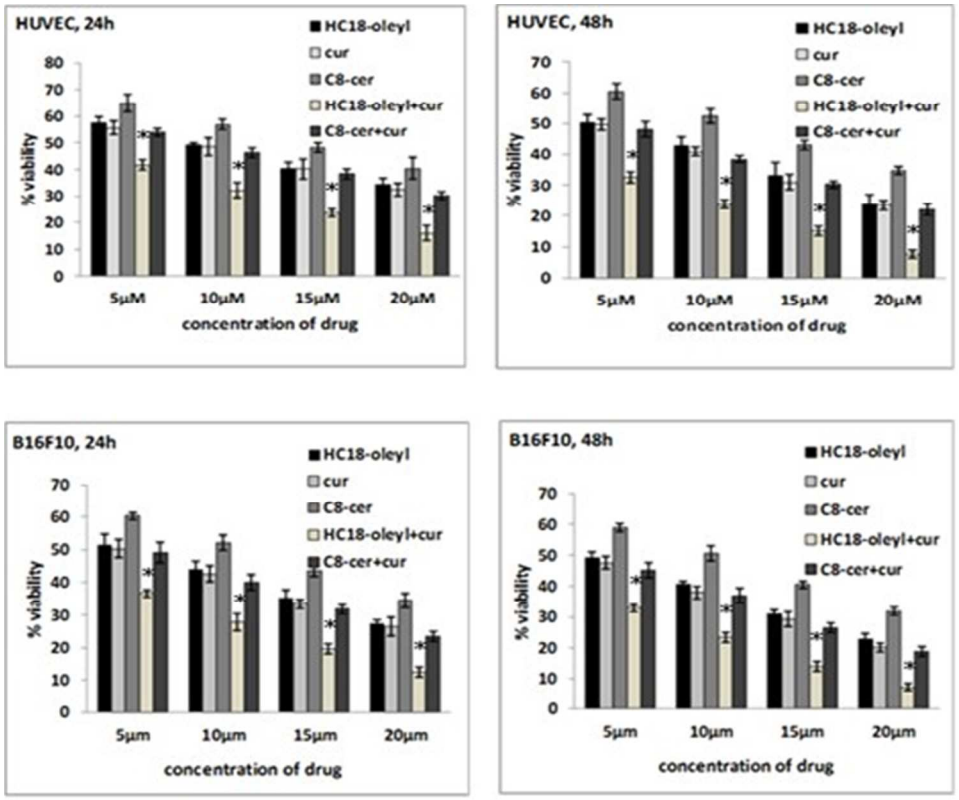


Figure S16. HC18-oleyl formulated in liposomes of pegylated RGDGWK-lipopeptide **1** is most potent cytotoxic agent among all the synthesized C8-ceramide analogs. B16F10 cells were treated with liposomal formulation of RGDGWK-lipopeptide **1** containing: ceramide analogs (5 μM, 10 μM, 15 μM, 20 μM) and both curcumin (2.5 μM, 5 μM, 7.5 μM, 10 μM) & ceramide analogs (2.5 μM, 5 μM, 7.5 μM, 10 μM). Relative cellular cytotoxicities of synthesized ceramide analogs either alone or in combination with curcumin were measured by MTT assay after 24 h and 48 h of treatment (*$P < 0.005$ vs. individual ceramides at corresponding concentration).

A.



B.

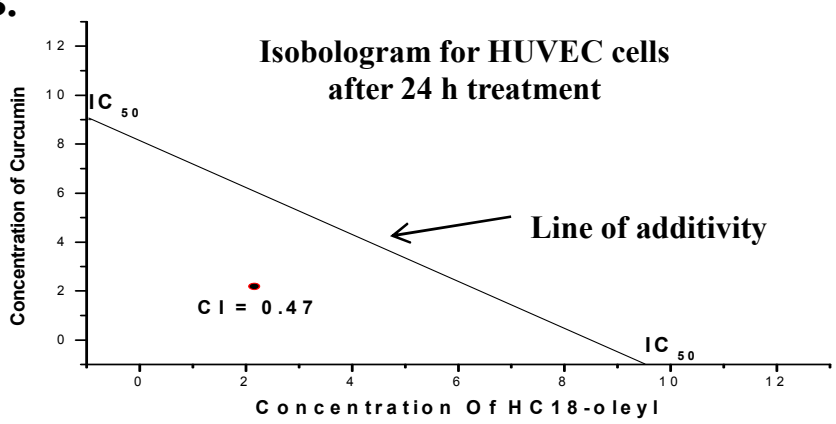


Figure S17. Curcumin and HC18-oleyl co-encapsulated in liposomes of pegylated RGDGWK-lipopeptide **1** show highest cytotoxicity in endothelial (HUVEC) and tumor (B16F10) cells in a dose dependent manner and work synergistically. **A.** HUVEC and B16F10 cells were treated with liposomally formulated: curcumin (5 μM, 10 μM, 15 μM, 20 μM), HC18-oleyl (5 μM, 10

μM, 15 μM, 20 μM), commercially available C8-cer (5 μM, 10 μM, 15 μM, 20 μM), both HC18-oleyl (2.5 μM, 5 μM, 7.5 μM, 10 μM) & curcumin (2.5 μM, 5 μM, 7.5 μM, 10 μM) and both C8-cer (2.5 μM, 5 μM, 7.5 μM, 10 μM) & curcumin (2.5 μM, 5 μM, 7.5 μM, 10 μM). MTT assays were performed after 24 h and 48h of treatment (*P < 0.005 vs. C8-cer at corresponding concentration). **B.** In isobologram for HUVEC cells after 24 h of treatment, combination index value (0.47) falls well below the line of additivity thereby showing synergistic actions of curcumin and HC18-oleyl encapsulated within the liposomes of pegylated RGDGWK-lipopeptide **1**.

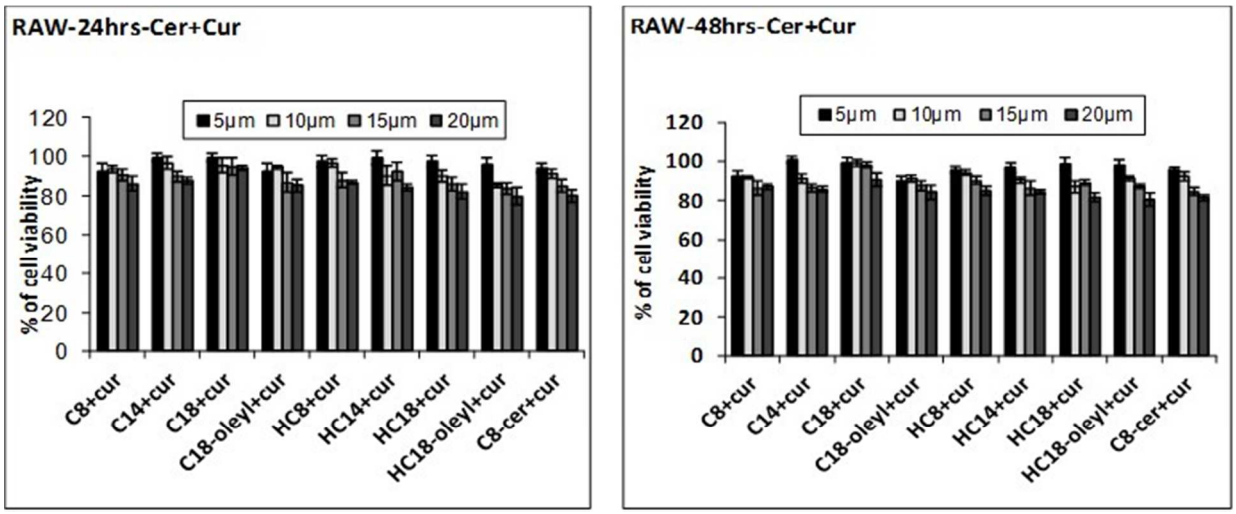


Figure S18. Curcumin and synthesized C8-ceramide analogs co-encapsulated in liposomes of pegylated RGDGWK-lipopeptide **1** show minimal cytotoxicity in non-cancerous (Raw) cell. Cells were treated with liposomes containing both curcumin (2.5 μM, 5 μM, 7.5 μM, 10 μM) & ceramide analogs (2.5 μM, 5 μM, 7.5 μM, 10 μM). MTT assays were performed after 24 h and 48h of treatment.

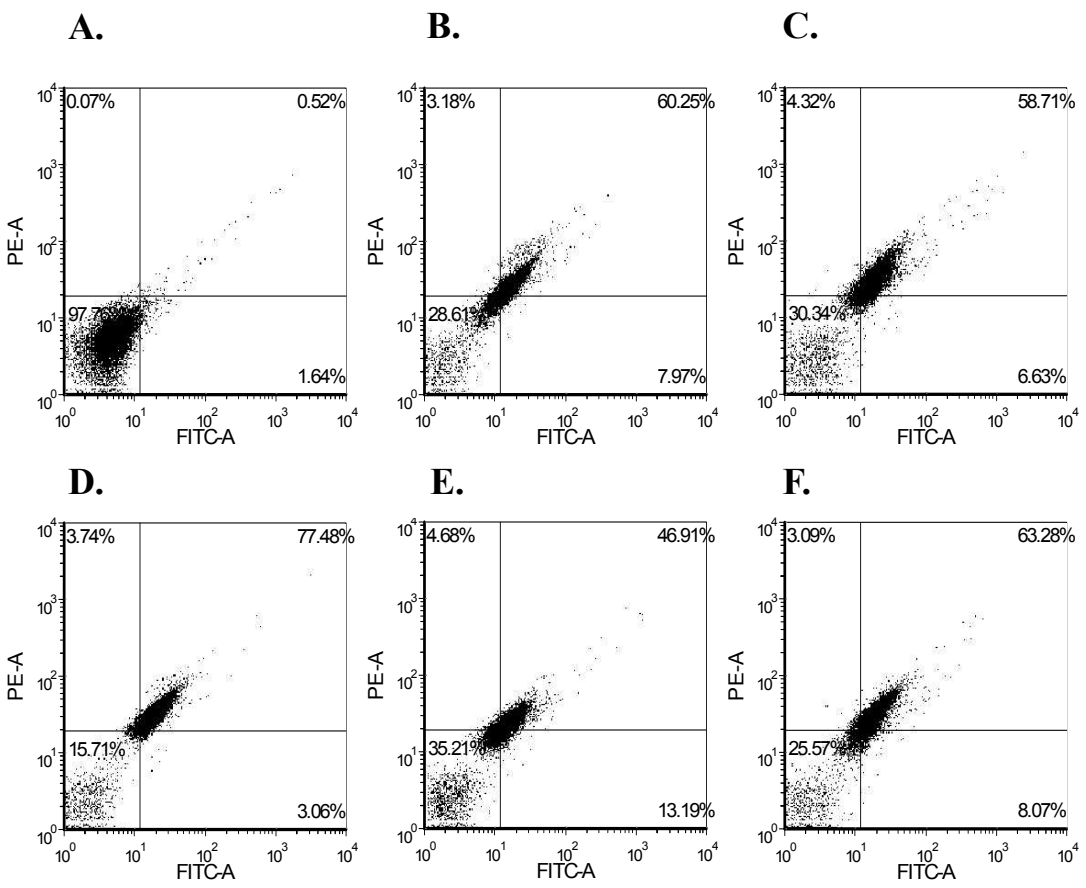


Figure 19. Liposomally bound curcumin & HC18-oleyl is more potent than liposomally bound curcumin & commercially available C8-cer in inducing apoptosis in B16F10 cells. Both untreated (**A**) and treated cells (**B**-**F**) were stained with FITC-Annexin V and propidium iodide (PI) for flow cytometric analysis of apoptosis. B16F10 cells were treated with liposomal formulations of pegylated RGDGWK-lipopeptide **1** containing: curcumin (**B**), HC18-oleyl (**C**), curcumin & HC18-oleyl (**D**), commercially available C8-cer (**E**), and curcumin & C8-cer (**F**) for 24 h. The horizontal and vertical axes represent cells labeled with FITC-Annexin V and PI respectively. Dots in the upper right quadrant represent late apoptotic population (positive for both annexin V and PI). Data shown here are representative of two separate experiments.

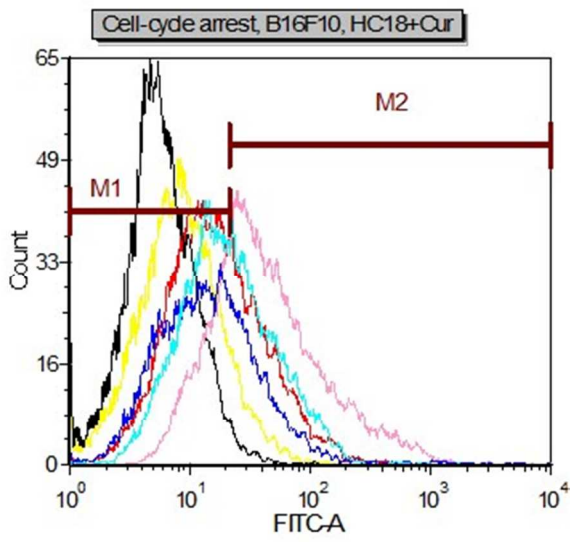


Figure S20. Treatment of B16F10 cells with liposomal formulations of pegylated RGDGWK-lipopeptide **1** containing both HC18-oleyl and curcumin leads to enhanced population of treated cells in G2/M phase. B16F10 cells synchronized at the G1/S boundary were released from the arrest and were treated with liposomal formulations of pegylated RGDGWK-lipopeptide **1** containing: curcumin, HC18-oleyl, C8-cer, both curcumin & HC18-oleyl and both curcumin & C8-cer. After 24 h of treatment, cells were trypsinized, fixed, permeabilized, incubated with primary antibody of cyciln B1 (a marker of G2/M phase) followed by incubation with FITC-conjugated secondary antibody and finally analyzed by flow cytometry. Overlap of FACS profiles for the cyclin B1 for cells treated with liposomal formulation of: C8-cer (yellow profile), HC18-oleyl (blue profile), curcumin (red profile), both curcumin & C8-cer (sky blue profile), both curcumin & HC18-oleyl (pink profile) and untreated cells (black profile).

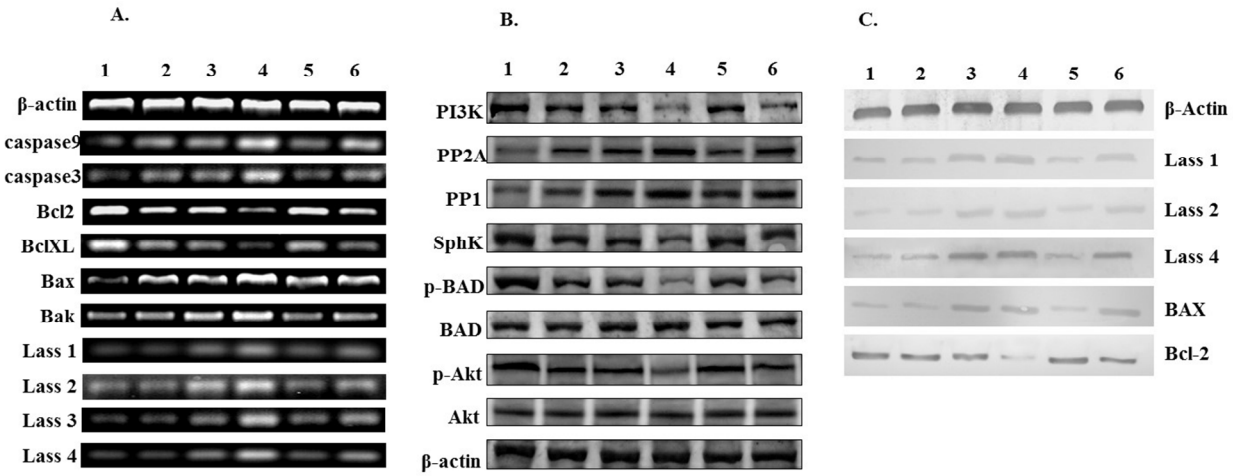


Figure S21. Liposomal formulations of curcumin & HC18-oleyl shows maximum effects in inhibiting expressions of proliferation and anti-apoptosis related genes in B16F10 cells at both mRNA and protein levels. mRNA levels (**A**) and protein expressions (**B & C**) of indicated genes involved in proliferation and apoptosis were measured by RT-PCR and Western blotting respectively. Lane 1, untreated cells; lane 2, cells treated with targeted liposomal HC18-oleyl; lane 3, cells treated with targeted liposomal curcumin; lane 4, cells treated with targeted liposome containing both curcumin & HC18-oleyl; lane 5, cells treated with targeted liposomal C8-cer; lane 6, cells treated with targeted liposome containing both curcumin & C8-cer

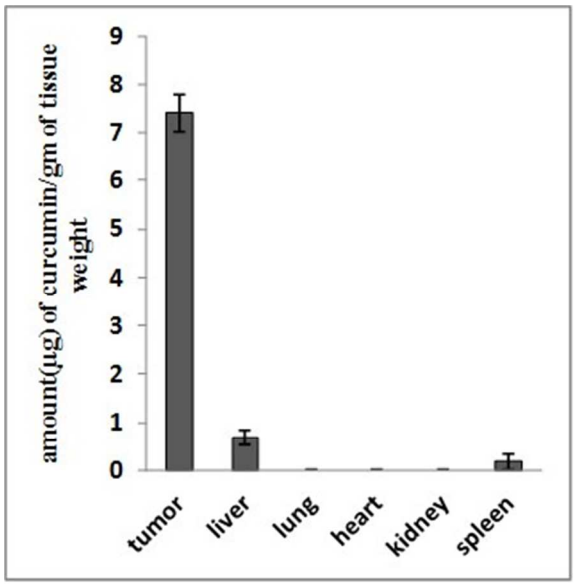


Figure S22. Intravenous administration of tumor vasculature targeting liposome of pegylated RGDGWK-lipopeptide **1** containing curcumin selectively accumulates to tumor tissue after 24 h.

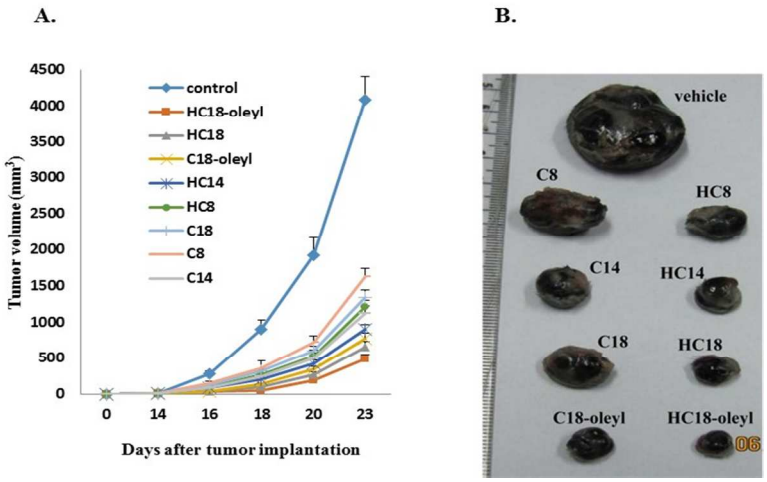


Figure S23. Among all the synthesized ceramide analogs HC18-oleyl is most potent to inhibit tumor growth. **A.** Relative tumor growth inhibition upon i.v. injected with liposomal formulations containing synthesized C8-ceramide analogs. **B.** Representative tumor sizes in each group on day 25 post tumor inoculation.

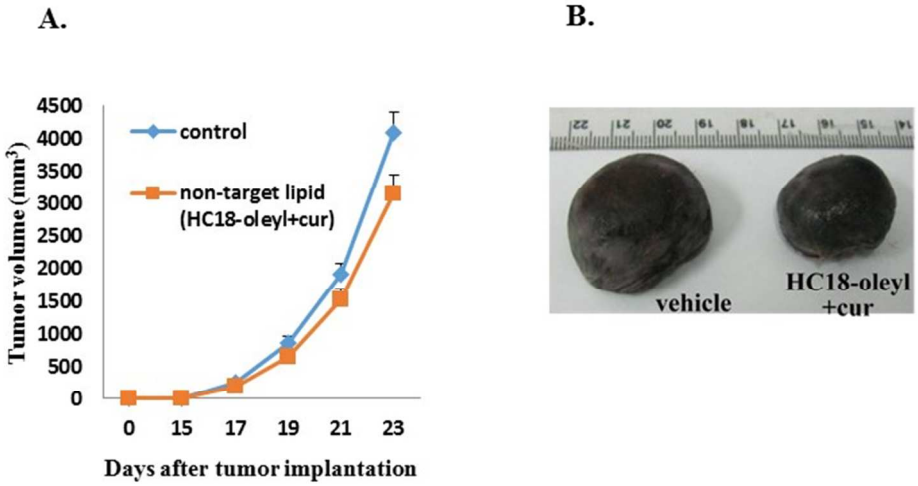


Figure S24. HC18-oleyl & curcumin formulated in non-targeting control liposomes of pegylated RGELFK-lipopeptide **2** inhibit tumor growth to a lesser extent. **A**. Tumor growth inhibition properties of non-targeted liposomal formulation containing both HC18-oleyl & curcumin. **B.** Representative tumor sizes in each group on day 25 post tumor inoculation.