Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

NO-Responsive Vesicles as Drug Delivery System

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Contents

General Experimental Section

Materials

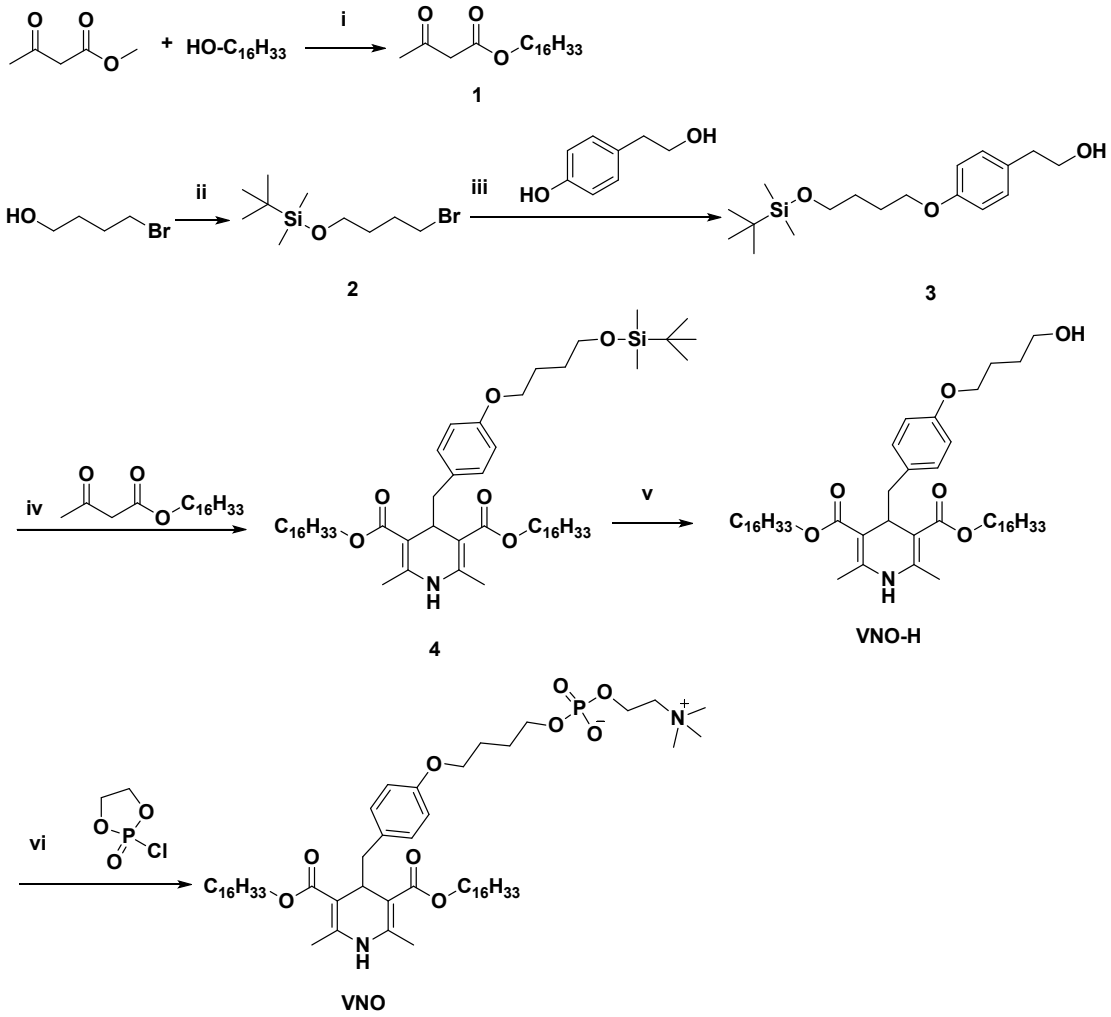
Compound **VNO** were maintained in a refrigerator at 4 ◦C. The nitric oxide (NO) stock solution in de-ionized water was prepared according to the literature[1]. Peroxynitrite ($ONOO^-$) was prepared following the reported method[2] and assayed by UV-vis spectroscopy ($\varepsilon_{302\ nm}$ = 1670 $L \cdot mol^{-1} \cdot cm^{-1}$). Singlet oxygen ($^1O_2$) was generated *in situ* by addition of NaClO to a solution containing 10 equiv. of H_2O_2. Hydroxyl radicals (·OH) was generated *in situ* by addition of Fe^{2+} to a solution containing 10 equiv. of H_2O_2 through Fenton reaction. Superoxide ($\cdot O_2^-$) was prepared by stirring KO_2 (1 mg) in dry DMSO (1 mL) for 10 min. Other reactive oxidative species (ROS), such as $NaNO_2$ and $NaNO_3$, were prepared by dilution of commercial suppliers in de-ionized water. Deionized water was used throughout all experiments. All other chemicals were purchased from commercial suppliers and used without further purification, unless indicated otherwise.

Instruments

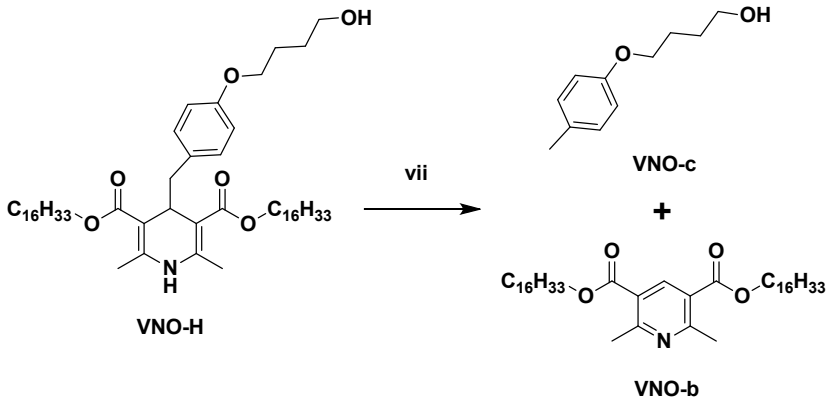
^1H NMR and ^{13}C NMR spectra were recorded on a 400 MHz Bruker Arance III spectrometer. Mass spectra were obtained from a Water LCT Premier XE spectrometer. Absorption spectra were recorded on a Cary-50 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence spectrophotometer. Determination of hydrodynamic diameters was performed on a Brookhaven Zeta Plus Partical Size and Zeta Potential Analyzer (USA). TEM images were obtained on a Philips CM-12 with Tungsten filament and operated at 120 kV.

Synthesis and Characterization

Synthetic Routes



Scheme S1. Synthesis of **VNO** Reagents: (i) H_2NSO_3H, (90%); (ii) TBSCl, imidazole, DCM, (92%); (iii) K_2CO_3, NaI, MeCN, (79%); (iv) 1) DMP, DCM, 2) $NH_3 \cdot H_2O$, EtOH, (35%); (v) TFA, DCM, (93%); (vi) 1) pyridine, toluene, 2) Me_3N, MeCN,(75%).



Scheme S2. Synthesis of **VNO-b and VNO-c.** Reagents: (vii) NO, DCM, (90% for VNO-b and 86% for VNO-c).

Synthetic Procedures

Hexadecyl 3-oxobutanoate (1). Compound **1** was synthesized according to the reported procedure[3]. White solid, 90% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.13 (t, *J* = 6.8 Hz, 2H), 3.45 (s, 2H), 2.27 (s, 3H), 1.64 (m, 2H), 1.26 (m, 26H), 0.88 (t, *J* = 6.6 Hz, 3H).

(4-Bromobutoxy)(tert-butyl)dimethylsilane (2). Compound **2** was synthesized according to the reported procedure[4]. Colorless oil, 92% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.59 (t, *J* = 6.1 Hz, 2H), 3.40 (t, *J* = 6.8 Hz, 2H), 1.89 (m, 2H), 1.61 (m, 2H), 0.84 (s, 9H), -0.01 (s, 3H).

2-(4-(4-(tert-butyldimethylsilyloxy)butoxy)phenyl)ethanol (3). To a mixture of compound **2** (26.7 g, 100 mmol), tyrosol (13.8 g, 100 mmol), K_2CO_3 (48.0 g, 400 mmol) and KI (1.7 g, 10 mmol) was added 300 mL MeCN, the reaction mixture was then refluxed for over 5 h until the reaction was reached completion. The solution was cooled to room temperature, filtered and concentrated in vacuum. The crude materials were further purified with column chromatography on silica gel (petroleum ether/ ethyl acetate = 4/1, v/v) to obtain product **3** as a colorless oil (25.6 g, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (d, *J* = 8.5 Hz, 2H), 6.79 (d, *J* = 8.6 Hz, 2H), 3.91 (t, *J* = 6.5 Hz, 2H), 3.77 (t, *J* = 6.5 Hz, 2H), 3.62 (t, *J* = 6.3 Hz, 2H), 2.75 (t, *J* = 6.5 Hz, 2H), 1.78 (tt, *J* = 8.6, 6.3 Hz, 2H), 1.68 – 1.57 (m, 2H), 0.84 (s, 9H), 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) 151.67, 130.52, 129.9, 114.54, 67.76, 63.67, 62.83, 38.33, 29.37, 26.0, 25.91, 18.33, -5.25; ESI-MS: m/z $C_{18}H_{32}O_3SiNa$ $[M+Na]^+$, calcd. 347.2,

found 347.3.

Dihexadecyl4-(4-(4-(tert-butyldimethylsilyloxy)butoxy)benzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (4). Compound **3** (16.2 g, 50 mmol) was dissolved in 100 mL of DCM, and then Dess-Martin periodinane (DMP, 23.3 g, 55 mmol) was added. After stirred at room temperature for another 1 h, the mixture was filtered through silica gel, and the solid residue was washed with 50 mL of DCM. The filtrate was combined, concentrated in vacuum to obtain a light yellow oil.

The oil and compound **1** (14.4 g, 44 mmol) were dissolved in 200 mL of EtOH, then 20 mL of ammonia (25% w.t., 266 mmol) was added. After refluxed under nitrogen atmosphere for 20 h, the solution was cooled to room temperature and concentrated in vacuum. The crude materials were further purified with column chromatography on silica gel (petroleum ether/ ethyl acetate = 6/1, v/v) to obtain product **4** as a white solid (14.9 g, 35% yield). Mp. 46~48 °C, ^{1}H NMR (400 MHz, MeOD δ 6.79 (d, *J* = 8.5 Hz, 2H), 6.63 (d, *J* = 8.6 Hz, 2H), 4.03 (t, *J* = 5.8 Hz, 1H), 3.95 – 3.77 (m, 6H), 3.63 (t, *J* = 6.2 Hz, 2H), 2.36 (d, *J* = 5.8 Hz, 2H), 2.11 (s, 5H), 1.79 – 1.68 (m, 2H), 1.60 (dq, *J* = 9.8, 6.4 Hz, 2H), 1.52 (p, *J* = 6.4 Hz, 4H), 1.20 (s, 48H), 0.83 (d, *J* = 7.6 Hz, 15H), 0.00 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.89, 157.36, 145.43, 145.40, 131.18, 130.94, 113.33, 101.64, 67.78, 63.74, 63.01, 62.85, 41.16, 35.42, 32.81, 31.92, 29.71, 29.69, 29.66, 29.64, 29.61, 29.60, 29.44, 29.41, 29.36, 28.77, 26.20, 25.98, 25.94, 25.75, 22.68, 19.09, 18.32, 14.11, -5.31. ESI-MS: m/z $C_{58}H_{103}NO_6SiNa$ $[M+H]^+$, calcd. 938.7627, found. 938.7620.

Dihexadecyl4-(4-(4-hydroxybutoxy)benzyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (VNO-H). Compound **4** (9.4 g, 10 mmol) was dissolved in 50 mL ofDCM, TFA (1.4 g, 12 mmol) was added dropwisely. After stirred at room temperature for another 30 min, 30 mL of saturated $NaHCO_3$ was added dropwise. The aqueous phase was then extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuum. The crude materials were further purified with column chromatography on silica gel (petroleum ether/ ethyl acetate = 3/1, v/v) to obtain product **VNO-H** as a white solid (7.6 g, 93% yield). Mp. 83~84°C,

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.89 (d, *J* = 8.5 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 5.24 (s, 1H), 4.18 (t, *J* = 5.0 Hz, 1H), 4.03 (ddt, *J* = 18.9, 16.4, 6.4 Hz, 6H), 3.71 (t, *J* = 6.2 Hz, 2H), 2.52 (d, *J* = 5.0 Hz, 2H), 2.14 (s, 5H), 1.92 – 1.80 (m, 2H), 1.74 (dq, *J* = 9.4, 6.8, 6.3 Hz, 2H), 1.63 (p, *J* = 6.8 Hz, 4H), 1.25 (s, 48H), 0.88 (t, *J* = 6.7 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.92, 156.98, 145.59, 131.43, 131.05, 113.45, 101.43, 67.75, 63.76, 62.58, 41.08, 35.39, 31.92, 29.71, 29.66, 29.64, 29.53, 29.36, 28.79, 26.20, 25.79, 22.69, 19.05, 14.12. ESI-MS: m/z $C_{52}H_{89}NO_6Na$ $[M+H]^+$, calcd. 824.6762, found. 824.6757.

4-(4-((3,5-bis(hexadecyloxycarbonyl)-2,6-dimethyl-1,4-dihydropyridin-4-yl)methyl)phenoxy)butyl 2-(trimethylammonio)ethyl phosphate (VNO). Compound **VNO-H** (4.1 g, 5 mmol) and imidazole (340 mg, 5 mmol) were dissolved in 15 mL of toluene. The solution was chilled to approximately 5 °C in an ice bath, and 2-chloro-1,3,2-dioxaphospholane-2-oxide (710 mg, 5 mmol) was added dropwisely. Then the mixture was increased to room temperature slowly, and kept the temperature for about 30 min. Filtered and the residue was washed with 5 mL of toluene. The filtrate was concentrated in vacuum to obtain a white solid.

The solids were mixed with Me_3N (20 mL, 2M in MeCN)in a 50 mL sealed vial, chilled to 80 °C and kept temperature for over 15 h. The solution was cooled to 0 °C, filtered and the residue was washed with 10 mL of ice-cold MeCN, then dried under reduced pressure to obtain product **VNO** as a white solid (5.2 g, 88% yield). Mp. 46~48 °C, ^{1}H NMR (400 MHz, MeOD) δ 6.86 (d, *J* = 8.3 Hz, 1H), 6.72 (d, *J* = 8.4 Hz, 1H), 4.26 (s, 1H), 4.14 (t, *J* = 5.4 Hz, 1H), 4.09 – 3.81 (m, 4H), 3.70 – 3.57 (m, 1H), 3.22 (s, 3H), 2.45 (d, *J* = 5.5 Hz, 1H), 2.16 (s, 2H), 1.92 – 1.74 (m, 2H), 1.62 (q, *J* = 6.7 Hz, 2H), 1.29 (s, 21H), 0.90 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, THF-*d*8) δ 167.50, 157.66, 147.58, 131.53, 131.26, 113.34, 99.70, 67.62, 64.89, 63.04, 59.49, 53.91, 41.23, 35.60, 32.19, 30.03, 30.01, 29.94, 29.75, 29.64, 29.30, 27.75, 26.59, 26.12, 22.88, 17.85, 13.79, 0.00.

ESI-MS: m/z $C_{57}H_{101}N_2O_9Na$ $[M+H]^+$, calcd.989.7317, found.989. 7314.

Dihexadecyl 2,6-dimethylpyridine-3,5-dicarboxylate (VNO-b) and 4-(p-tolyloxy)-

butan-1-ol (VNO-c). Compound **VNO-H** (824 mg, 1 mmol) was dissolved in 20 mL of DCM. Nitrogen monoxide was blew into the solution for over 20 min (~5 mL/ min). Then concentrated in vacuum, and the crude materials were further purified with column chromatography on silica gel (petroleum ether/ ethyl acetate = 8/1, v/v) to obtain product **VNO-b** as a white solid (580 mg, 90% yield) and **VNO-c** as a colorless oil (156 mg, 86% yield). VNO-b was also synthesized VNO and NO by suing the same method. 87% yield.

VNO-b: Mp. 83~84 °C,^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H), 4.32 (t, *J* = 6.7 Hz, 4H), 2.85 (s, 6H), 1.77 (p, *J* = 6.9 Hz, 4H), 1.25 (s, 59H), 0.88 (t, *J* = 6.7 Hz, 7H).^{13}C NMR (101 MHz, $CDCl_3$) δ 165.67, 161.90, 140.66, 122.75, 65.25, 31.61, 29.38, 29.36, 29.34, 29.28, 29.22, 29.04, 28.94, 28.31, 25.73, 24.65, 22.37, 13.80. ESI-MS: m/z $C_{47}H_{74}NO_4$ $[M+H]^+$, calcd. 644.5612, found 644.5608.

VNO-c[5]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (d, *J* = 8.2 Hz, 2H), 6.81 (d, *J* = 8.1 Hz, 2H), 3.99 (t, *J* = 5.7 Hz, 2H), 3.64 (t, *J* = 6.2 Hz, 2H), 2.31 (s, 3H), 2.10 – 1.83 (m, 4H).

CMC Measurement

Aqueous solutions of **VNO** at different concentrations, ranging from 0.1 mg/ L to 250 mg/ L were prepared. 10 μL of pyrene solution (200 mg/ L, acetone) was added to each sample solution of **VNO** (4 mL).The solutions were then sonicate for 10 min before their fluorescencent spectra were recorded. The intensity ratio I_{373}/ I_{393} was plotted as a function of the logarithm of the concentration of **VNO** (Figure S1). The concentration at which the value of I_{373}/ I_{393} suddenly drop, was measured the CMC of **VNO.**

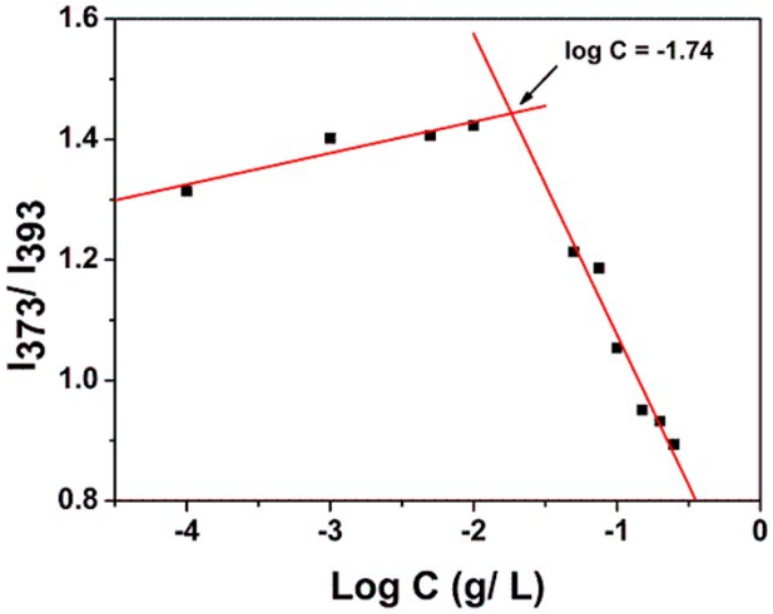
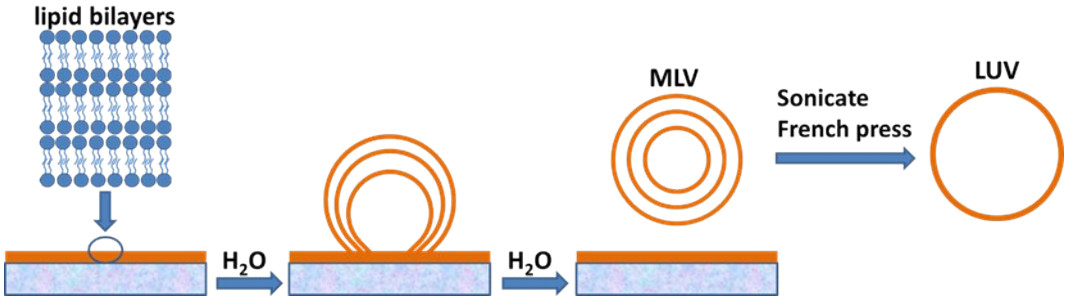


Fig. S1 Plot of I_{373}/ I_{393} ratios as a function of log c of **VNO**.

Vesicles Preparation

VNO (5.0 mg, 5 μmol) was added into a 50 mL round-bottom bottle and was dissolved in 10 mL of DCM. The solvent was slowly removed in vacuum to produce a lipid film, followed by adding 1 mL of carboyfluorescein (CF, 200 mM, PBS buffer solution, in the presence of 1 drop of 5 M NaOH as a co-solvent) was added. The mixture was vortexed for 2 minutes to form multilamellar vesicles (MLVs), and then sonicated at 50 °C for 10 min to turn MLVs to large unilamellar vesicles (LUVs). The resulting dispersion of LUVs was extruded 20 times through a polycarbonate extrusion membrane (100-nm pore-size) to obtain uniform-size vesicles (Scheme S3). Finally, the untapped CF was removed with gel chromatography using a Sephadex G-50 column eluted with PBS buffer and the obtained vesicle solution was adjusted volume to 500 mL to obtain the vesicle stock suspension (10μM for the concentration of **VNO**). The diameter of vesicles was approximately 138 nm which was determined by DLS.



Scheme S3. The mechanism of the formation of the vesicles.

^{1}H NMR Analysis

Compound VNO-H and VNO-c were dissolved separately in DMSO-d_6 at 1 mM concentration to give the stock solutions. The solutions were mixed with DMSO-*d*6 and de-ionized water to give the solutions at 100 μM concentration (80% DMSO and 20% water) and their ^{1}H NMR spectra were recorded. However, VNO-b is totally insoluble and no peaks were observed. Then Different equiv. of the NO stock solution (1.9 mM, de-ionized water) were added to the VNO-H stock solution separately, and DMSO-d_6 with de-ionized water were used to adjust the concentration of VNO-H to 100 μM (80% DMSO + 20% water). The solutions were kept at 37 °C for 30 min before their ^{1}H NMR spectra were recorded.

1 equiv. of the NO stock solution (1.9 mM, de-ionized water) and other ROSs stock solution (1 mM, de-ionized water) were added to the VNO-H stock solution separately for the selectivity assay.

MS Analysis

1 equiv. of NO stock solution was added to the vesicle solution and kept for another 30 min at 37 °C. The reaction mixture was then characterized with MS without any purification.

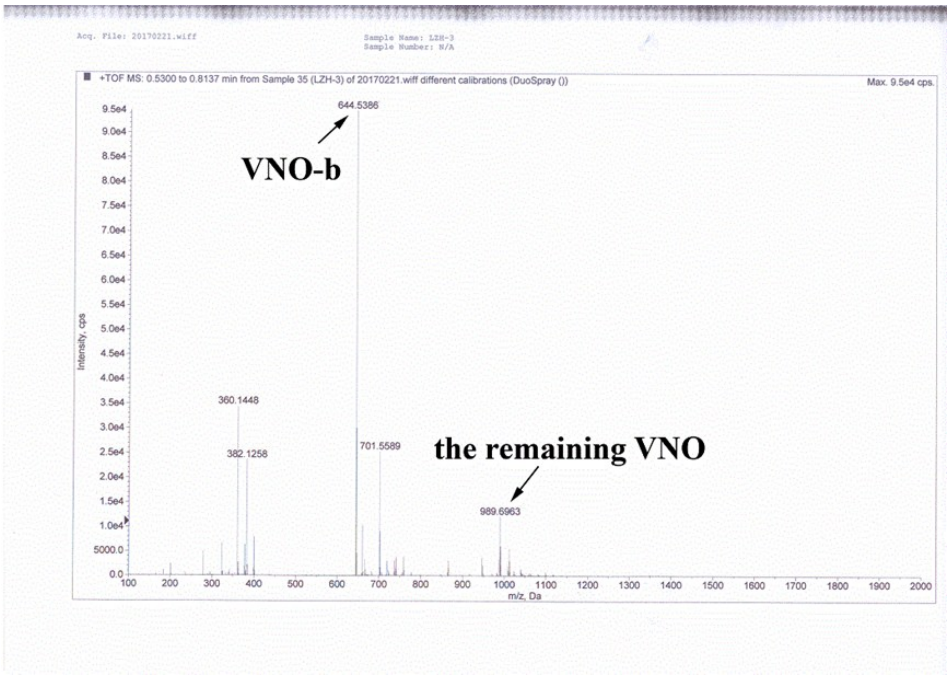


Fig. S2 MS spectrum of the reaction mixture of VNO with nitro oxide (1 equiv.).

DLS Measurement

The mean diameter of vesicles for all experiments was determined by dynamic light scattering (DLS). Each sample was measured three times (5 min for every time) at a fixed angle of θ = 173 °.Test temperature was 25 °C.

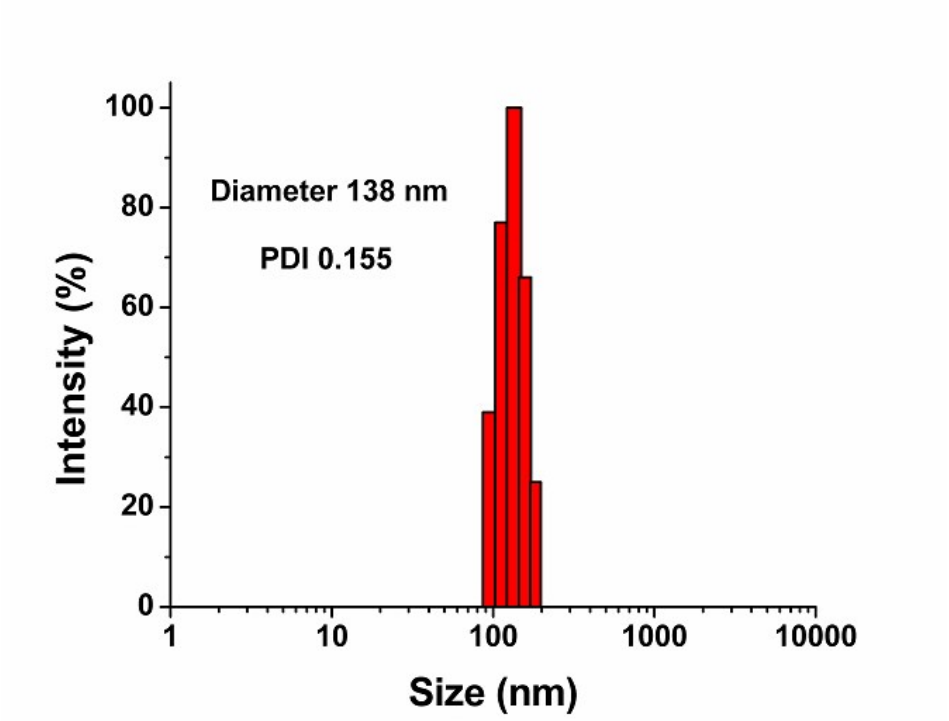


Fig. S3 Particle size distributions of **VNO** aggregates in PBS buffer (50 mM, pH = 7.4)

TEM Imaging

One drop of the vesicle stock suspension was added onto a glow-discharged EM grid and kept for 10 min, and the residual solution was removed with a piece of filter paper. Then one drop of the sodium phosphotungstate solution ($Na_3PO_4 \cdot 12WO_3 \cdot 18H_2O$, 2%, de-ionized water) was added onto the glow-discharged EM grid and kept for 10 min,and the residual solution was removed with a piece of filter paper. The grid was dried in vacuum for 10 min before TEM pictures were recorded.

Fluorescence Analysis

Suspensions vesicles were prepared by transferring appropriate aliquots of the trick suspension which was just purified through the Sephadex G-50 column to volumetric flasks and thendiluting them to certain volumes using PBS buffer and NO stock solution (1.9 mM, de-ionized water).

All the fluorescence spectral properties of the vesicles were obtained with 1.0-cm quartz cuvettes. The slit width was 5 nm for both excitation and emission. The photon multiplier voltage was 600 V. λ_{ex} was 490 nm. Test temperature was 37 °C. Every fluorescent titration experiment was performed atleast three times for calculating error bars.

Cell Culture

RAW 264.7 cells were incubated at 37 °C under a humidified atmosphere that contains 5% CO_2 and in DMEM medium with 10% FBS. For imaging studies, cells were seeded in glass bottom cell culture dishes (Nest) containing 1 mL of DMEM and incubated for 24 h. Before imaging, cells were washed with 1 mL of PBS 5 times. Bright field and fluorescence images were taken with a 40× objective lens.

Cell Cytotoxicity

The cells were grown in 96-well plates (about 10^4 cells per well) and incubated in 5% CO_2/ 95% air atmosphere at 37 °C for 24 h. Then the cells were treated with 5 μM, 10

μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM of compound VNO and VNO-b and were incubated for 12 h respectively. MTT solution (20 μL, 5 mg/ mL, PBS) was then added to each well and kept for another 4 h. 100 μL of DMSO was added to each well after the remaining MTT solution was removed. Absorbance at 490 nm was measured on a plate reader.

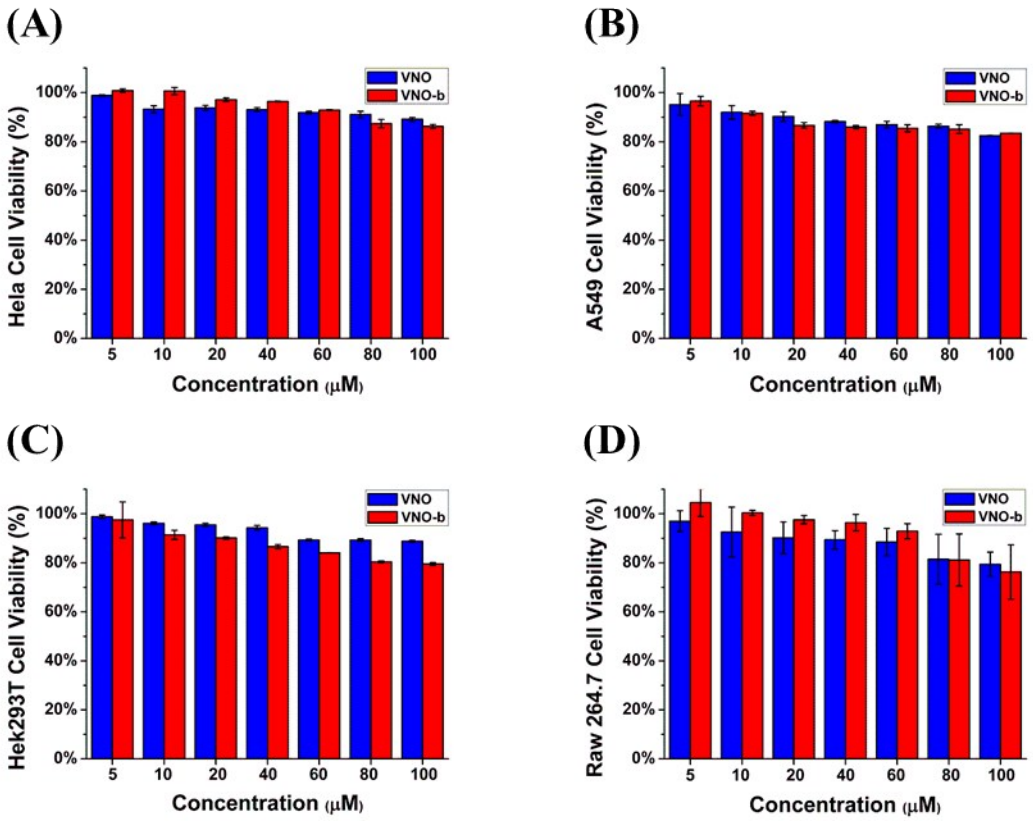
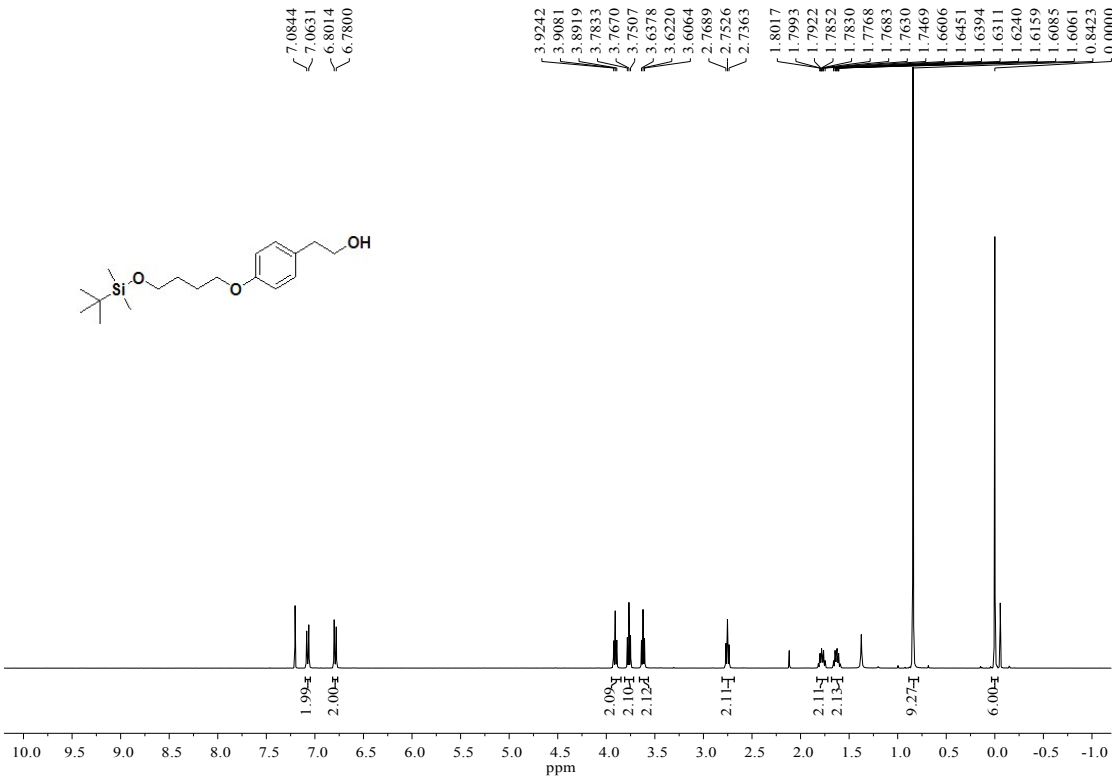
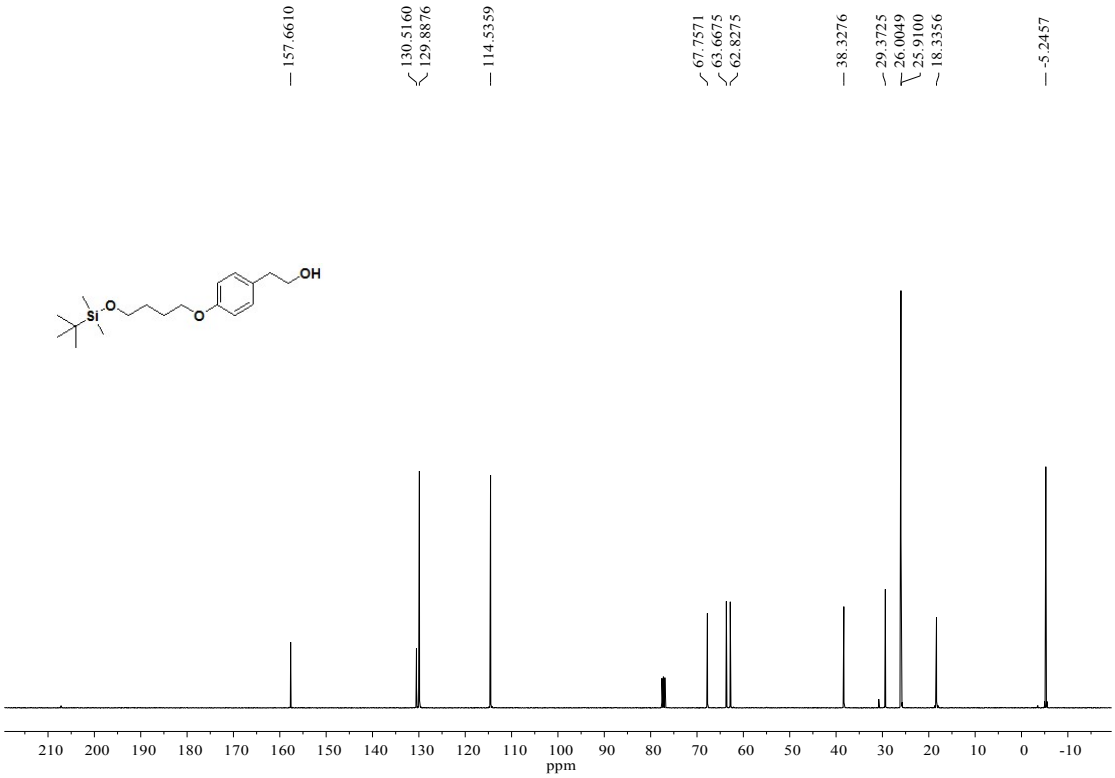


Fig. S4 *In vitro* cytotoxicity after being treated with compound **VNO** and **VNO-b** at different concentrations using (A) Hela, (B) A549, (C) Hek293T, (D) Raw246.7 cells. The results were determined by the MTT assay.

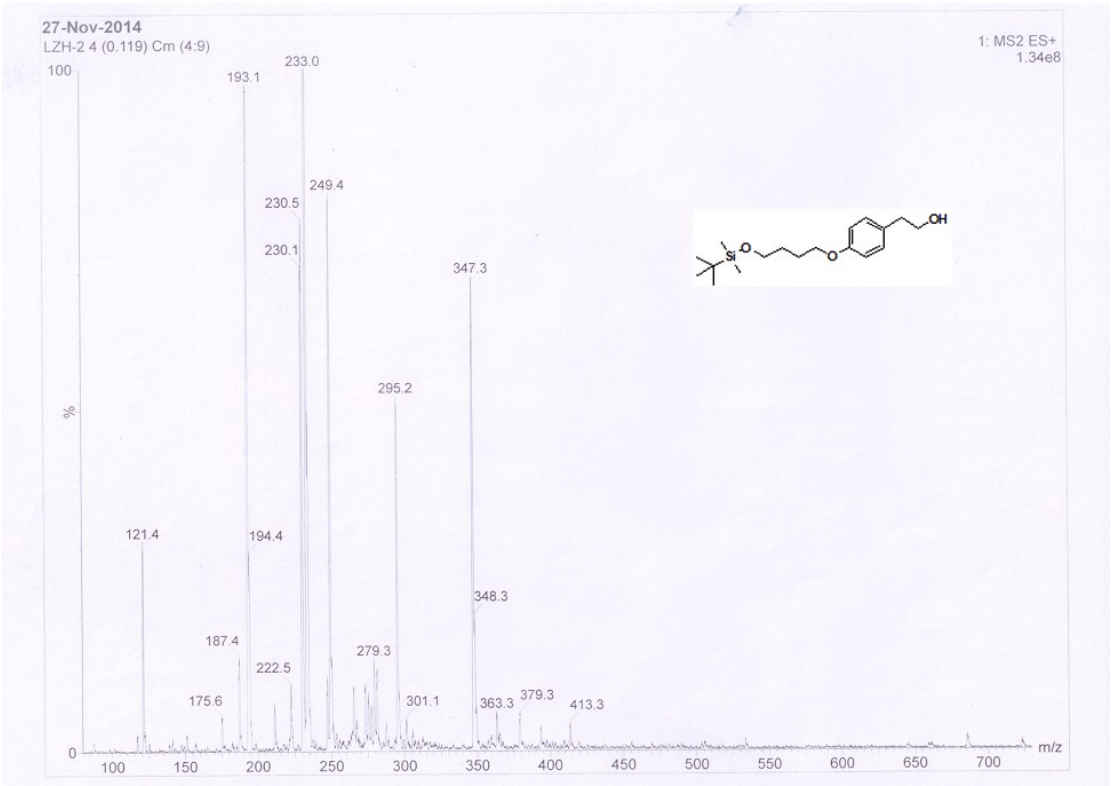
Spectra of the compounds



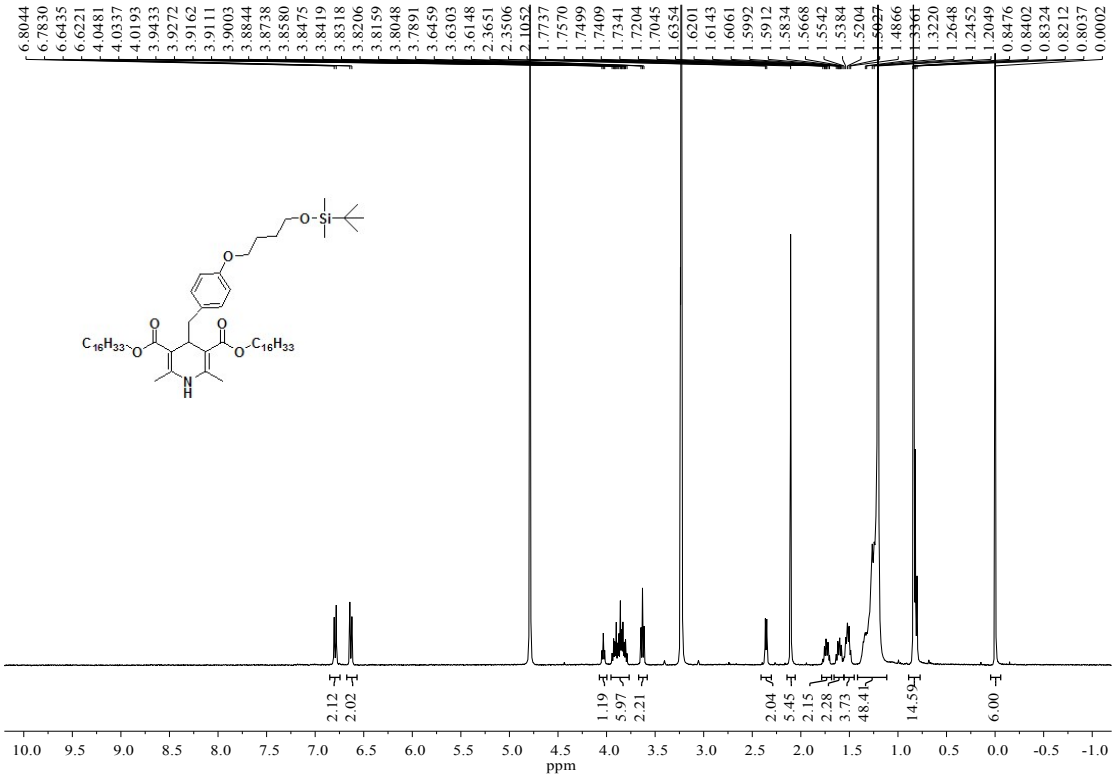
^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **3**



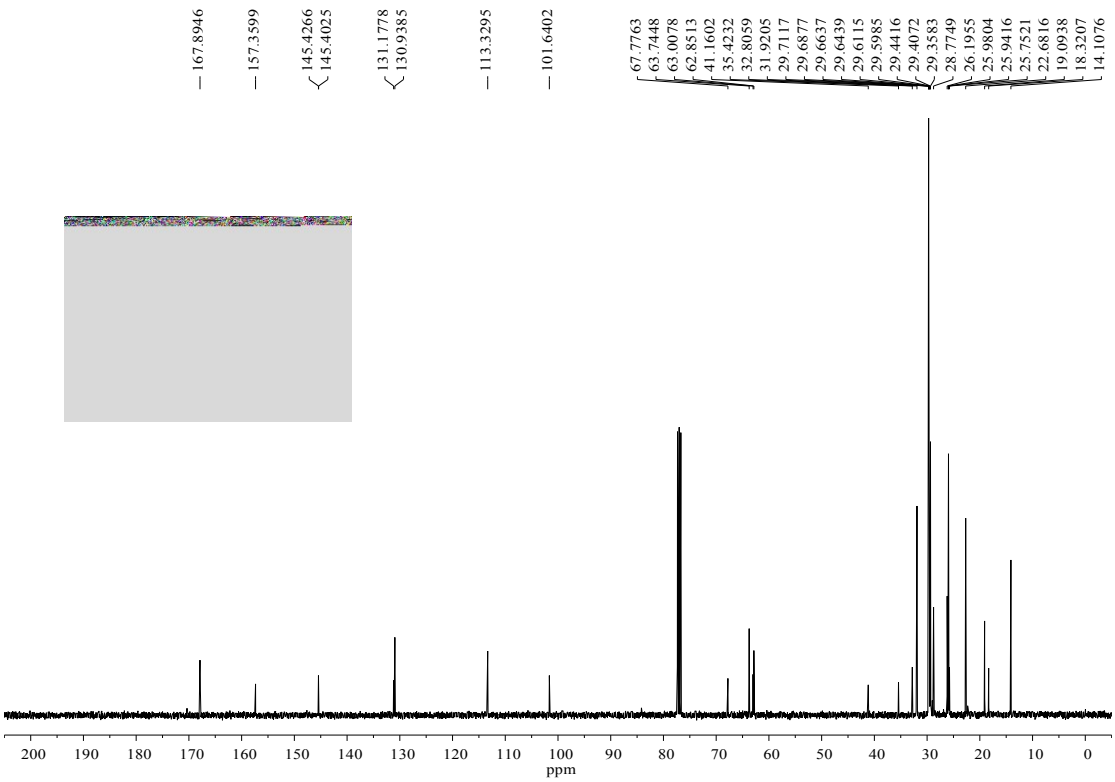
^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of compound **3**



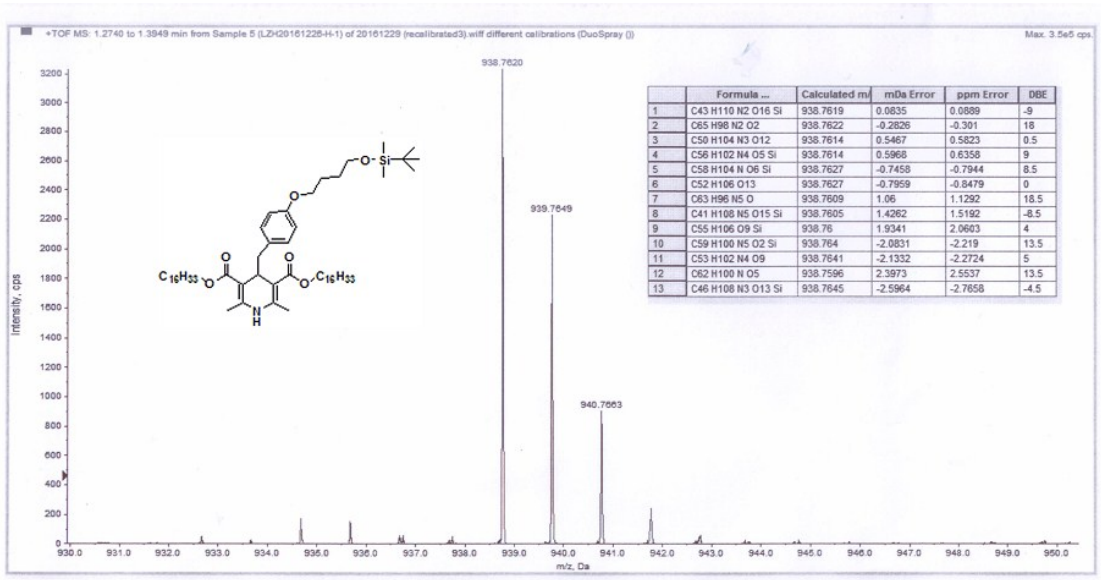
MS spectrum of compound **3**



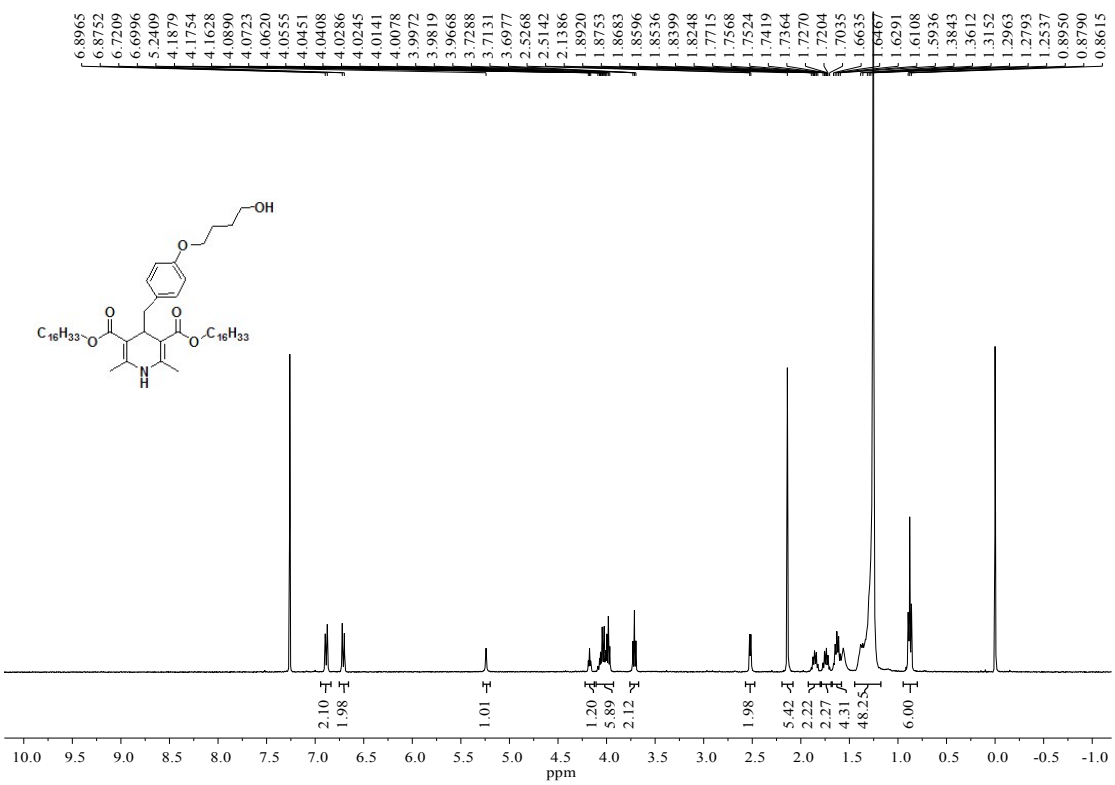
^{1}H NMR spectrum (400 MHz, MeOD, 298 K) of compound **4**



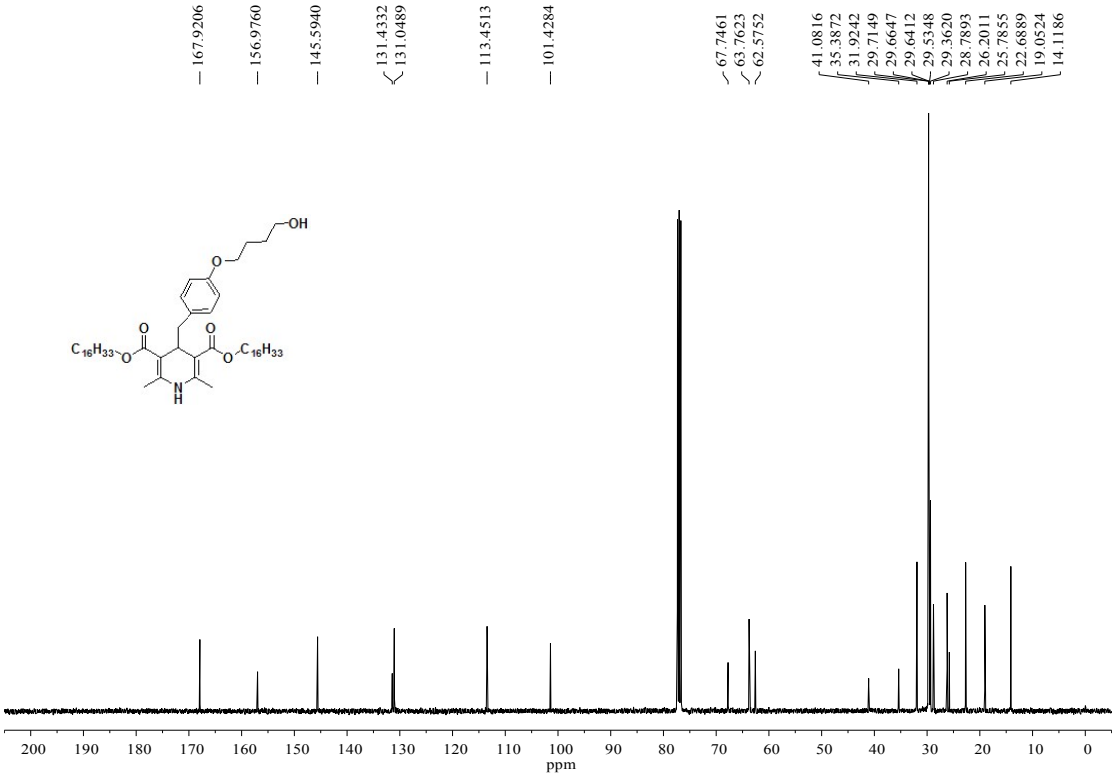
^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of compound **4**



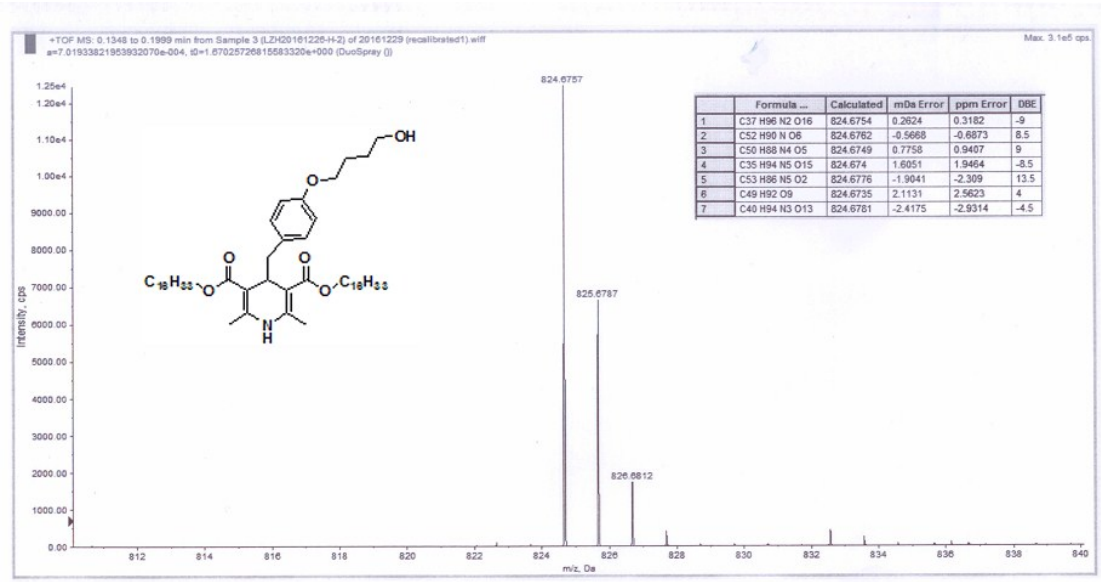
HR-MS spectrum of compound **4**



^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **VNO-H**

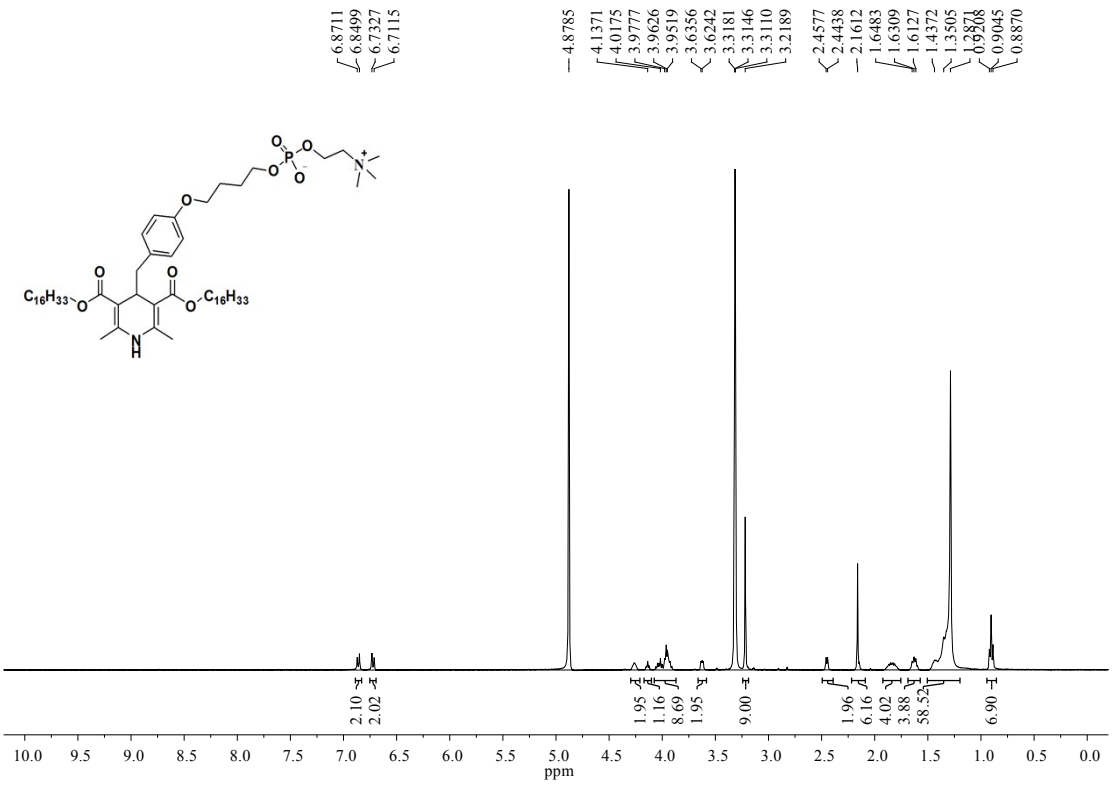


^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of compound **VNO-H**

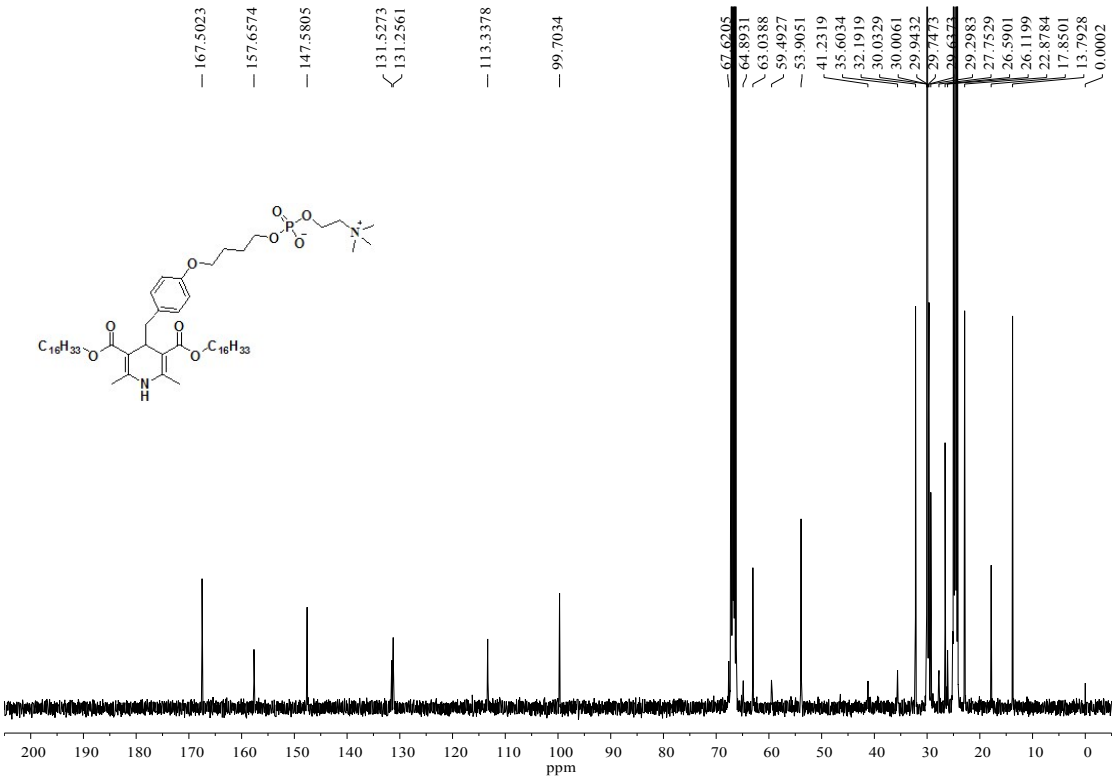


	Formula ...	Calculated	mDa Error	ppm Error	DBE
1	C37 H96 N2 O16	824.6754	0.2624	0.3182	-9
2	C52 H90 N O6	824.6782	-0.5668	-0.6873	8.5
3	C50 H88 N4 O5	824.6749	0.7758	0.9407	9
4	C35 H94 N5 O15	824.674	1.6051	1.9464	-8.5
5	C53 H86 N5 O2	824.6776	-1.9041	-2.309	13.5
6	C49 H92 O9	824.6735	2.1131	2.5623	4
7	C40 H94 N3 O13	824.6781	-2.4175	-2.9314	-4.5

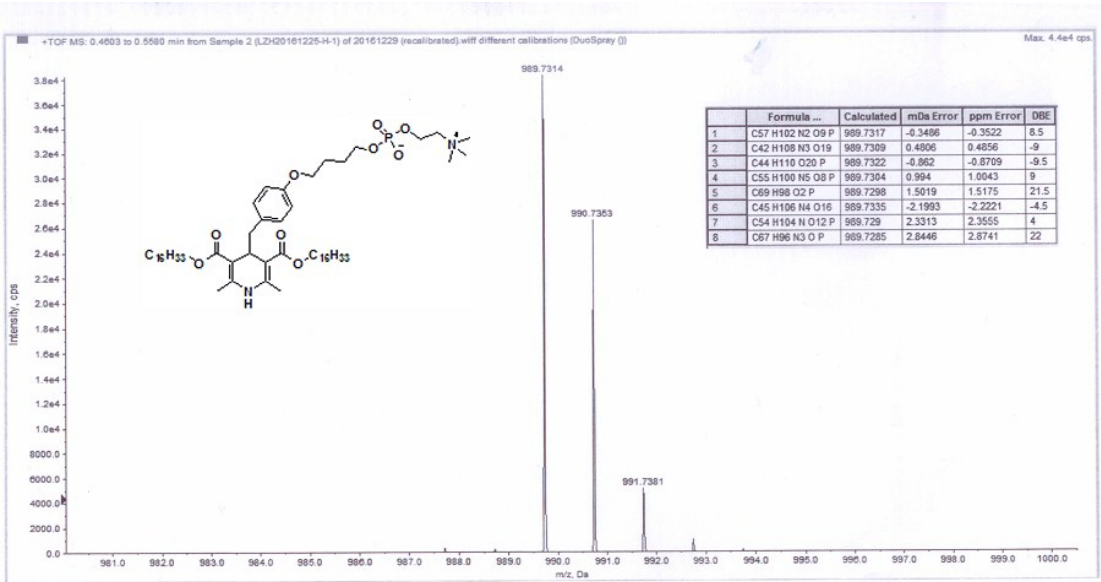
HR-MS spectrum of compound **VNO-H**



^{1}H NMR spectrum (400 MHz, MeOD, 298 K) ofcompound**VNO**

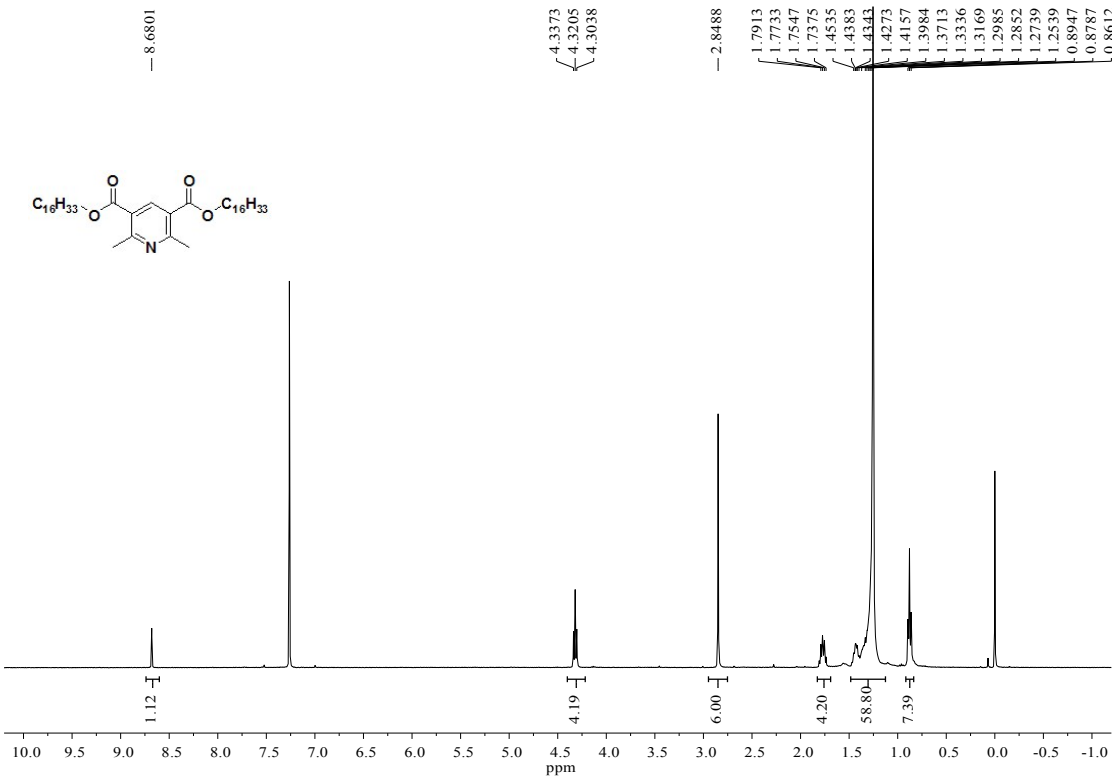


^{13}C NMR spectrum (100 MHz, THF-*d*8, 298 K) of compound **VNO**

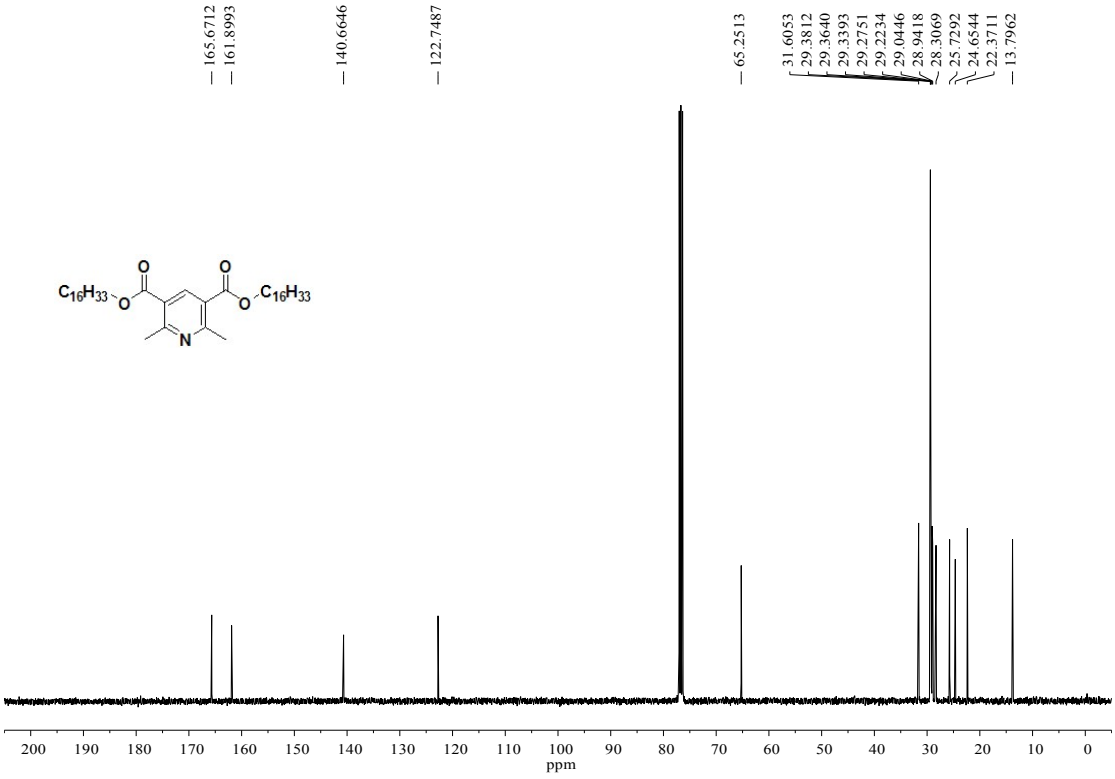


	Formula ...	Calculated	mDa Error	ppm Error	DBE
1	C57 H102 N2 O9 P	989.7317	-0.3486	-0.3522	8.5
2	C42 H108 N3 O19	989.7309	0.4806	0.4856	-9
3	C44 H110 O20 P	989.7322	-0.862	-0.8709	-9.5
4	C55 H100 N5 O8 P	989.7304	0.994	1.0043	9
5	C69 H98 O2 P	989.7298	1.5019	1.5175	21.5
6	C45 H106 N4 O16	989.7335	-2.1993	-2.2221	-4.5
7	C54 H104 N O12 P	989.729	2.3313	2.3555	4
8	C67 H96 N3 O P	989.7285	2.8446	2.8741	22

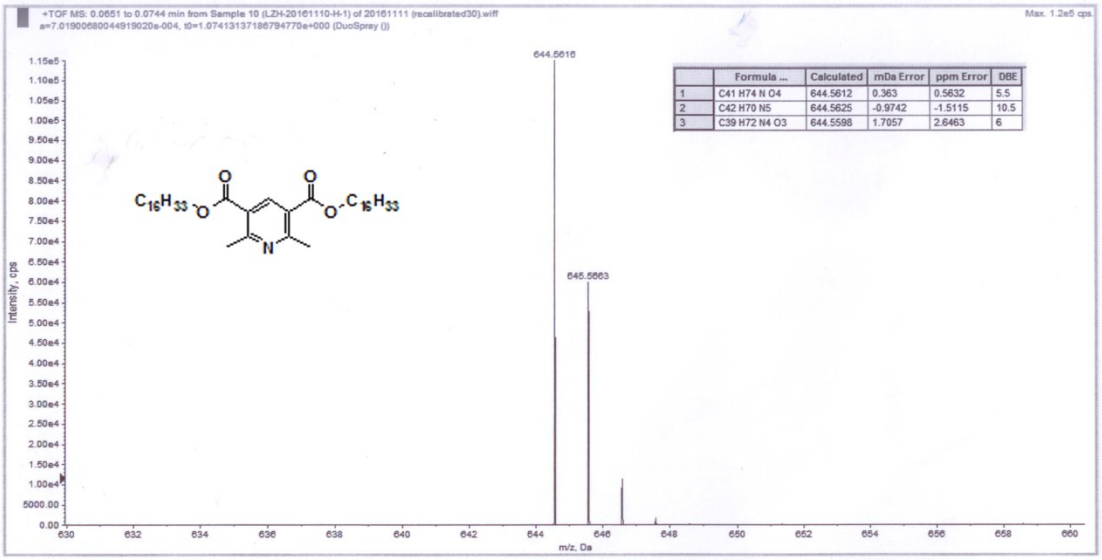
HR-MS spectrum of compound **VNO**



^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **VNO-b**



^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of compound **VNO-b**



HR-MS spectrum of compound **VNO-b**

References

1. Huang, K.-J.; Wang, H.; Ma, M.; Zhang, X.; Zhang, H.-S., Real-time imaging of nitric oxide production in living cells with 1,3,5,7-tetramethyl-2,6-dicarbethoxy-8-(3',4'-diaminophenyl) -difluoroboradiaza-s-indacence by invert fluorescence microscope. *Nitric Oxide* **2007,***16*, 36-43.

2. Uppu, R. M.; Pryor, W. A., Synthesis of Peroxynitrite in a Two-Phase System Using Isoamyl Nitrite and Hydrogen Peroxide. *Analytical Biochemistry* **1996,***236*, 242-249.

3. Treptow, T. G. M.; Figueiro, F.; Jandrey, E. H. F.; Battastini, A. M. O.; Salbego, C. G.; Hoppe, J. B.; Taborda, P. S.; Rosa, S. B.; Piovesan, L. A.; Montes D'Oca, C. D. R.; Russowsky, D.; Montes D'Oca, M. G., Novel hybrid DHPM-fatty acids: Synthesis and activity against glioma cell growth in vitro. *Eur. J. Med. Chem.* **2015,***95*, 552-562.

4. Fuerstner, A.; Mlynarski, J.; Albert, M., Total Synthesis of the Antiviral Glycolipid Cycloviracin B1. *J. Am. Chem. Soc.* **2002,***124*, 10274-10275.

5. Zheng, Y.; Zou, W.; Luo, L.; Chen, J.; Lin, S.; Sun, Q., Ligand-free Cu-catalyzed O-arylation of aliphatic diols. *Rsc Advances* **2015,***5*, 66104-66108.