Supplementary Material (ESI) for Chemical Communications

Supporting Information for:

Fluorescent labeling of human mesenchymal stem cells by thiophene fluorophores conjugated to a lipophilic carrier

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Abbreviations: TFA (trifluoroacetic acid), TF (tiophene fluorophore)

Contents

Full synthetic and experimental details

General Instrumentation and Reagents

Solvents and most of the starting materials were purchased from Aldrich and Alfa Æsar. High resolution mass spectra (HRMS) were obtained with a LTQ Orbitrap hybrid mass spectrometer with an electronspray ionization probe (Thermoscientific, San Jose, CA) by direct infusion from a pump syringe to confirm correct molar mass and high purity of compounds. The ^{1}H and ^{13}C NMR (500 MHz and 125 MHz, respectively) spectra were recorded on a Bruker AC 500 spectrometer. Chemical shifts are expressed as parts per million from tetramethylsilane. Splitting patterns have been designated as follows: s (singlet), d (doublet), t (triplet) and m (multiplet). Coupling constants (J values) are listed in hertz (Hz). Reactions were monitored by HPLC using reverse phase analytical column (Thermo, RP C18 250 x 4.6 mm, 5µ) on a Waters^{TM} 600E pump and a photodiode array detector Waters^{TM} 996. Data were monitored using a Waters Millenium software. Semipreparative reverse phase column (Thermo, RP C18 250 x 10 mm, 5µ) was used in order to purify the reaction products. Both for analytical and semipreparative HPLC we used a gradient from 20 to 100% of CH_3CN in H_2O both containing 0.1% of TFA with UV detection at 254 nm and 350 nm.

Human mesenchymal stem cells culture and characterization

Primary human mesenchymal stem cell (MSC) cultures were established from patients undergoing elective surgery at Istituto Ortopedico Rizzoli after obtaining informed consent. Briefly, a 10 mL bone marrow sample was aspirated from the anterior iliac crest. Mononucleated cells were isolated in a density gradient and resuspended in α-modified minimum essential medium (α-MEM; BioWhittaker, Lonza, Verviers, Belgium) containing 20% fetal bovine serum (FBS; BioWhittaker, Lonza, Verviers, Belgium) and 1% glutamax (Gibco-Invitrogen, Paisley, Scotland). All the mononucleated cells were plated in a 150 cm^2 culture flask and incubated in a humidified atmosphere at 37°C with 5% CO_2. Non-adherent cells were discarded after 3 days and adherent cells were cultured for further expansion. When cultured flasks became near confluent (70-80%), cells were detached by mild trypsinization (TripLe Select, Gibco-Invitrogen, Paisley, Scotland) and reseeded onto new plates at 1/3 density for continued passage. Media were changed every 3 to 4 days. Cell number and viability was assessed for each experiment by using a NucleoCounter (Chemometec, Allerød, Denmark) according to the procedure provided by the manufacturer.

MSC were recognized by their ability to proliferate in culture with attached, spindle-shape morphology. Further identification of MSC was performed by cytofluorimetric analysis of a panel of cell surface markers. MSC at passage 2 were labelled with following monoclonal antibodies: CD34-phycoerythrin (PE), CD45-allophycocyanin (APC), CD90-phycoeryththrin-cyanine 5 (PC5), and CD105-PE (all from Beckman Coulter, Fullerton, CA, USA). Control samples were labelled with isotype-matched irrelevant antibodies (Beckman Coulter). Briefly, cells were trypsinized and aliquoted at a concentration of 0.5×10^6 cells/ml, fixed in ethanol 70% and stained for 30 min with either conjugated specific antibodies or istotype-matched control mouse immunoglobulin G, at recommended concentrations. Labelled cells were washed twice and resuspended in FACS buffer. The analysis was performed using a FC500 flow cytometer (Beckman Coulter). At passage 2 cultured cells displayed a distinct phenotypic profile characteristic of hMSC: CD34-, CD45-, CD90+ (99.3% ± 0.4%), and CD105+ (99.0% ± 0.6%) .

Experiments were conducted with hMSCs from 3 different donors' mesenchymal stem cells of bone marrow. The cells of passages 4 or 5 were used throughout the study.

Live cell labeling

For living cell observations, hMSCs were seeded in a 24-well tissue culture plate at a density of 50000 cells per well in 400 μl complete culture medium. The cells had a 40-50% confluence before incubation. TFs powder dyes were dissolved in DMSO in order to obtain a stock solution of 40 mg/ml and then they were administered to cells by adding the appropriate dilution in phosphate buffered saline (PBS) to obtain the final concentration. Different concentrations (0.2, 0.1, and 0.05 mg/ml) and different time of incubation (15, 30 and 60 minutes) were tested to select the minimum incubation time and concentrations that effectively labeled hMSCs. At the end of incubation period unbound TF was removed (3 washes with PBS), and we continued to culture TF-labelled cells for up to 72 hours in standard culture condition.

Where it was indicated, 10 minutes before the end of TF treatment 5 μg/ml of Hoechst 33342 (Molecular Probe-Invitrogen, Paisley, Scotland) was added to TF solution in order to label nuclei. Image J software was used to composite merged images.

Digital fluorescence microscopy.

Fluorescence microscopy experiments were performed on a NIKON ECLIPSE TE 2000-U (NIKON, Japan) inverted microscope equipped with a 100 W mercury arc lamp for fluorescence excitation. The filter to detect TF fluorescence consisted of an 455/35 nm excitation filter, and a 515 nm emitter filter (DM 500 nm). Digital images were taken with a CCD camera (Nikon digital camera DXM 1200F) using ACT-2U Image software.

Cellular distribution of compound 7

hMSCs were seeded in a 12-well tissue culture plate containing glass coverslips at a density of 40000 cells per well in 800 μl complete culture medium. After incubation with compound **7 (0.1 mg/mL; 60 minutes)**, cells were washed three times with PBS and then fixed in a 4% paraformaldehyde solution in PBS at room temperature (RT) for 20 minutes.
For plasma membrane and Golgi apparatus staining, after fixation cells were washed twice with PBS, blocked with 1% bovine serum albumin in PBS for 1 hour and incubated with the purified mouse anti-human CD90 antibody (BD Pharmigen, San Jose, California, USA) solution (1:50 in blocking solution, overnight, 4°C) or with the purified mouse anti-GM130 antibody (BD Transduction Laboratories, San Jose, California, USA) solution (1:100 in blocking solution, 1 hour, RT). A second incubation with anti-mouse IgG Cy3 conjugate (Sigma-Aldrich, Saint Louis, Missouri, USA) took place (1:2500 in blocking solution, 1 hour, RT). Last, the samples were covered with mounting medium (Prolong Gold antifade reagent, Molecular Probe-Invitrogen) and visualized under the confocal microscope.
For nucleus staining, after fixation cells were washed twice with PBS and once with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). Cells were then incubated in 100 μg/mL DNase-free RNase for 20 minutes at 37°C. After washing with 2× SSC, cells were incubated with a solution of 1μg/mL propidium iodide (PI) in 2× SSC for 5 minutes at RT. The cells were washed 3 times with 2× SSC , mounted with antifade reagent and visualized under the confocal microscope.
For mitochondrial staining, after compound **7** incubation, cells were stained with 50 nM MitoTracker orange CMTMRos (Molecular Probe-Invitrogen) for 30 minutes at 37°C. Cells were washed three times with PBS and fixed in a 4% paraformaldehyde solution in PBS at RT for 20 minutes. The samples were covered with mounting medium and visualized under the confocal microscope.

Confocal laser scanning microscopy

Confocal microscopy analysis was obtained by a BioRad Radiance 2000 laser scanning system mounted on a Nikon Eclipse TE300 inverted-based microscope with a 60x 1.40NA oil immersion objective. Sequential excitation at 405 nm for compound 7 and 568 nm for Cy3 was provided by blue diode and argon-krypton lasers, respectively. The emission signal was separated by using a dichroic mirror (500 nm) followed by barrier filters BP515/30 and LP580 for collecting green and red in channels one and two, respectively. The confocal pinhole was set to 1.7-2.0 Airy disk. Optical x-y sections were obtained with 2.5x optical zoom at increments of 0.3 μm in the *z*-axis and were digitized by a line frequency of 166 Hz and a scanning mode format of 512 x 512 pixels and 256 gray levels (intensity resolution 8 bits). Vertical x-z sections were obtained across the specimen by scanning a single line at increments of 0.15 μm at different z-axis depths along one transverse x-axis. The image processing and the volume rendering were performed using ImageSpace software (Molecular Dynamics, Inc.) running on a Indigo workstation (Silicon Graphics, Mountain View, CA.

The colocalization of the fluorochromes was evaluated by comparing the equivalent pixel positions in each of the acquired images (optical sections), using LaserPix software (BioRad). Briefly, a two-dimensional scatter plot diagram of the individual pixels from the paired images was generated. Pixels with intensity values greater than 150 grey levels (on a scale from 0 to 255) were selected for both detectors, to calculate the colocalization binary maps that indicate regions containing highly colocalized signals. Overlap of the signals (in yellow) corresponds to the areas of colocalization. To evaluate the level of overlap of the signals, avoiding the typical limitations of fluorescence imaging, such as efficiency of immunoreaction, sample photobleaching and photomultiplier quantum efficiency, the coefficients k1 and k2[1] were calculated on acquired optical sections. These coefficients split the value of colocalization into the two separate parameters that depend on the sum of the products of the intensities of two channels, the coefficients k1 and k2 being sensitive to differences in the intensity of green and red signals, respectively. Relative quantification of colocalized fluorescent signals was performed on 30 cells using overlap coefficient k1 and was expressed as a percentage of the amount of green signal in the areas of colocalization as

[1] E. M. M. Manders, F. J. Verbeek and A. Aten, *J. Microsc*. 1993, **169,** 375.

previously described.[2] The statistical significance of the difference between the experimental points was evaluated by Student's t test.

[2] M. Riccio, M. Dembic, C. Cinti and S. Santi, *Humana, New York*, 2004, pp. 171–177.

Experimental Details

Synthesis of (4-aminobutyl)triphenylphosphonium amine (6). Commercially available 4-bromobutyl)triphenylphosphonium bromide (**5**) (100 mg, 0.252 mmol) was solubilized in 5 mL of methanol and NH_{3gas} was bubbled in the solution during 15 sec. The reaction was then left stirring at room temperature overnight. After evaporation of the solvent the desired product was obtained as a white solid and was employed in the following reactions without further purification: yield 82.5 mg (98%); retention time 7.4 min; ^{1}H NMR ($CDCl_3$) δ 1.60-1.80 (m, 2H), 2.10-2.20 (m, 2H), 3.43 (t, J = 6.1 Hz, 2H), 3.70-3.85 (m, 2H) and 7.50-7.80 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 19.0, 20.2, 35.8, 41.9, 118.0, 130.4, 131.2 and 135.0; mass spectrum (ESI), *m/z* $(M+H)^+$ 335.18 (calculated 335.18).

General procedure for the synthesis of compounds 7-10. To a vial containing 4.60 µmol of compounds **1-4**[3] was added a solution of 3.00 mg (8.98 µmol) of compound **6** in 100 µL of DMF, followed by 20 µL of Et_3N. The reaction mixture was stirred at room temperature. After 4 h a 5-µL aliquot of the reaction mixture was diluted with 100 µL 1:2 CH_3CN–H_2O, and was analyzed by HPLC using an analytic C_{18} reversed-phase column (250 x 4.6 mm). The column was washed with 20 → 100% acetonitrile in H_2O containing 0.1% of TFA, over a period of 30 min at a flow rate of 1 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 400 µL of 1:2 CH_3CN – H_2O, and purified using a semipreparative C_{18} reversed-phase column. After lyophilization of the appropriate fractions compounds **7-10** were obtained as bright yellow solids.

5''-Methylsulfanyl-[2,2';5',2'']tert-thiophene-5-(4-triphenylphosphonium)butylamide (7). Yield 2.20 mg (73%); retention time 27.0 min; ^{1}H NMR (CD_3OD) δ 1.60-1.70 (m, 2H), 1.75-1.85 (m, 2H), 2.46 (s, 3H), 3.35-3.45 (m, 4H) 6.93 (d, *J* = 3.5 Hz, 1H), 7.05 (d, *J* = 3.5 Hz, 1H), 7.08 (s, *J* = 3.5 Hz, 1H), 7.14 (d, *J* = 3.5 Hz, 1H), 7.19 (d, *J* = 4 Hz, 1H), 7.39 (d, *J* = 4 Hz, 1H), 7.60-7.65 (m, 6H), 7.68-7.73 (m, 6H) and 7.75-7.80 (m, 3H); ^{13}C NMR (CD_3OD) δ 20.8, 22.3, 31.7, 31.8, 39.3, 119.9, 120.6, 125.6, 126.0, 126.2, 127.5, 131.9, 132.0, 135.2, 135.3, 136.7, 138.7, 138.9, 139.4, 140.0, 143.3, 147.2, 153.5, 160.6, 160.7, 164.5 and 166.2; mass spectrum (ESI), *m/z* 654.11957 $(M)^+$ ($C_{36}H_{33}NOPS_4$ requires 654.11771).

3''-Methylsulfanyl-[2,2';5',2'']tert-thiophene-5-(4-triphenylphosphonium)butylamide (8). Yield 1.90 mg (63%); retention time 25.1 min; ^{1}H NMR (CD_3OD) δ 1.55-1.65 (m, 2H),

[3] M. Zambianchi, A. Barbieri, A. Ventola, L. Favaretto, C. Bettini,M. Galeotti, G. Barbarella† *Bioconj. Chem.*, 2007, **18**, 1004.

1.75-1.80 (m, 2H), 2.50 (s, 3H), 3.33-3.43 (m, 4H) 6.95 (d, J = 3.5 Hz, 1H), 7.09 (d, J = 4.0 Hz, 1H), 7.13 (d, J = 4.0 Hz, 1H), 7.41 (d, J = 4.0 Hz, 1H), 7.58-7.65 (m, 2H), and 7.67-7.85 (m, 15H); ^{13}C NMR (CD_3OD) δ 20.9, 23.4, 29.9, 34.5, 38.1, 119.3, 120.6, 124.3, 125.7, 129.7, 130.8, 130.9, 131.7, 134.0, 134.1, 135.5, 137.5, 138.3, 139.3, 142.1, 147.2, 148.0, 153.4, 157.2, 159.0, 161.0, 164.5, 166.2; mass spectrum (ESI), *m/z* 654.11926 $(M+H)^+$ ($C_{36}H_{33}NOPS_4$ requires 654.11771).

5'-Methylsulfanyl-[2,2']bithiophenyl-5-(4-triphenylphosphonium)butylamide (9). Yield 2.10 mg (80%); retention time 22.4 min; ^{1}H NMR (CD_3OD) δ 1.62-1.72 (m, 2H), 1.75-1.85 (m, 2H), 2.42 (s, 3H), 3.35-3.45 (m, 4H), 7.06 (d, J = 5.0 Hz, 1H), 7.18 (d, J = 4.0 Hz, 1H), 7.33 (d, J = 5.0 Hz, 1H), 7.43 (d, J = 4.0 Hz, 1H) and 7.60-7.80 (m, 15H); ^{13}C NMR (CD_3OD) δ 20.8, 23.1, 26.7, 31.1, 39.3, 114.8, 117.4, 119.5, 119.7, 120.4, 121.8, 125.5, 126.0, 126.5, 128.2, 131.0, 131.9, 132.0, 132.1, 135.2, 135.3, 136.7, 136.8, 145.4, 147.8, 153.2, 156.7, 159.3, 162.3, 165.8, 167.3; mass spectrum (ESI), *m/z* 572.13135 $(M)^+$ ($C_{32}H_{31}NOPS_3$ requires 572.12999).

5'''-Methylsulfanyl-[2,2';5',2'';5'',2''']quaterthiophene-5-(4-triphenylphosphonium)butylamide (10). Yield 3.19 mg (75%); retention time 32.9 min; ^{1}H NMR (CD_3OD) δ 1.65-1.75 (m, 2H), 1.80-1.90 (m, 2H), 2.46 (s, 3H), 2.85-2.95 (m, 2H), 3.35-3.45 (m, 2H), 6.97 (d, J = 3.5 Hz, 1H), 7.08 (d, J = 5 Hz, 1H), 7.10 (d, J = 5 Hz, 1H), 7.17 (d, J = 3.5 Hz, 1H), 7.20 (d, J = 5 Hz, 1H), 7.31 (d, J = 5 Hz, 1H), 7.45 (d, J = 5 Hz, 1H), 7.51 (d, J = 5 Hz, 1H) and 7.60-7.85 (m, 15H); ^{13}C NMR (CD_3OD) δ 20.0, 21.5, 21.9, 39.1, 118.5, 119.2, 125.9, 128.0, 130.8, 130.9, 134.0, 134.1, 135.7, 147.3, 151.1, 152.9, 158.4, 160.6, 164.5, 168.2 and 172.0; mass spectrum (ESI), *m/z* $(M)^+$ 736.10547 ($C_{40}H_{35}NOPS_5$ requires 736.10543).

[2,2';5',2'']tert-thiophene-5-(4-triphenylphosphonium)butylamide (13). Yield 4.8 mg (72%); retention time min 27.3 min; ^{1}H NMR (CD_3OD) δ 1.65-1.75 (m, 2H), 1.80-1.85 (m, 2H), 2.29 (t, J = 8.0 Hz, 2H), 2.68 (t, J = 7.0 Hz, 2H), 2.80 (t, J = 7.0 Hz, 2H), 2.89 (t, J = 7.0 Hz, 2H), 3.38 (t, J = 7.0 Hz, 2H), 4.16 (t, J = 6.5 Hz, 2H), 6.71 (d, J = 3.5 Hz, 1H), 6.95-7.00 (m, 3H), 7.02 (d, J = 3.5 Hz, 1H), 7.13 (d, J = 3.5 Hz, 1H), 7.26 (d, J = 5.5 Hz, 1H), 7.65-7.75 (m, 12H) and 7.78-7.85 (m, 3H); ^{13}C NMR (CD_3OD) δ 19.0, 25.7, 30.2, 32.1, 38.8, 39.0, 43.5, 51.2, 119.8, 120.5, 124.7, 124.9,125.0, 125.2, 125.3, 125.8, 126.2, 126.8, 127.9, 129.5, 131.9, 132.0, 134.4, 135.2, 135.3, 136.7, 137.3, 137.4, 138.0, 138.5, 159.0, 160.5, 163.2,

163.6, 169.2, 169.5, 177.6, 178.1; mass spectrum (ESI), *m/z* $(M)^+$ 744.13128 ($C_{39}H_{39}NO_2PS_5$ requires 744.13165).

^{1}H NMR and ^{13}C NMR Data

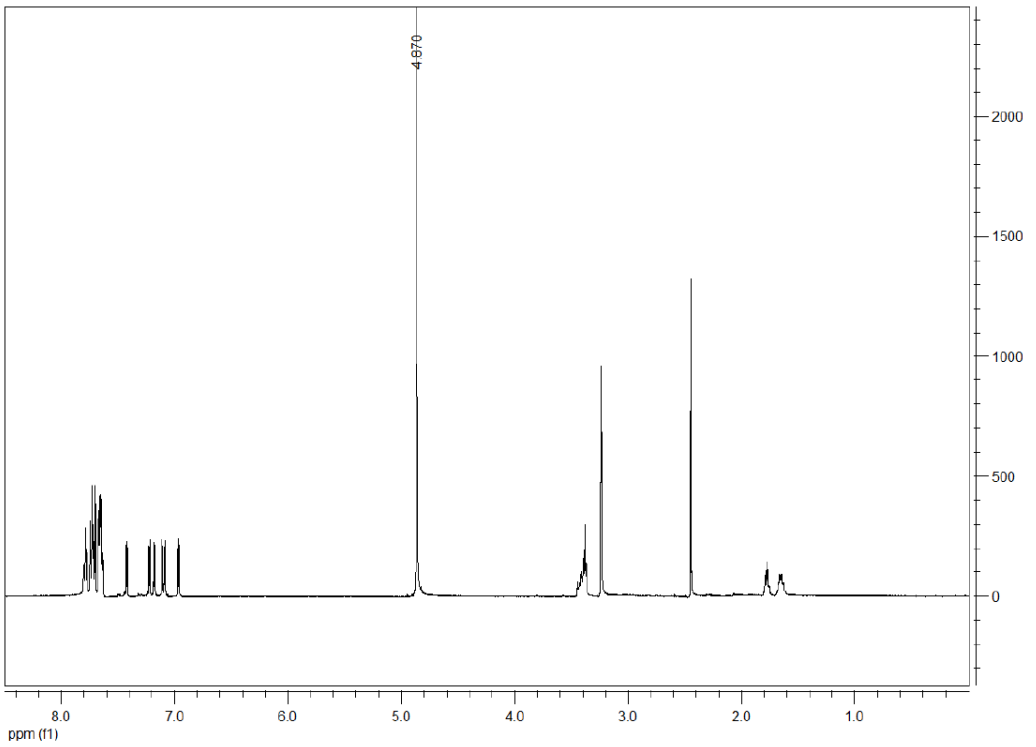

Figure S4. ^{1}H NMR of compound **7** in CD_3OD.

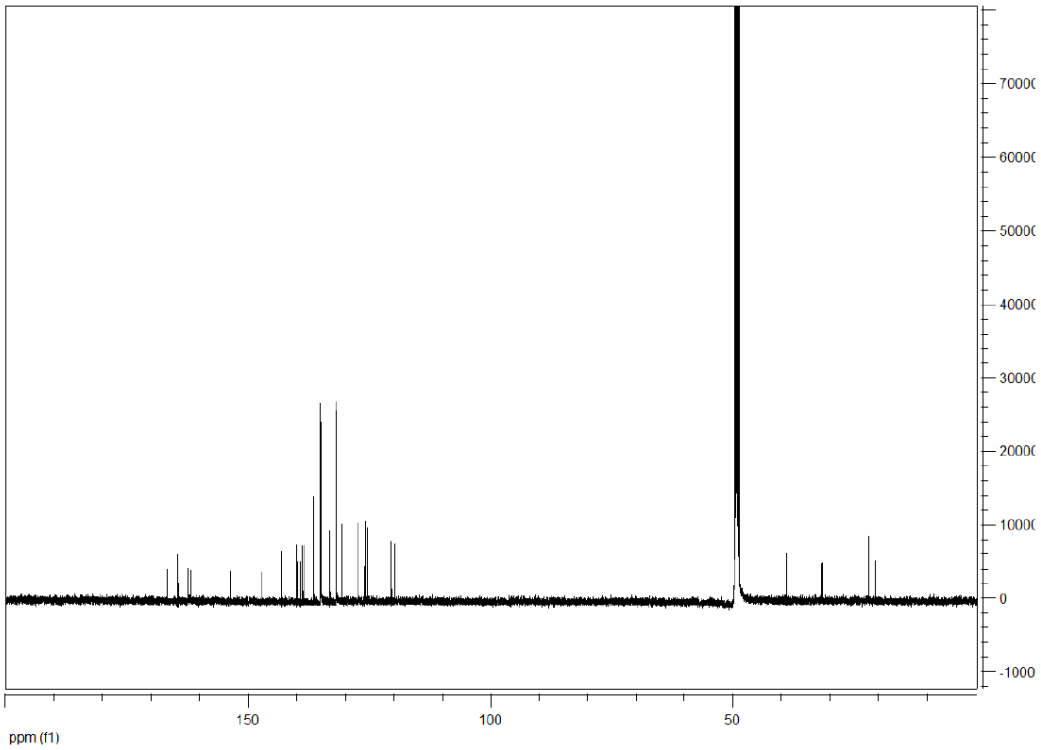

Figure S5. ^{13}C NMR of compound **7** in CD_3OD

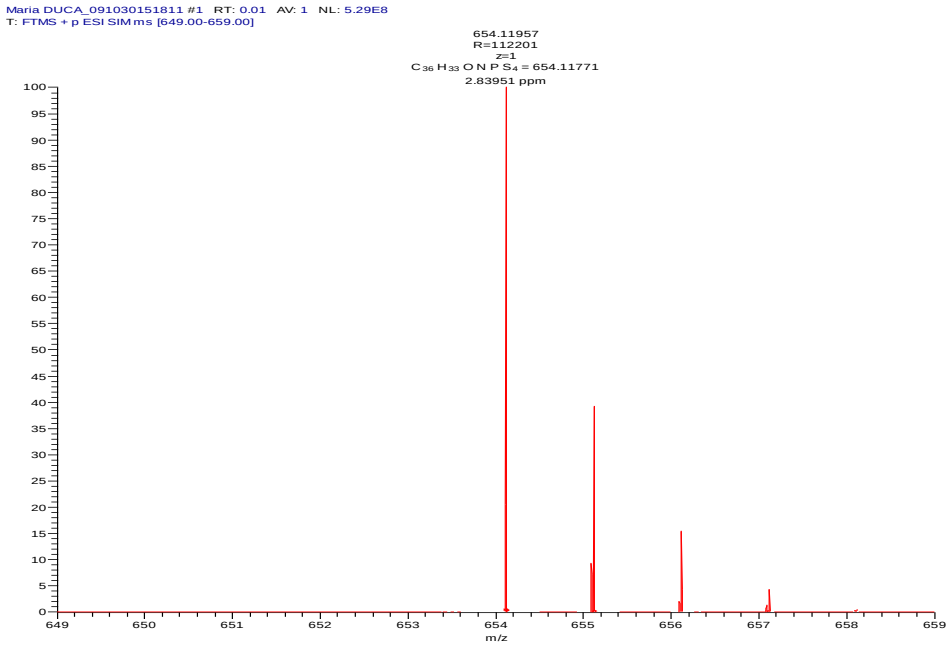

Figure S6. HRMS (ESI) spectra of compound **7**.

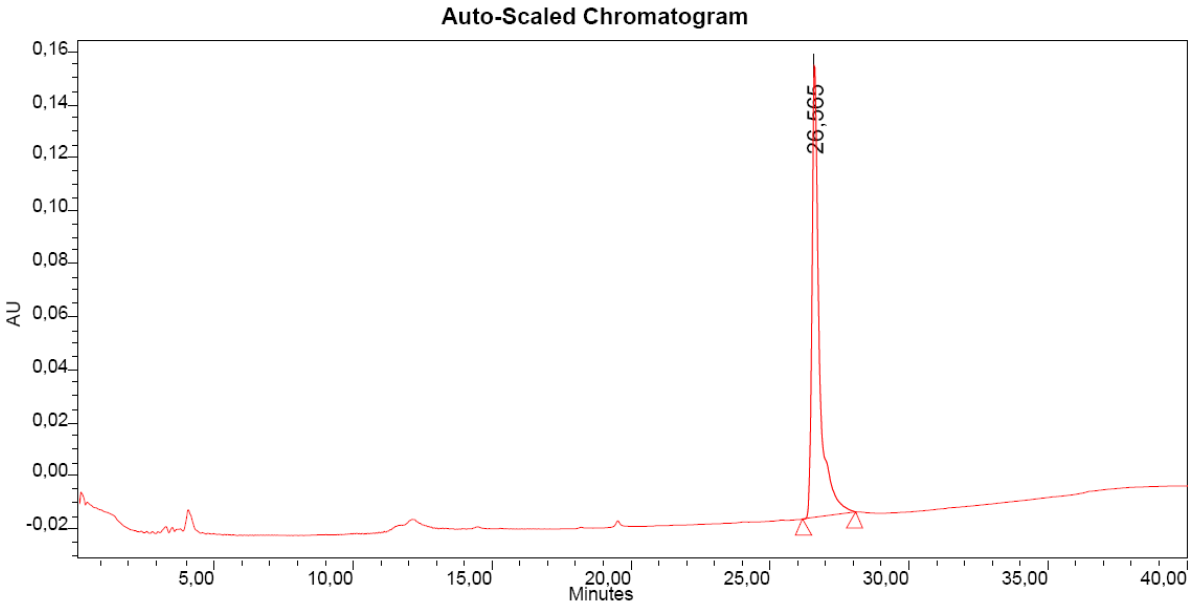

Figure S7. HPLC trace at 254 nm of the purified compound **7**.

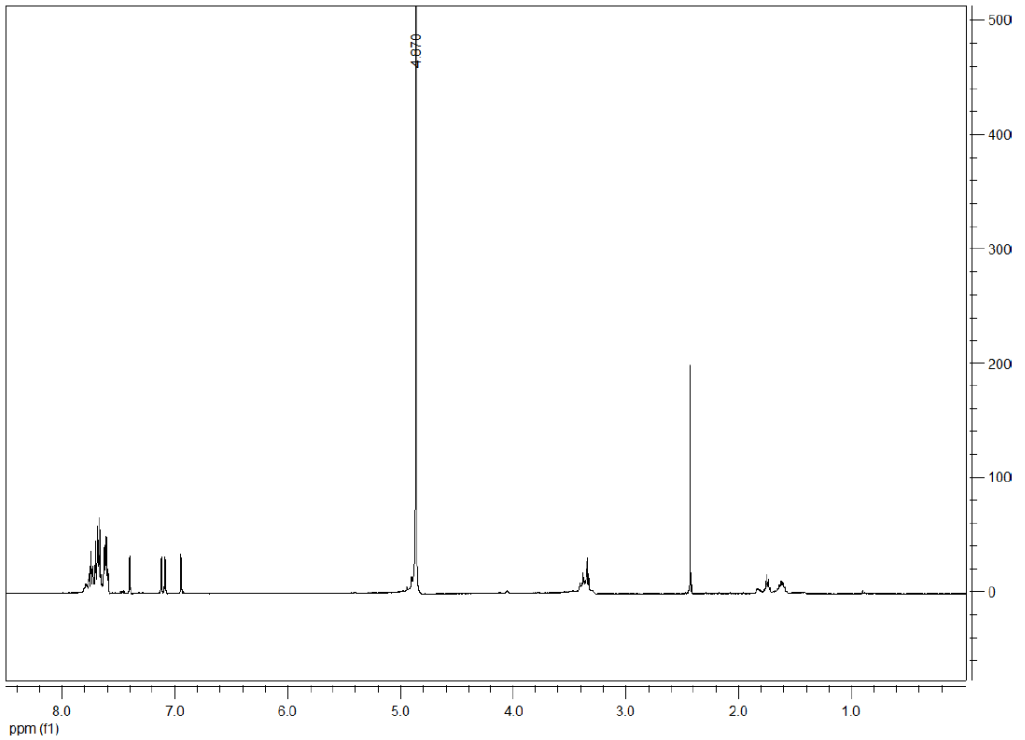

Figure S8. ^{1}H NMR of compound **8** in CD_3OD.

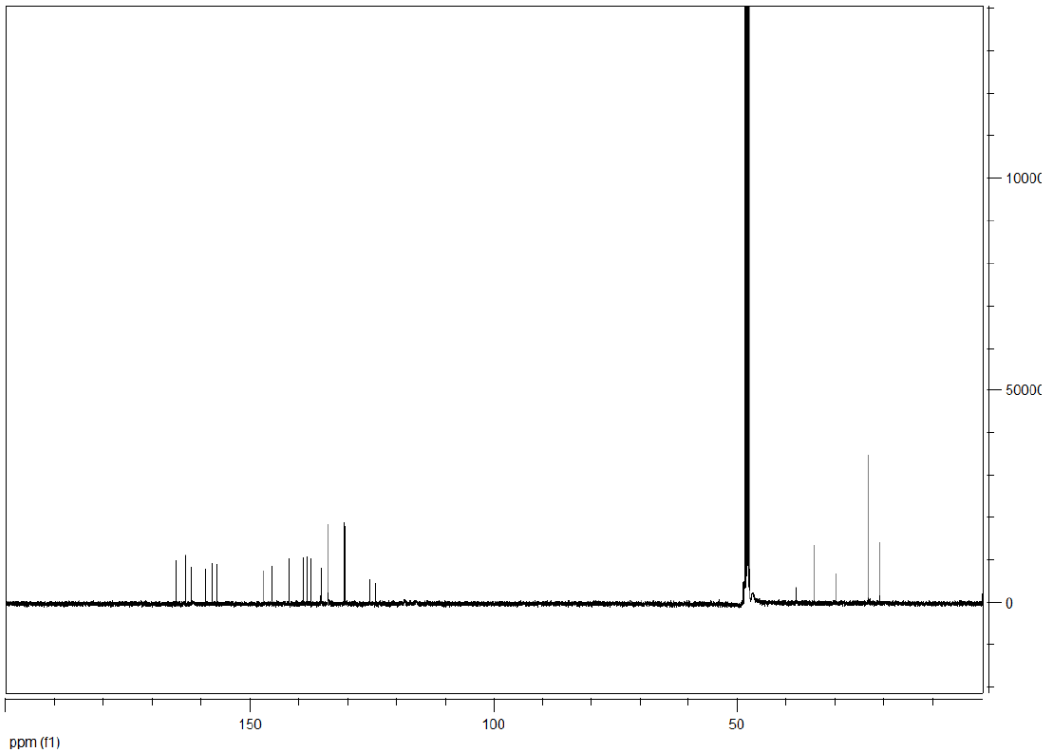

Figure S9. ^{13}C NMR of compound **8** in CD_3OD.

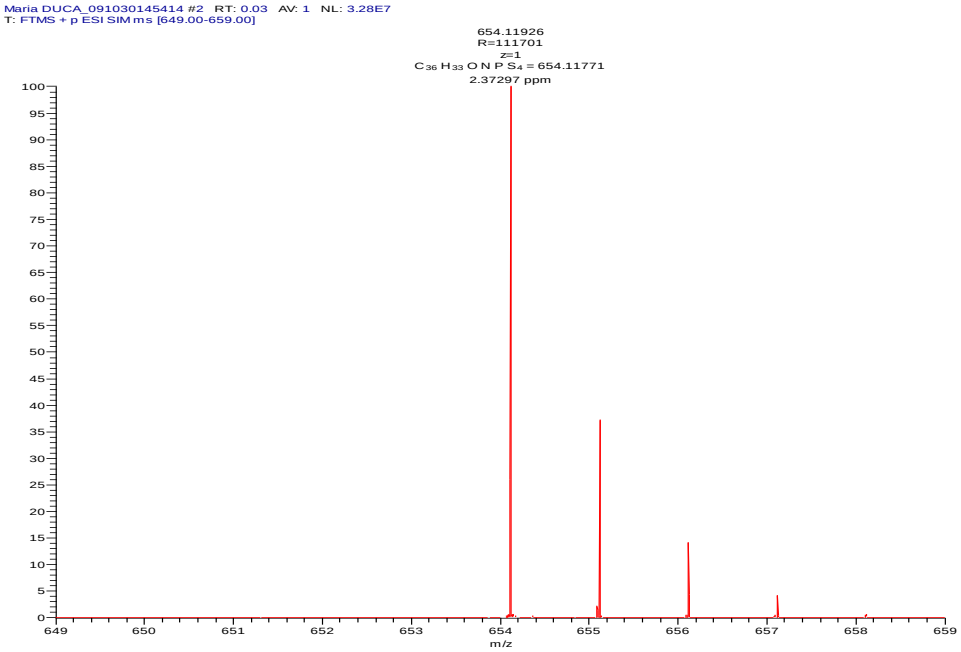

Figure S10. HRMS (ESI) spectra of compound **8**.

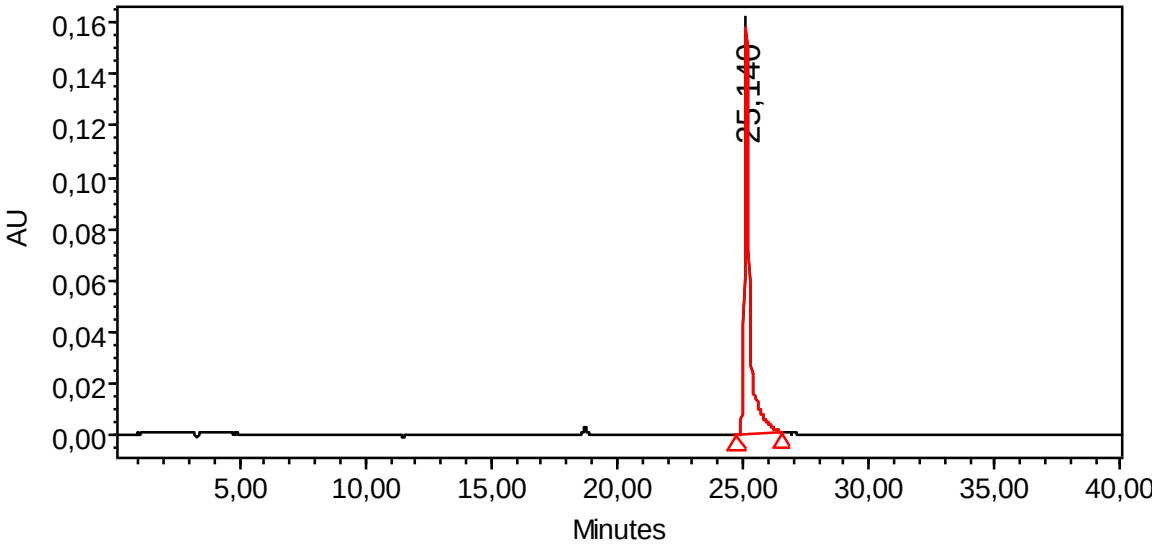

Figure S11. HPLC trace at 254 nm of the purified compound **8**.

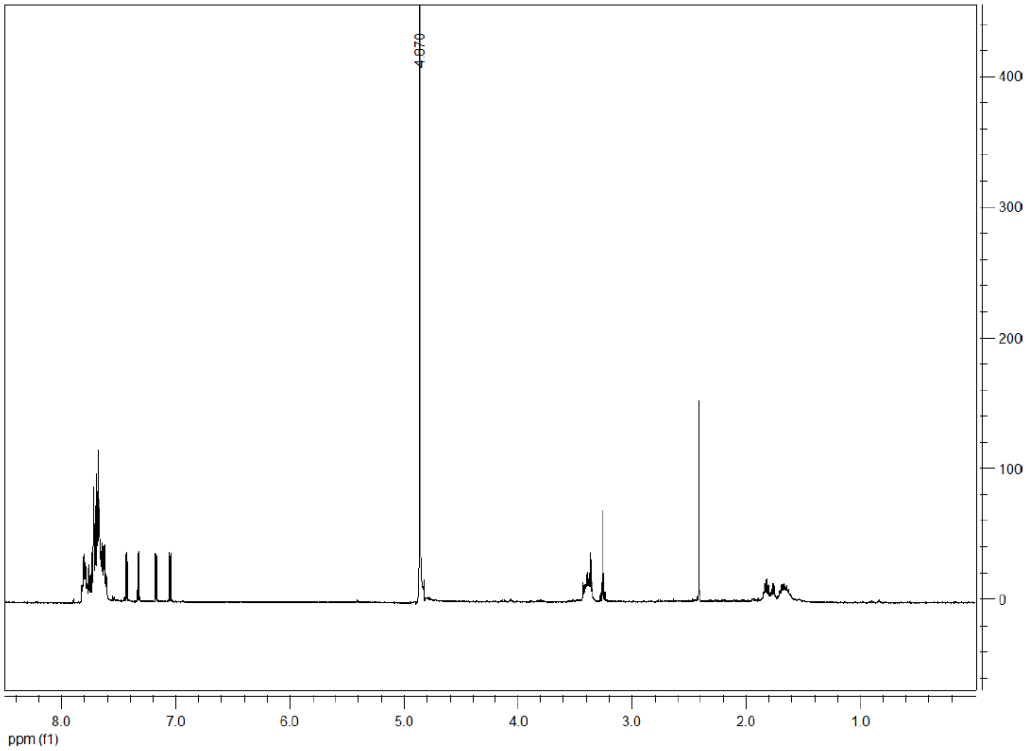

Figure S12. ^{1}H NMR of compound **9** in CD_3OD.

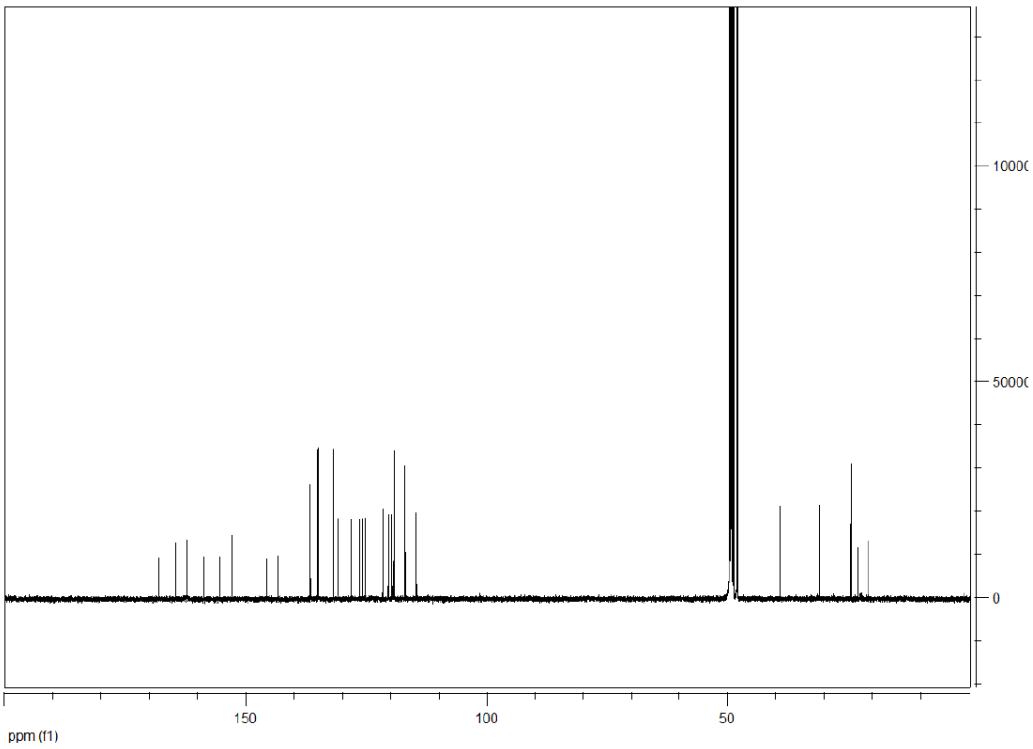

Figure S13. ^{13}C NMR of compound **9** in CD_3OD.

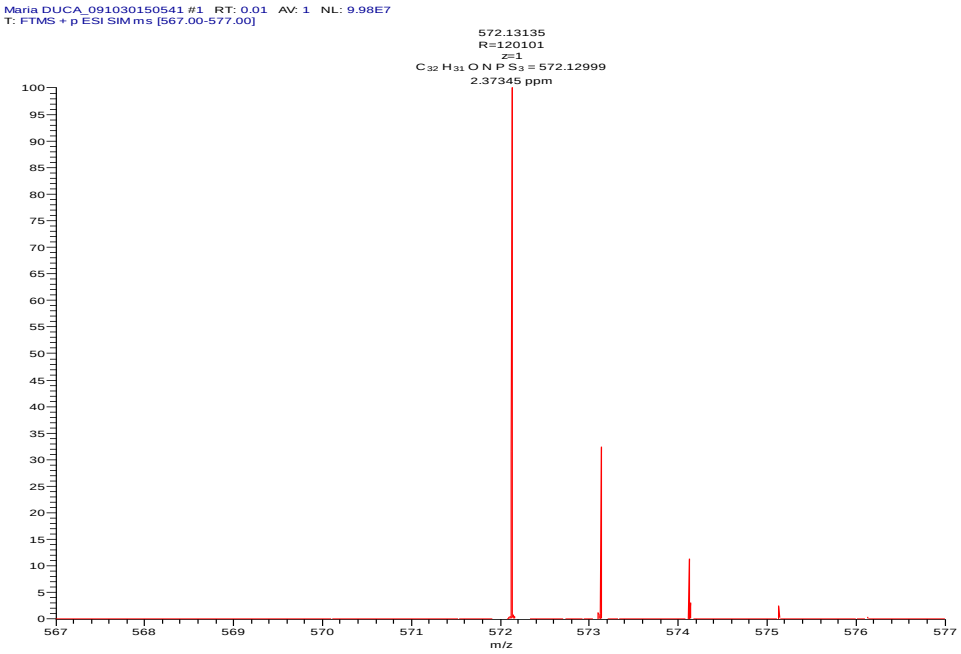

Figure S14. HRMS (ESI) spectra of compound **9**.

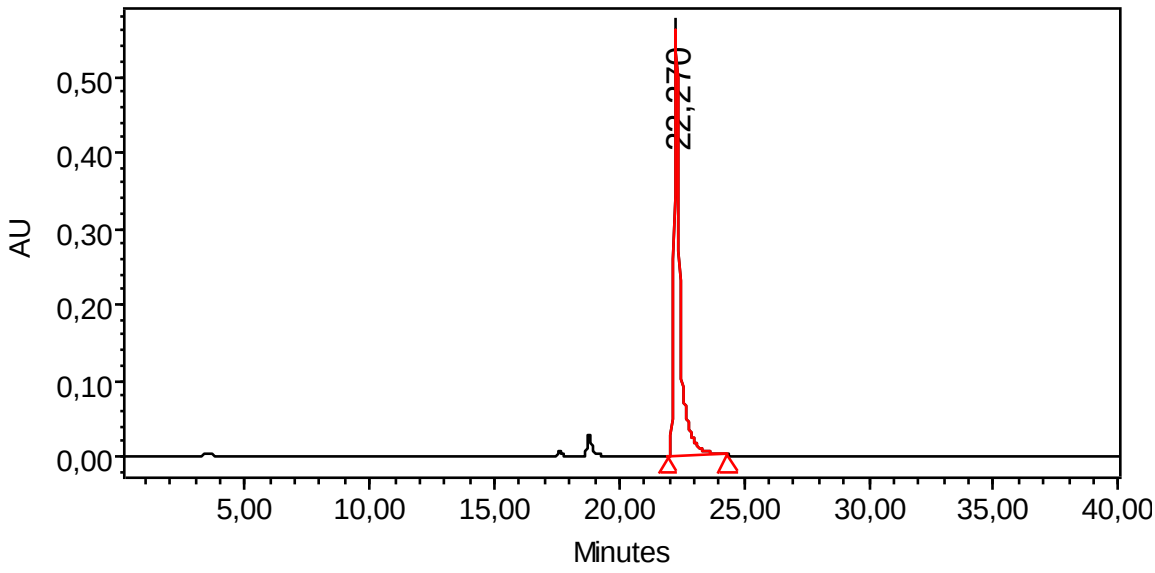

Figure S15. HPLC trace at 254 nm of purified compound **9**.

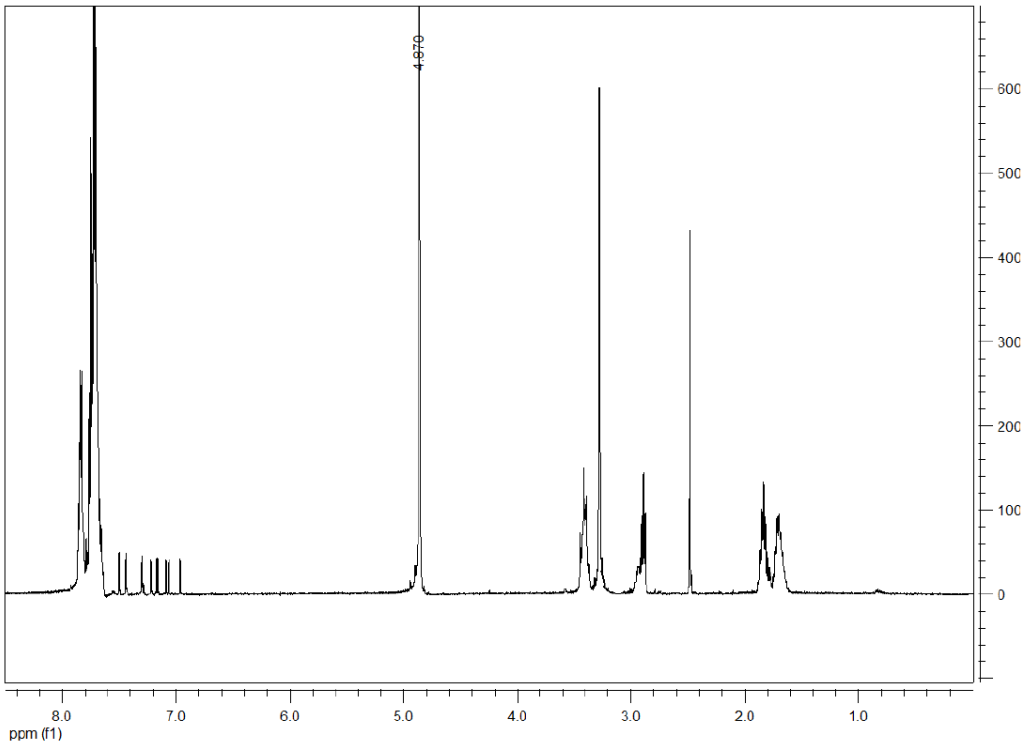

Figure S16. ^{1}H NMR of compound **10** in CD_3OD.

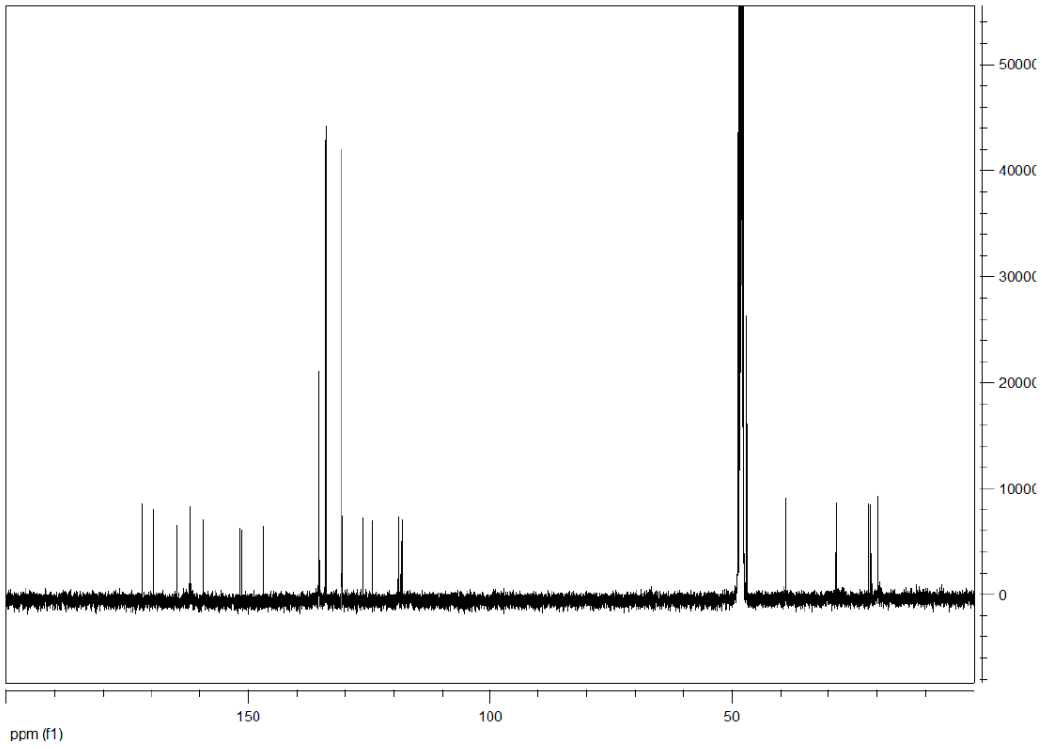

Figure S17. ^{13}C NMR of compound **10** in CD_3OD.

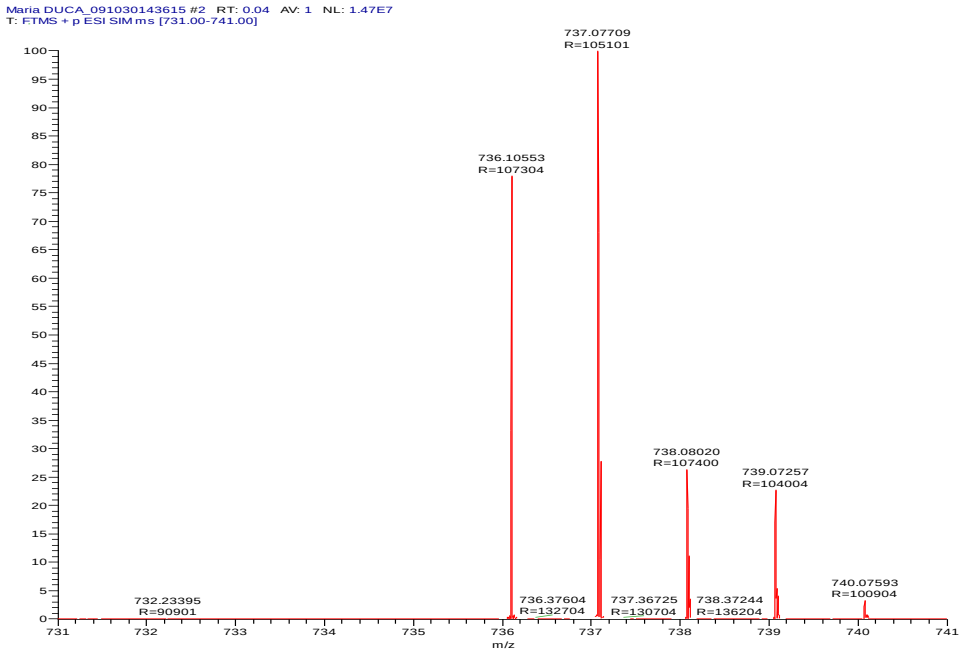

Figure S18. HRMS (ESI) spectra of compound **10**.

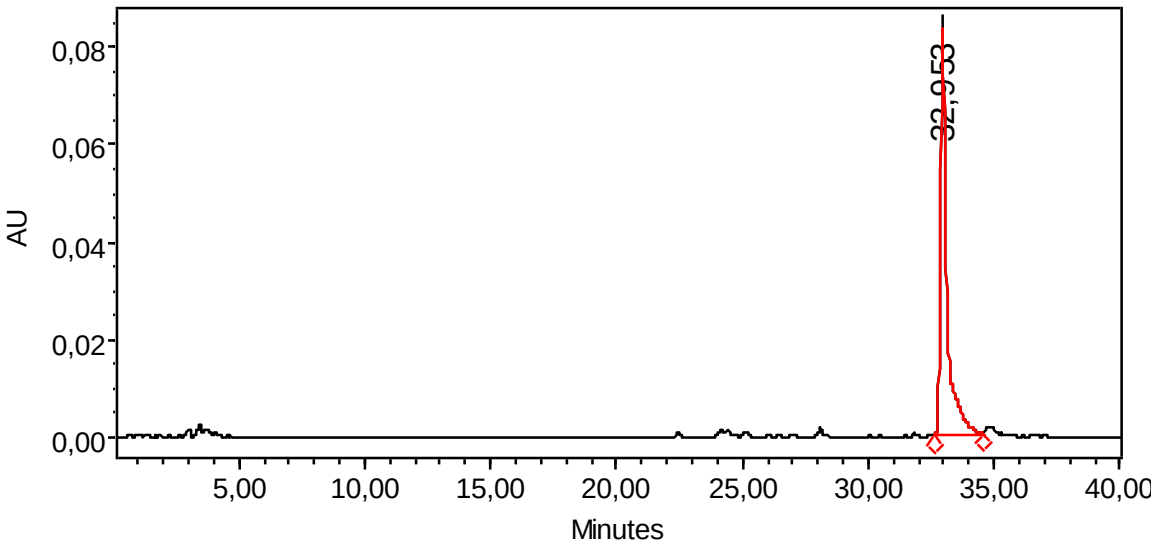

Figure S19. HPLC trace at 254 nm of purified compound **10**.

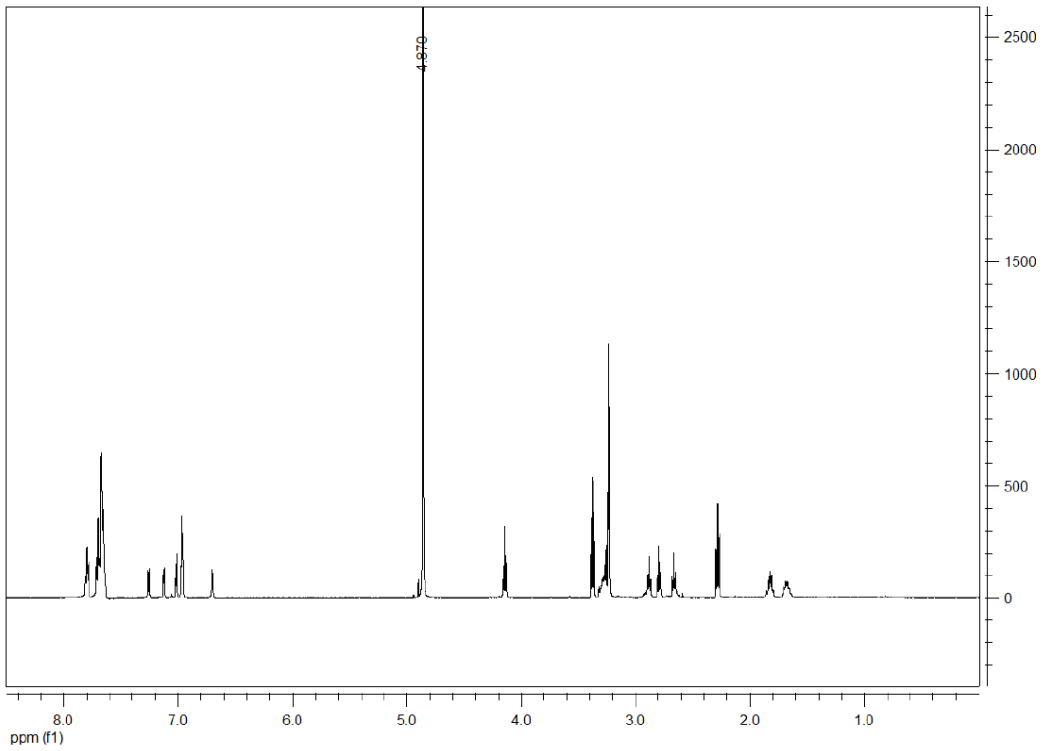

Figure S20. ^{1}H NMR of compound **13** in CD_3OD.

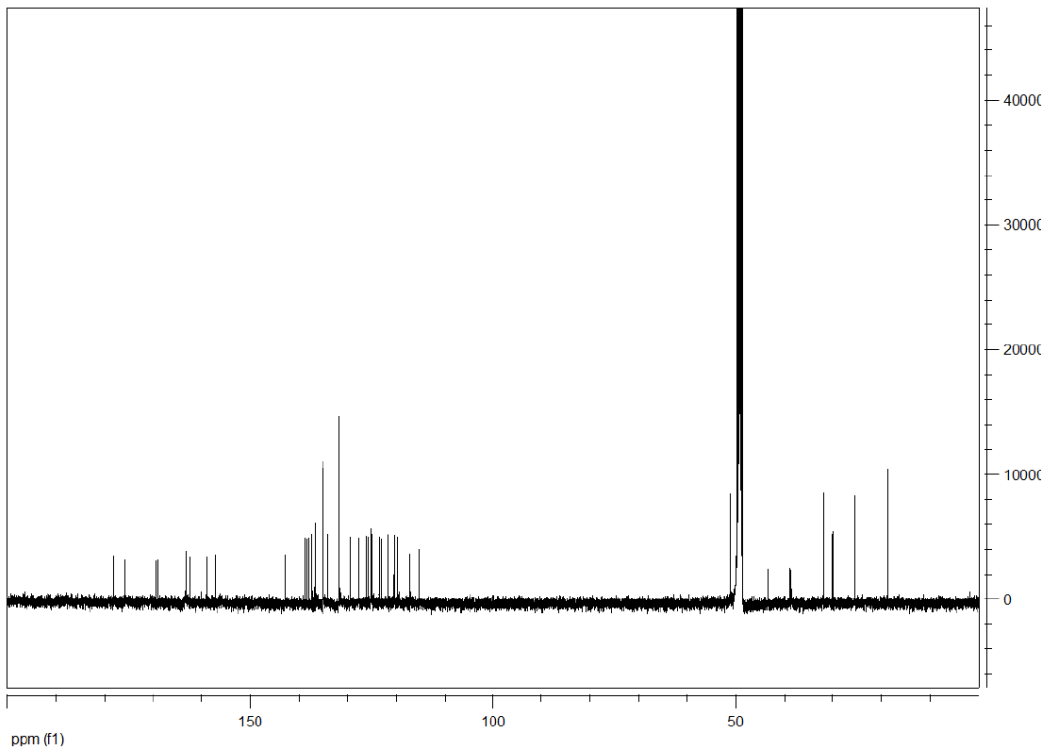

Figure S21. ^{13}C NMR of compound **13** in CD_3OD.

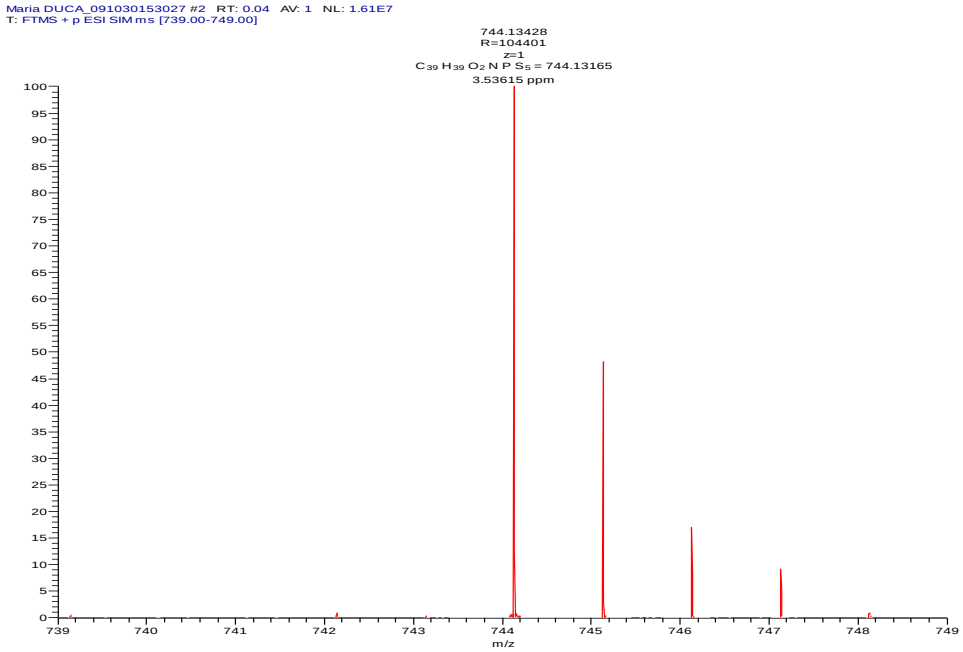

Figure S22. HRMS (ESI) spectra of compound **13**.

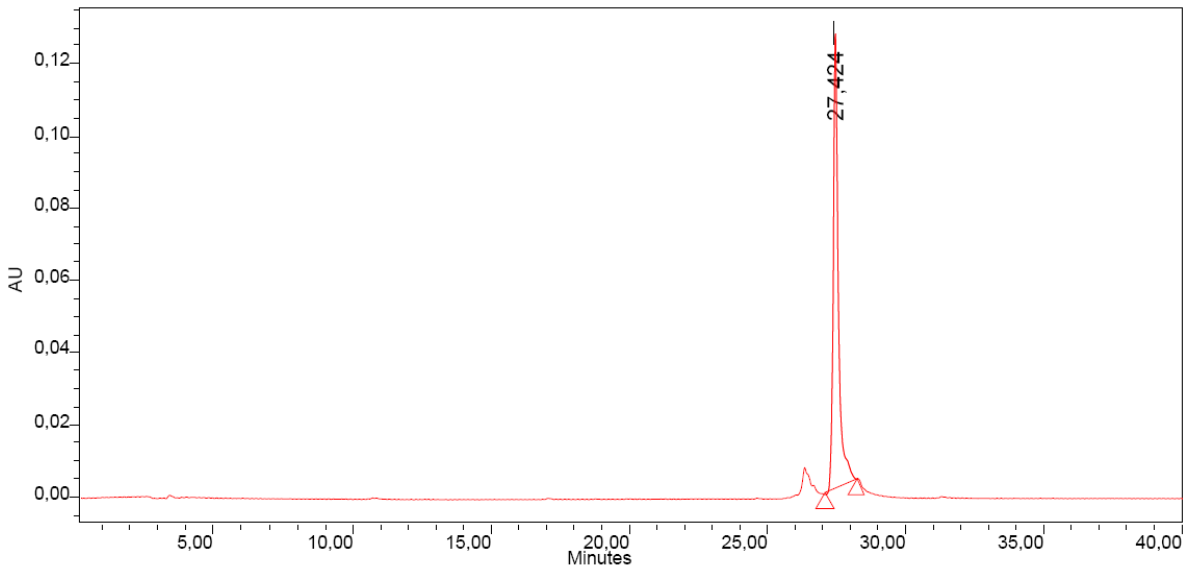

Figure S23. HPLC trace at 254 of purified compound **13**.

Supplementary Data

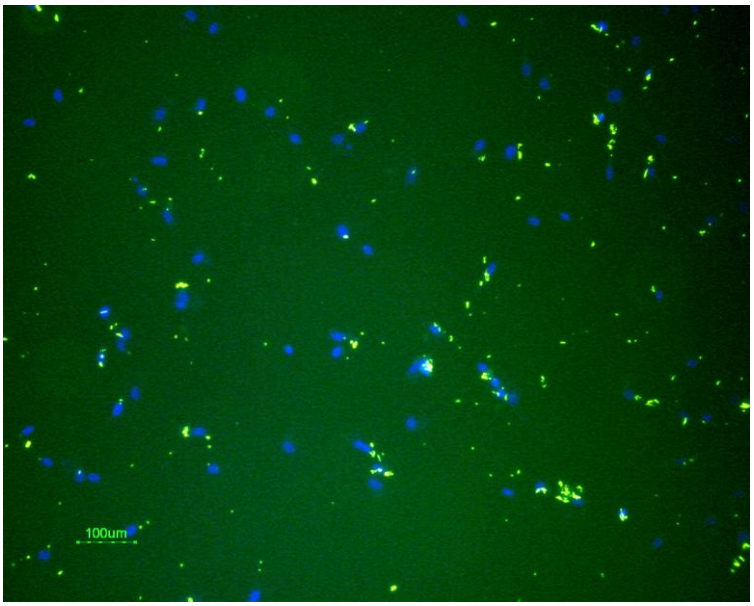

Figure S1. Living human mesenchymal stem cells (hMSCs) treated with 0,05 mg/ml compound **1** (green color) for 60 minutes at 37 °C and with Hoechst 33342 for 10 minutes to detect nuclei (blu color). Merged image is shown.

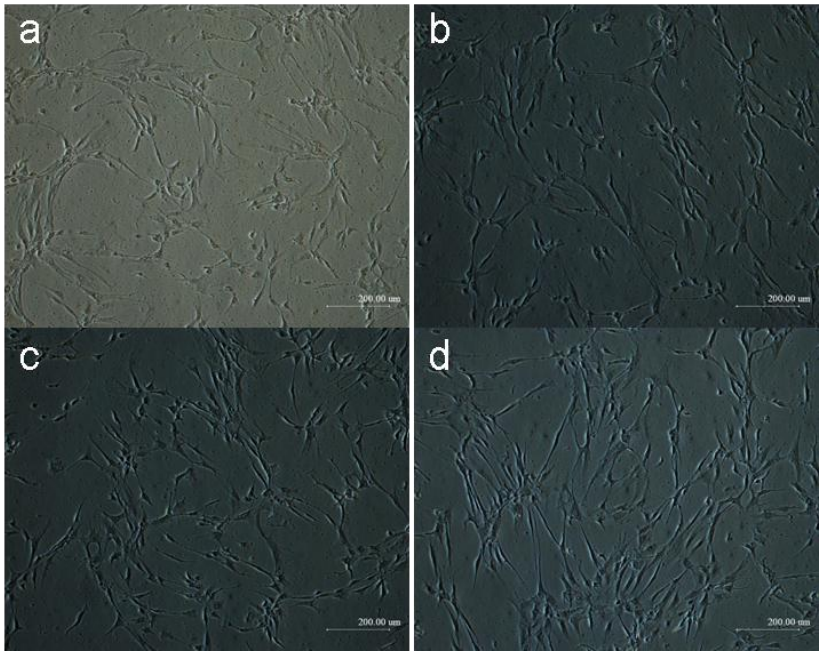

Figure S2. Phase-contrast microscopy control images of living hMSCs labelled with compound **7** (0.1 mg/mL) as reported in figure 2. (a) hMSCs immediately after 60 minute-incubation. After the removal of extracellular conjugates, TF-labeled hMSCs were continuously cultured and monitored at 24 (b), 48 (c) and 72 hours (d).

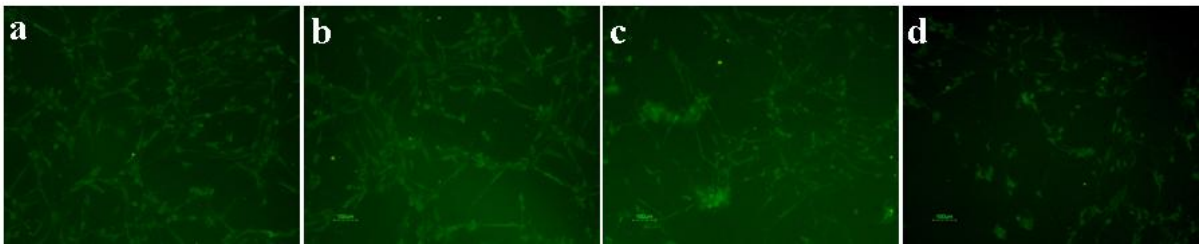

Figure S3. Living human mesenchymal stem cells (hMSCs) labeled with compound **13** (0.2 mg/mL). (a) hMSCs immediately after 60 minute-TF incubation. Following the removal of extracellular conjugates, TF-labeled hMSCs were continuously cultured and monitored for 24 hours (b), 48 hours (c), and 72 hours (d).