SUPPLEMENTARY MATERIAL

α-Glucosidase Inhibitors Based on Oleanolic Acid for the Treatment of Immunometabolic Disorders

Anastasiya V. Petrova [1], Denis A. Babkov [2,*], Elmira F. Khusnutdinova [1], Irina P. Baikova [1], Oxana B. Kazakova [1,*], Elena V. Sokolova [2] and Alexander A. Spasov [2]

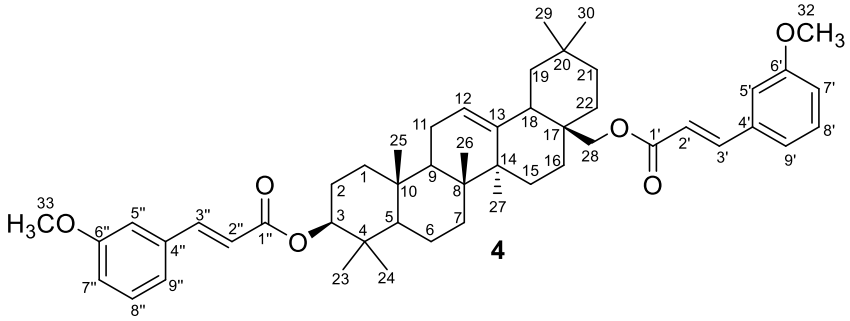
[1] Ufa Institute of Chemistry, Ufa Federal Research Centre, Russian Academy of Science, 71 Pr., Oktyabrya Ufa 450054, Russia; ana.orgchem@gmail.com (A.V.P.); elmah@inbox.ru (E.F.K.); poljr@anrb.ru (I.P.B.)

[2] Scientific Center for Innovative Drugs, Volgograd State Medical University, 39, Novorossiyskaya st., Volgograd 400087, Russia; sokolova210795@gmail.com (E.V.S.); aspasov@mail.ru (A.A.S.)

* Correspondence: denis.a.babkov@gmail.com (D.A.B.); obf@anrb.ru (O.B.K.)

Table of contents

S1. NMR, IR, and mass spectra of compounds **4**, **5**, **14**, **17**, **23**, and **26**



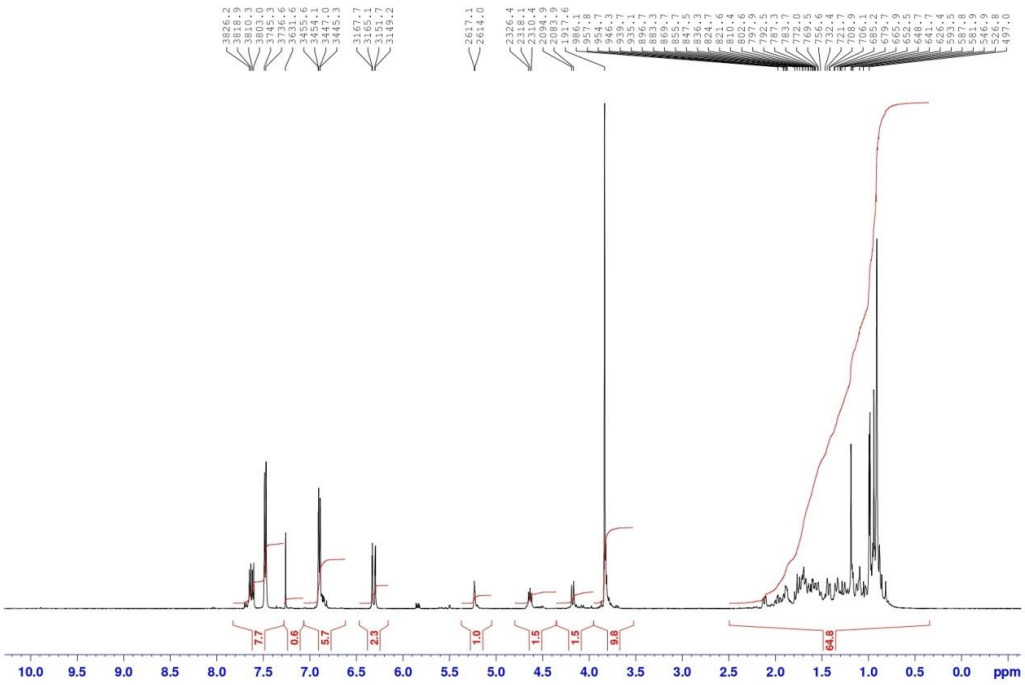


Figure S1 ^{1}H NMR spectrum of compound **4**

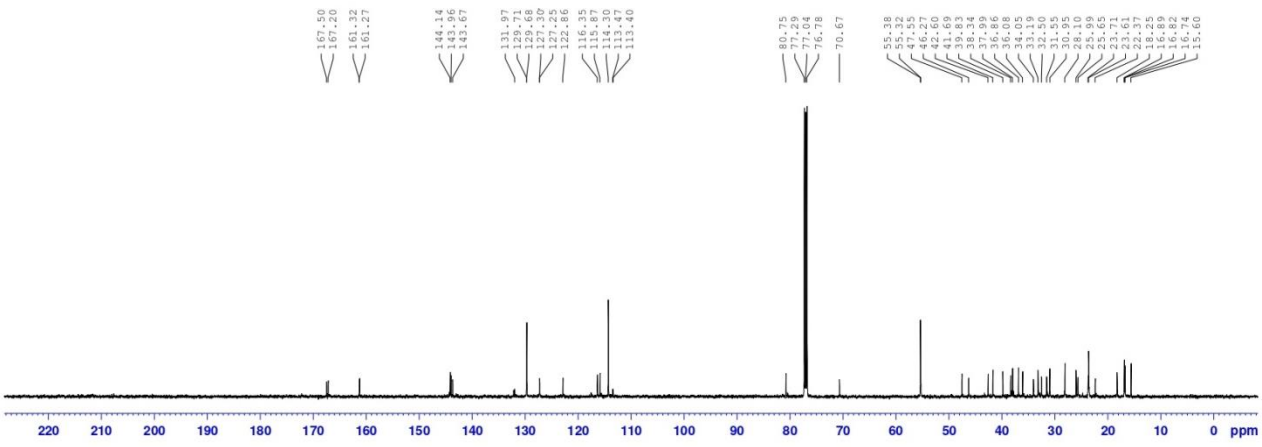


Figure S2. ^{13}C NMR spectrum of compound **4**

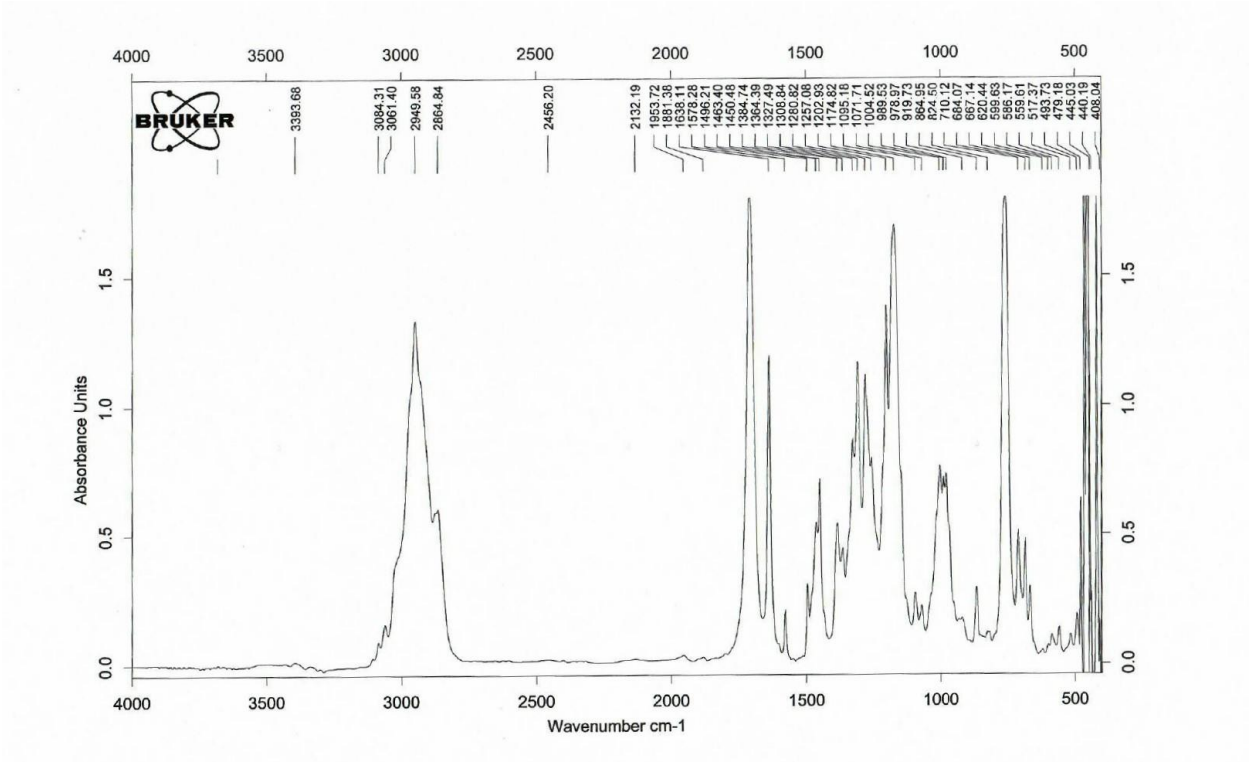


Figure S3. IR spectrum of compound **4**

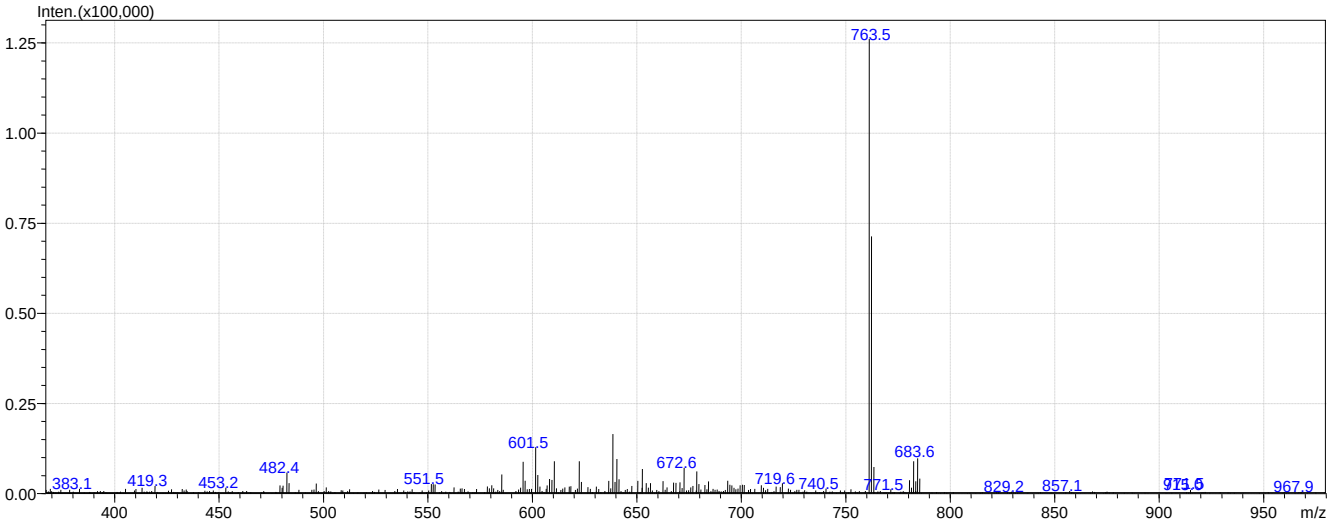


Figure S4. Mass spectrum of compound **4**

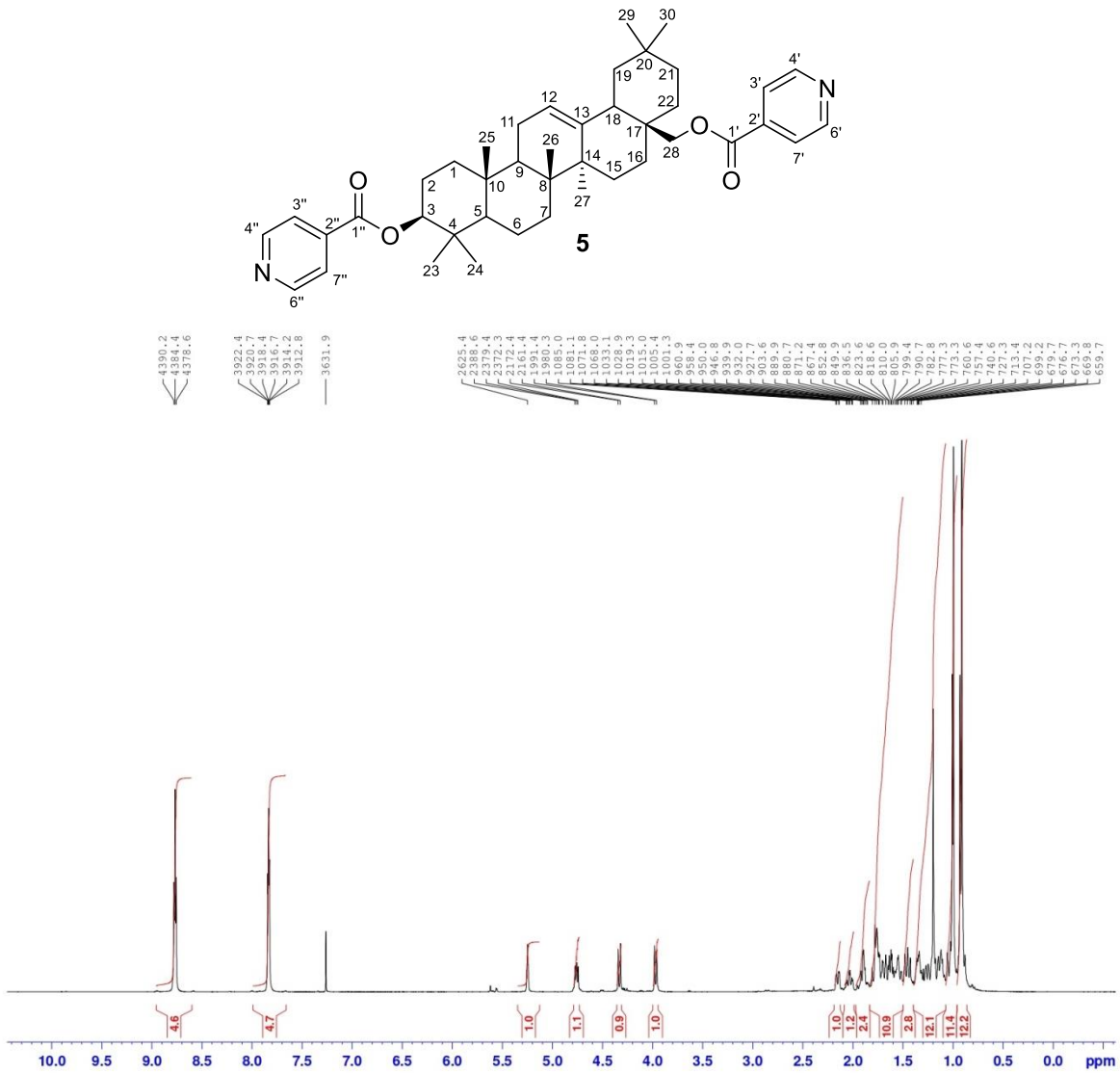


Figure S5. ^{1}H NMR spectrum of compound **5**

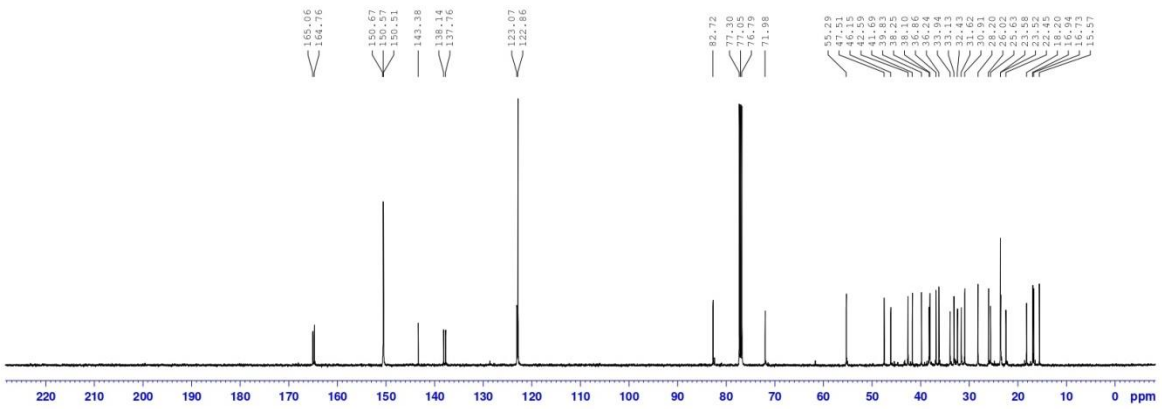


Figure S6. ^{13}C NMR spectrum of compound **5**

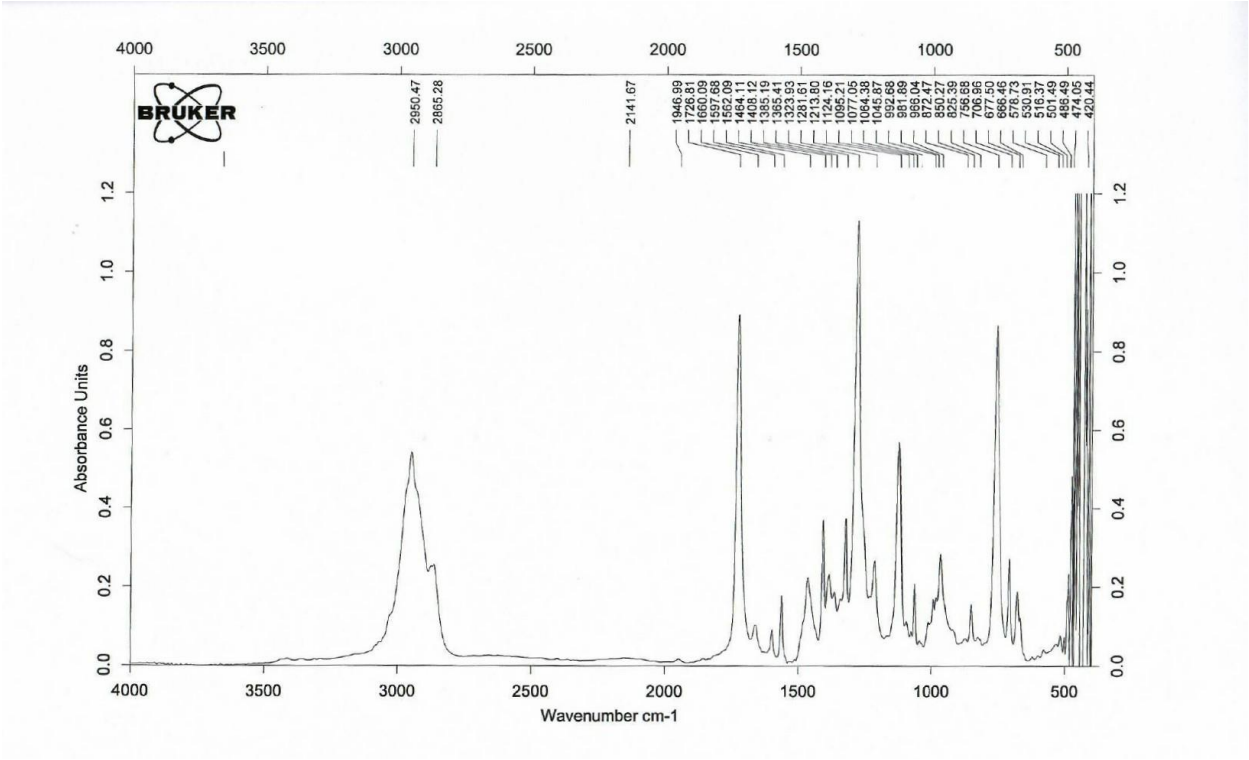


Figure S7 IR spectrum of compound **5**

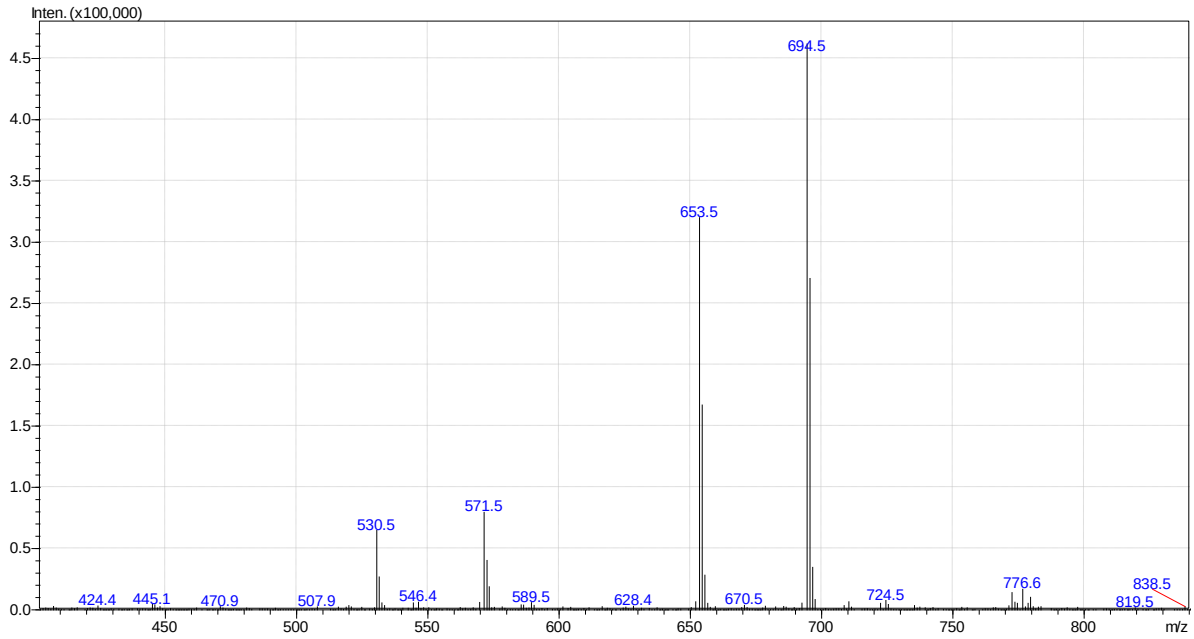
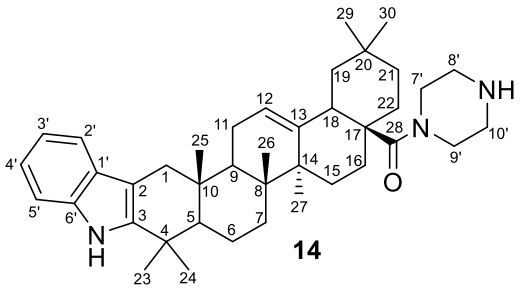


Figure S8. Mass spectrum of compound **5**



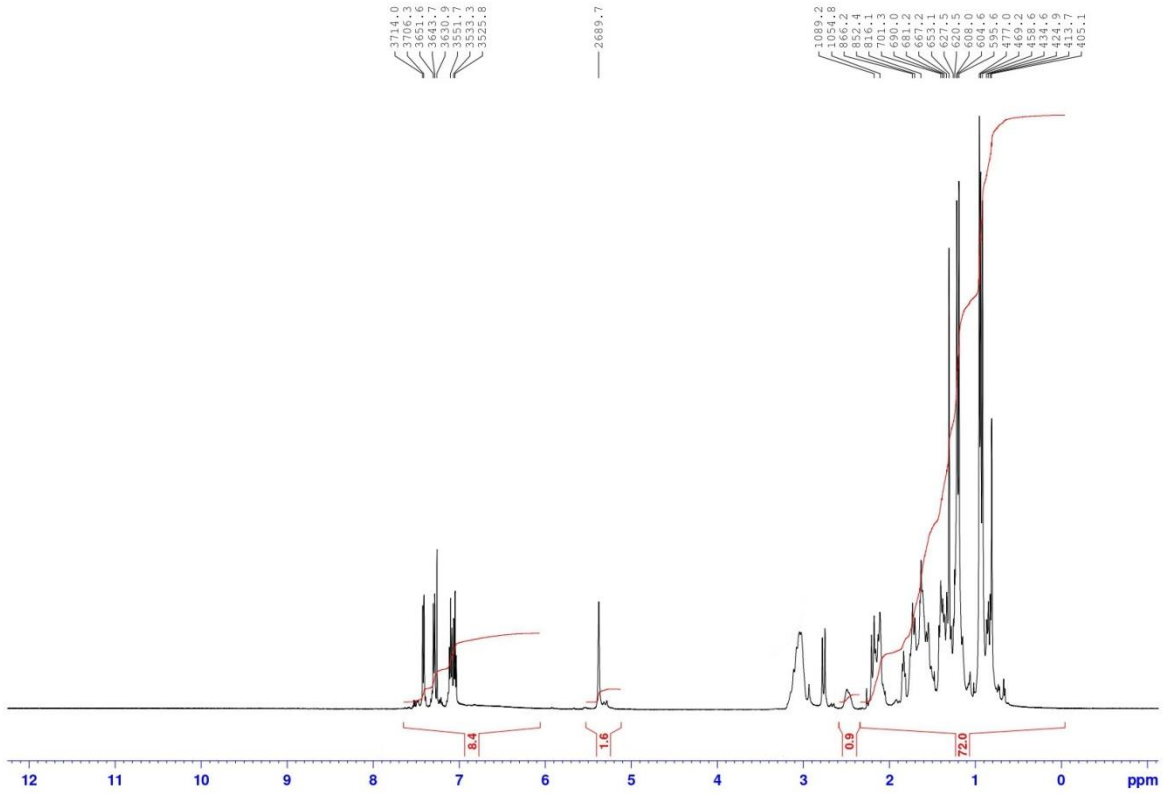


Figure S9. ^{1}H NMR spectrum of compound **14**

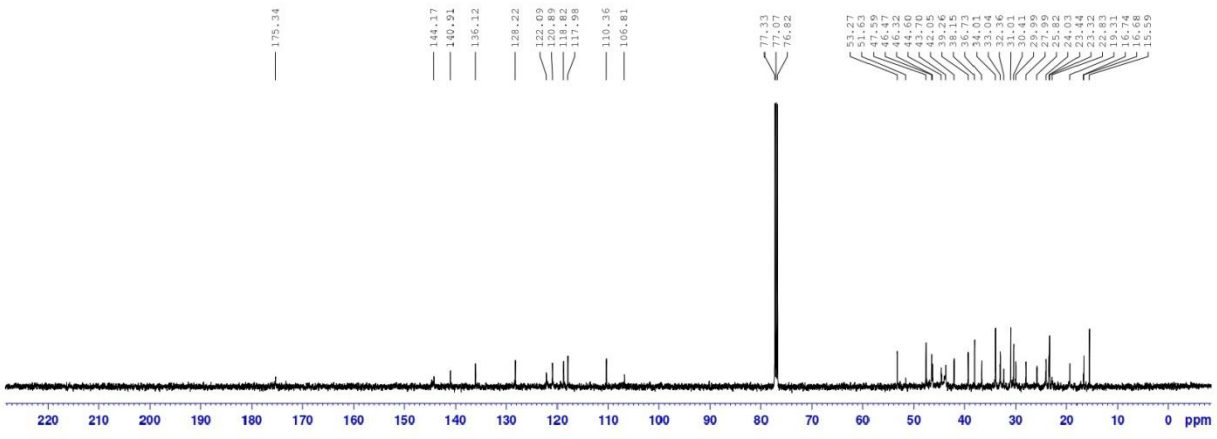


Figure S10. ^{13}C NMR spectrum of compound **14**

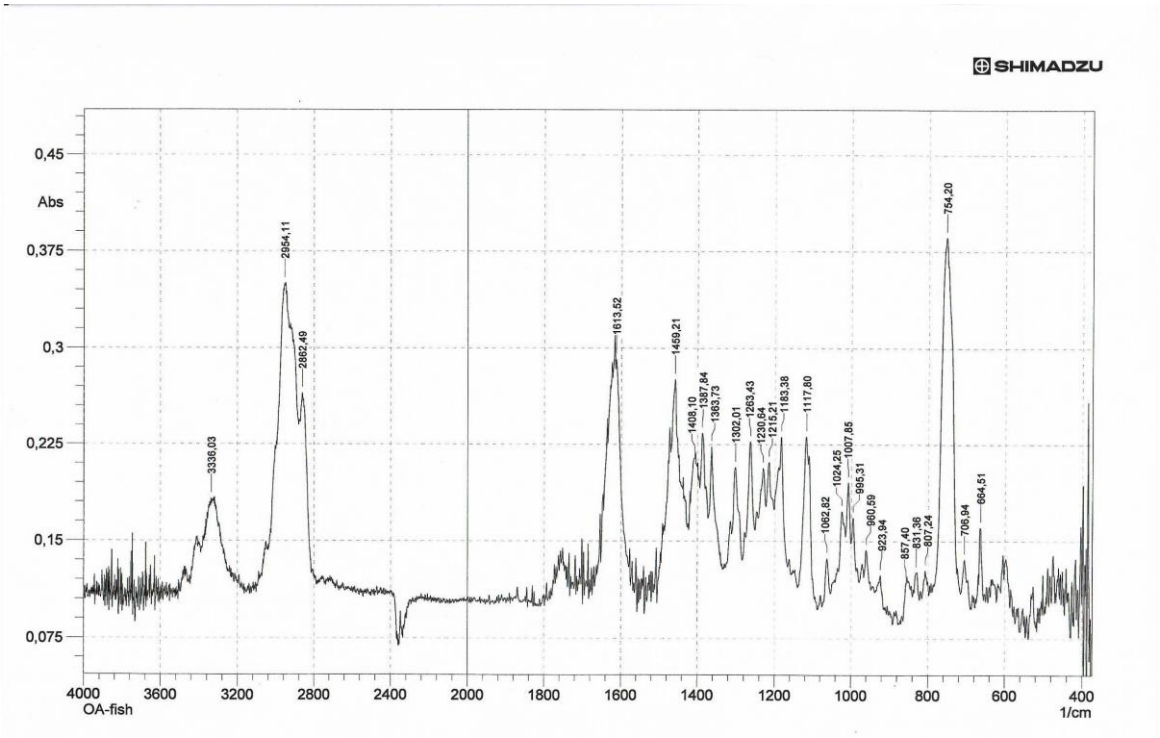


Figure S11. IR spectrum of compound **14**

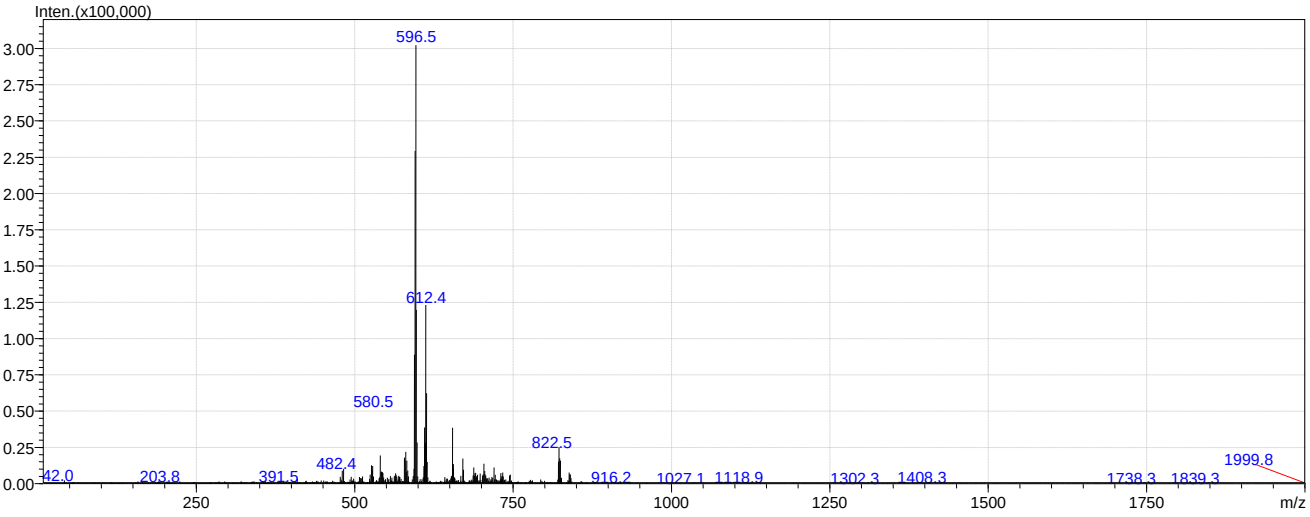
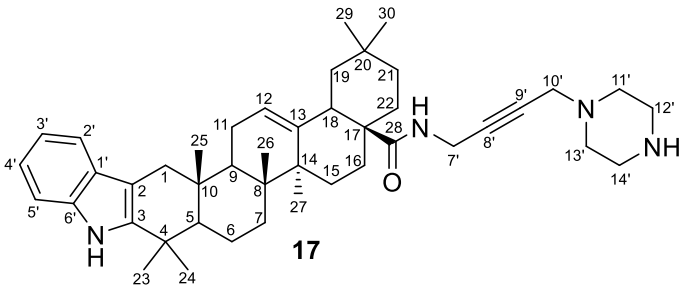


Figure S12. Mass spectrum of compound **14**



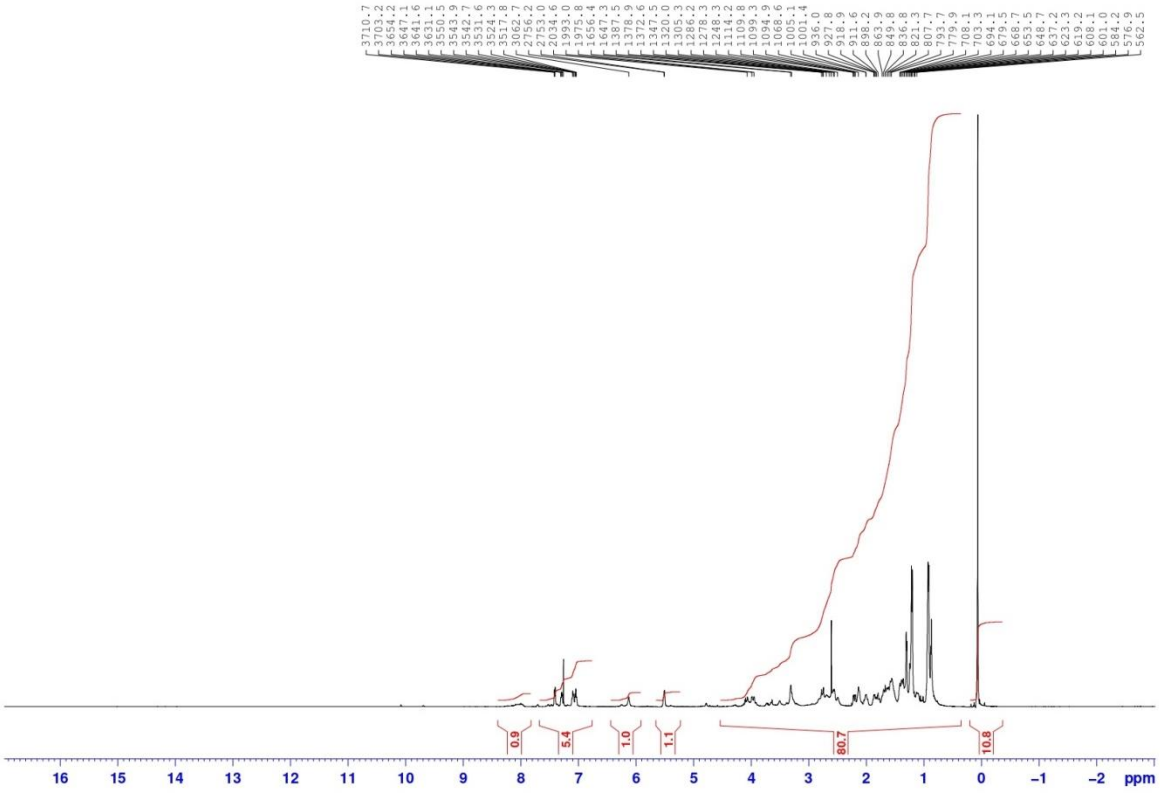


Figure S13. ^{1}H NMR spectrum of compound **17**

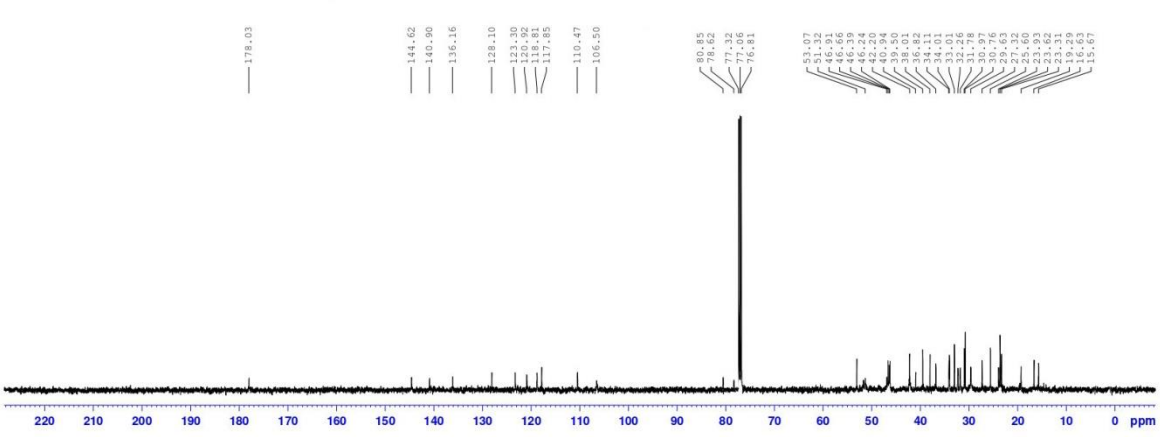


Figure S14. ^{13}C NMR spectrum of compound **17**

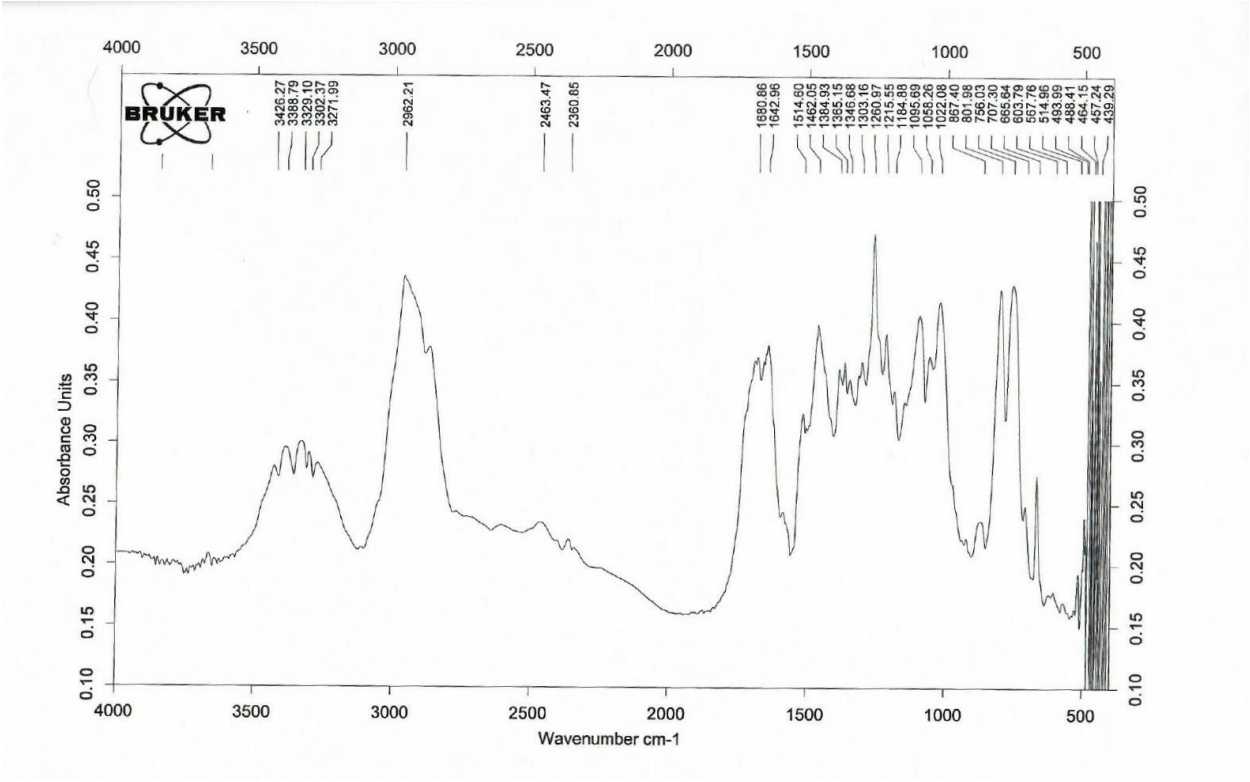


Figure S15. IR spectrum of compound **17**

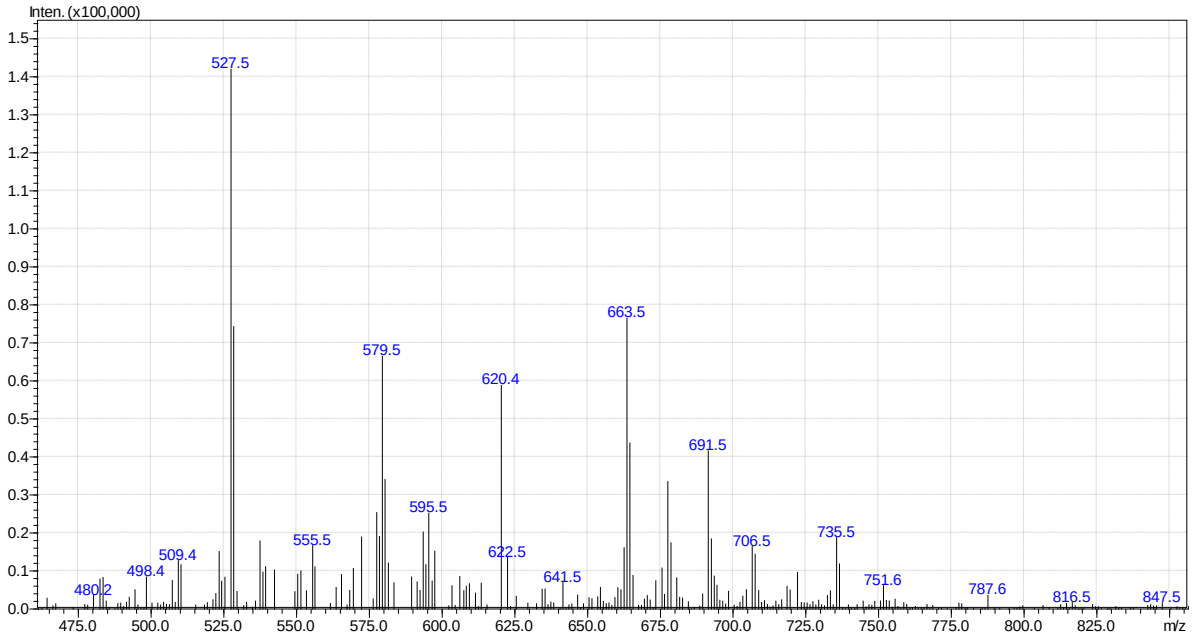


Figure S16. Mass spectrum of compound **17**

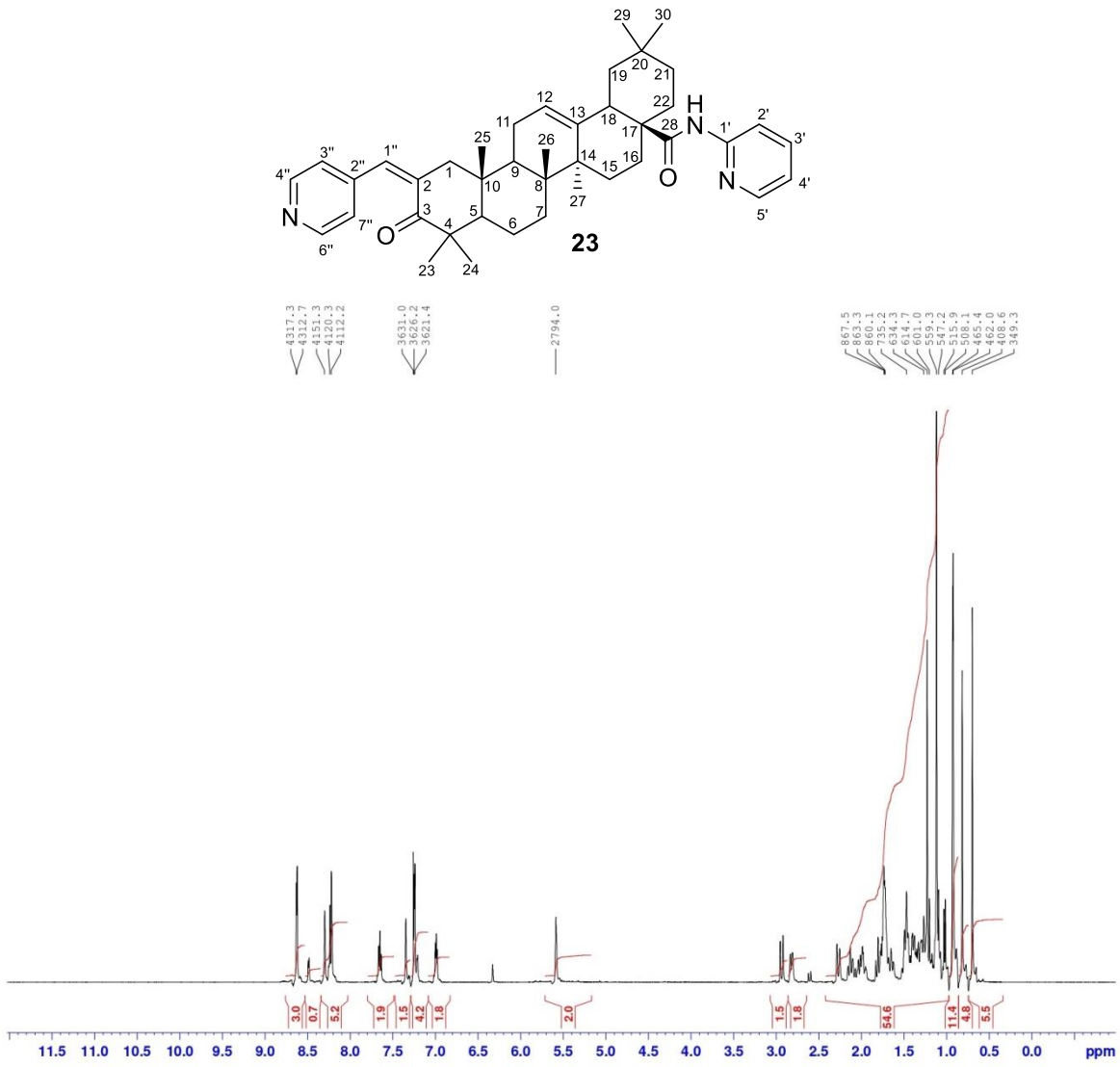


Figure S17. ^{1}H NMR spectrum of compound **23**

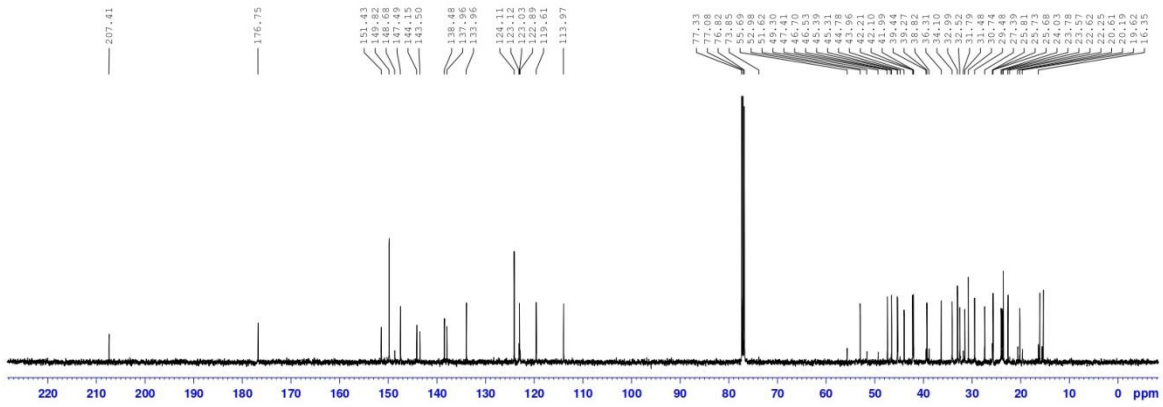


Figure S18 ^{13}C NMR spectrum of compound **23**

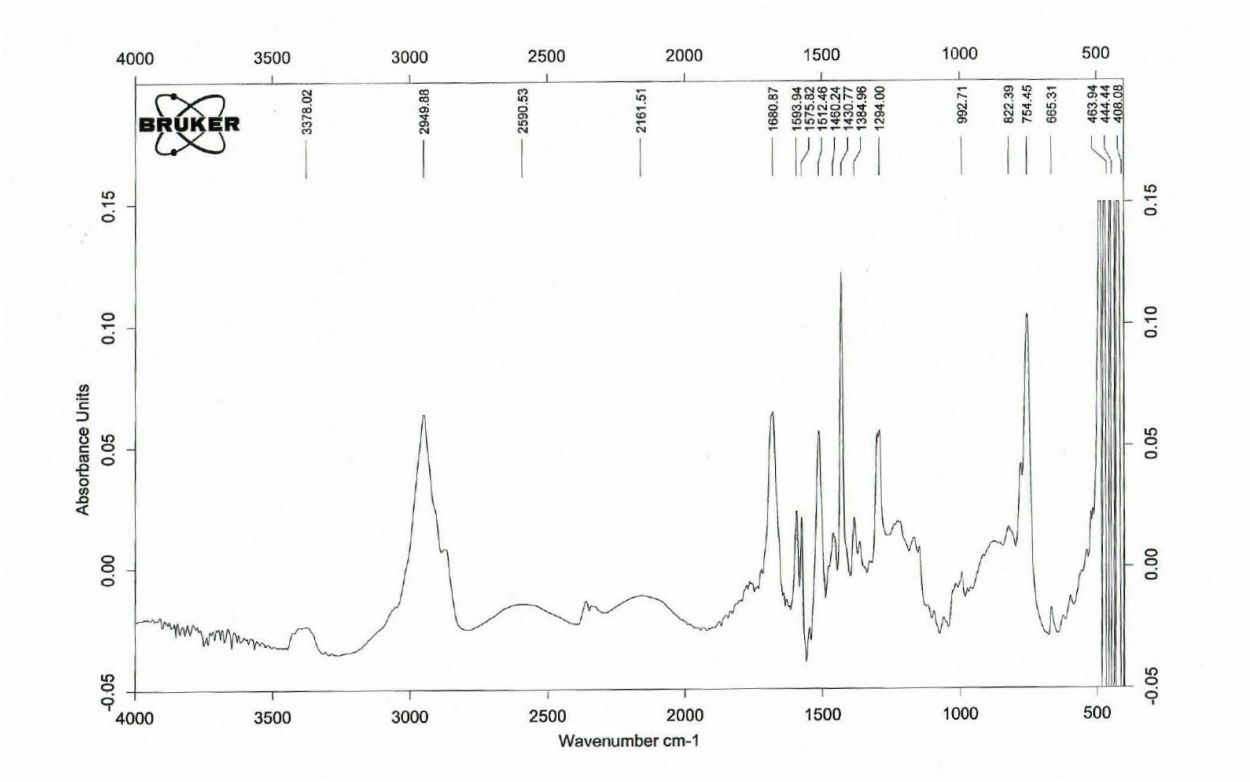


Figure S19. IR spectrum of compound **23**

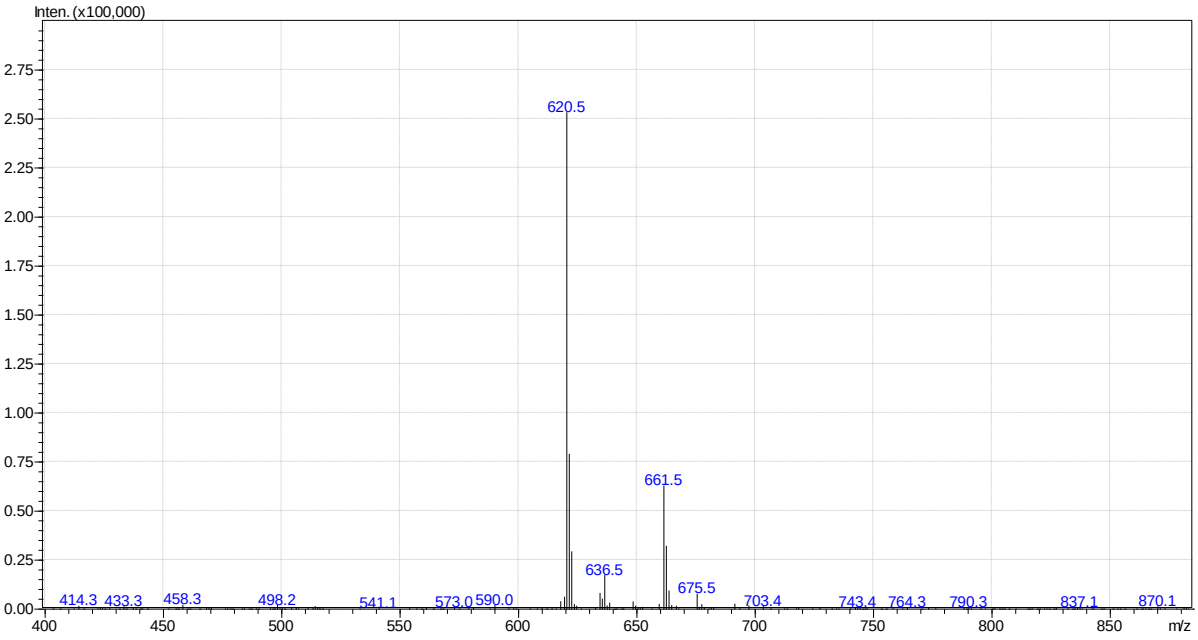


Figure S20. Mass spectrum of compound **23**

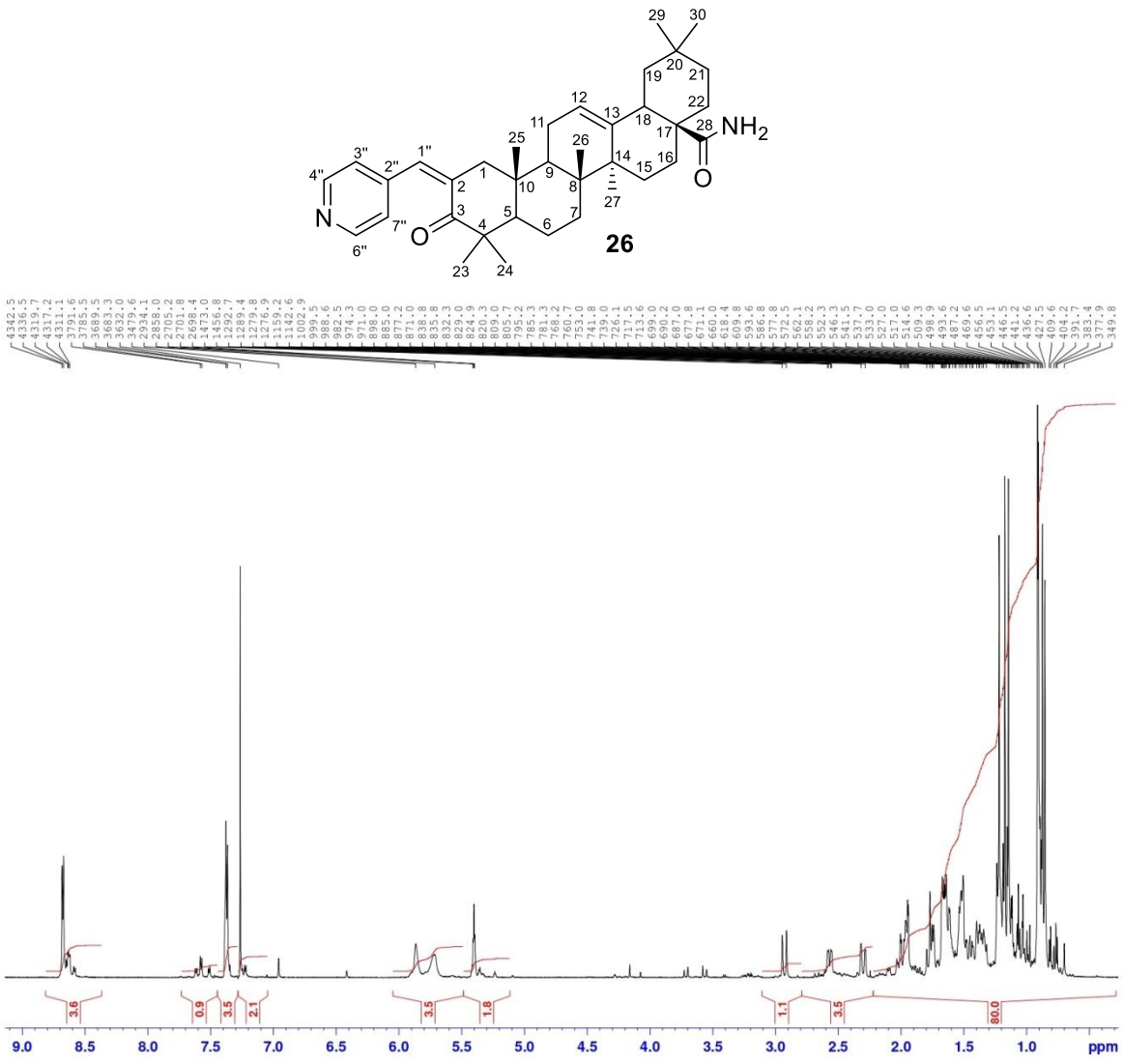


Figure S21. ^{1}H NMR spectrum of compound **26**

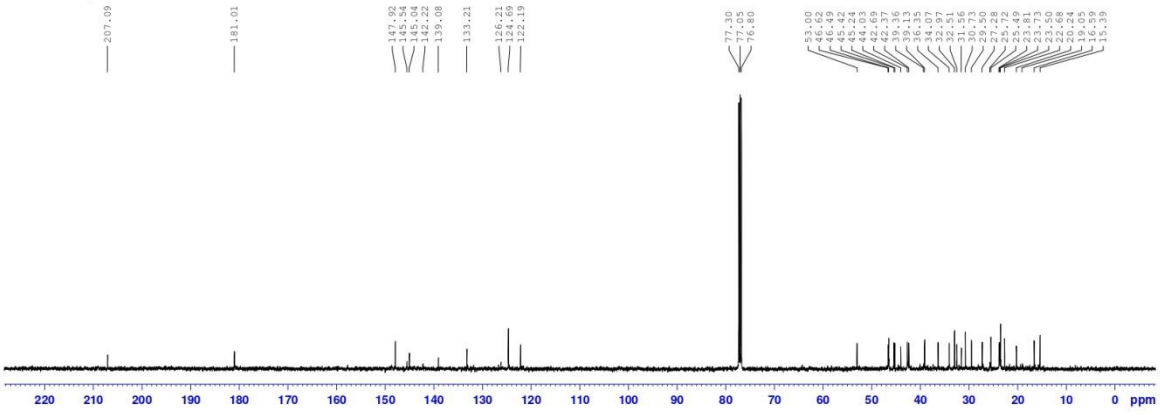


Figure S22. ^{13}C NMR spectrum of compound **26**

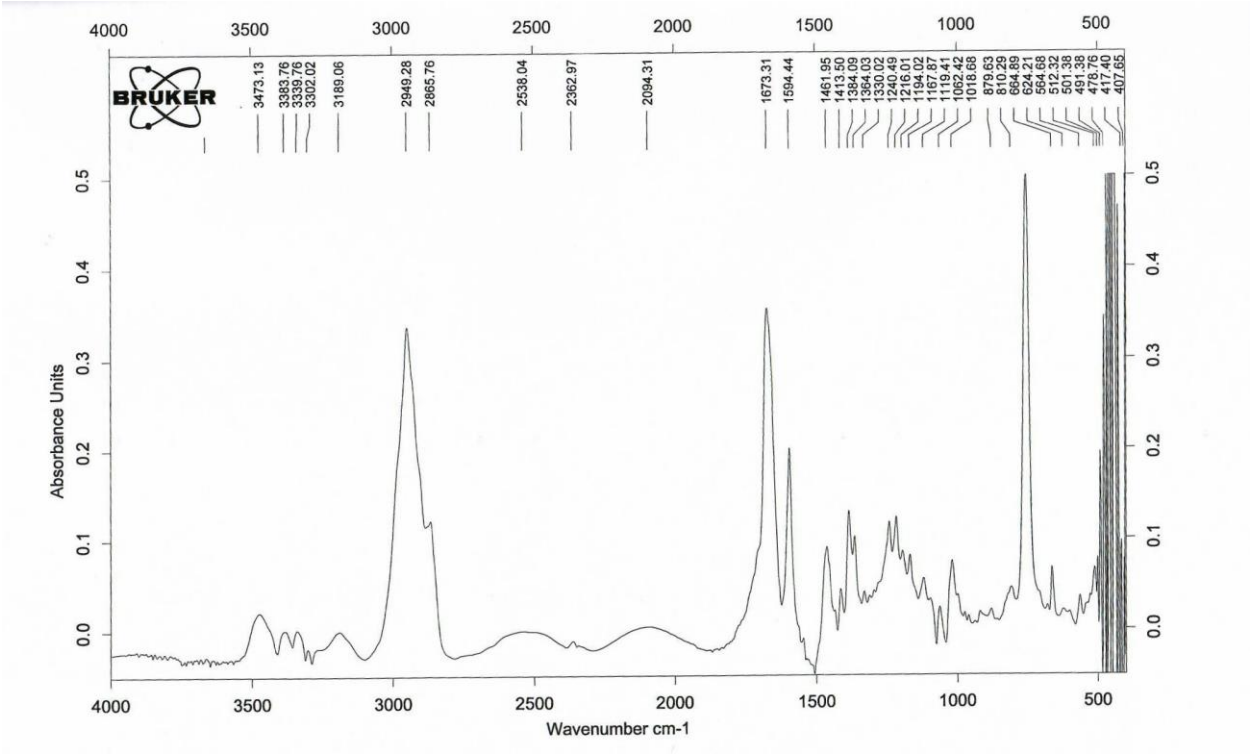


Figure S23. IR spectrum of compound **26**

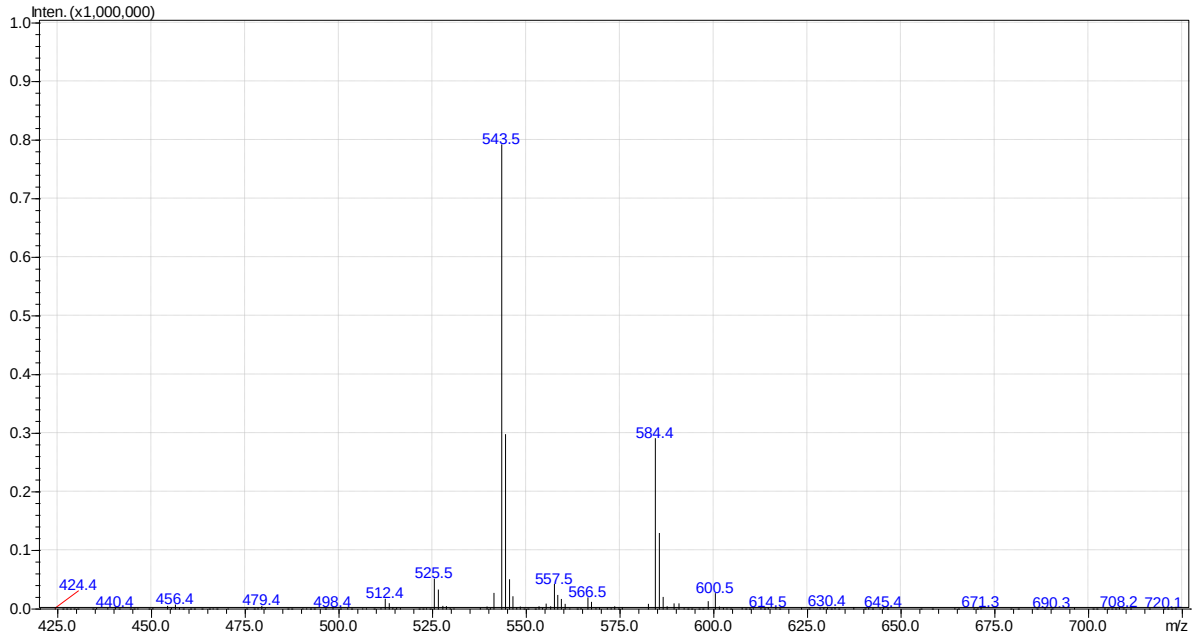


Figure S24. Mass spectrum of compound **26**

S2. Biological evaluation

S2.1 α-Glucosidase assay

In a 96-well flat-bottom transparent plate, 25 μL of the enzyme solution (EC 3.2.1.20, expressed in *S. cerevisiae*, Sigma #G5003, final concentration 0.12 U/mL) was incubated with 50 μL of test compounds in 68 mM phosphate buffer (pH 6.8) at 37°C for 5 min. Then, 25 μL of 4 mM para-nitrophenyl-α-D-glucopyranoside (Sigma #N1377), was added and increase in absorbance was recorded for 15 min at 400 nm wavelength with Infinite M200 PRO microplate reader (Tecan, Austria). Test compounds were replaced with 68 mM phosphate buffer (pH 6.8) in negative control experiments [49,50]. Acarbose was used as a positive control [51].

Mouse small intestine homogenate was used as an enzyme source. The small intestinal tissues homogenize in PBS (pH 6.8) with 14 mM NaCl (1:10 w/v), centrifuged for 12 min at 14 000 g, supernatant was collected, diluted 2-fold and used as the enzyme solution. Further reaction was carried out as with a yeast enzyme.

S2.2. PTP1B inhibition assay

Protein tyrosine phosphatase 1B activity was determined using active human full-length PTP1B (Sigma #SRP0215, USA) and *p*-nitrophenyl phosphate (pNPP, Sigma #SRE0026, USA) as a substrate according to a published procedure [52]. An increase in the absorption of the reaction mixture at a wavelength of 405 nm was measured using an Infinite M200 PRO microplate reader (Tecan, Austria). Experimental inhibitor NSC-87877 (8-hydroxy-7-[(6-sulfo-2-naphthyl)azo]-5-quinolinesulfonic acid, >98% purity, Tocris Bioscience) was used as a positive control [53].

S2.3 Cellular assays

S2.3.1 Isolation and treatment of peritoneal macrophages

Peritoneal macrophages (PM) were isolated from the peritoneal exudate of C57bl/6j mice. To accumulate PM, 1 ml of 3% peptone solution was injected intraperitoneally. After 3 days the mice were euthanized by cervical dislocation. Cells of peritoneal exudate were obtained by aseptically washing the abdominal cavity with 5 ml of sterile Hanks's solution (+4–6 °C) without calcium and magnesium ions. The total number and viability of cells were assessed in a Goryaev counting chamber (Russia) with a 0.4% trypan blue staining (Sigma-Aldrich, USA). The cell concentration was adjusted to 1.0×10^6 cells/ml in DMEM (Gibco) supplemented with 2 mM *L*-glutamine (Gibco), 10% heat-inactivated fetal bovine serum (BioClot, Germany), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) and plated 200 μl/well in 96-well transparent plates (SPL Life Sciences Co., Ltd., Korea). It was left for 2 h at 37°C in a humidified atmosphere with 5% CO_2, after which the wells were washed to remove non-adherent cells. After 24

hours of incubation, 20 μl of the supernatant was removed, and 20 μl of solutions of test compounds were added 30 min before *E. coli* O127:B8 LPS (100 ng/ml final concentration).

S2.3.2 Assay of nitric oxide (NO)

Nitrite anion (a stable end product of NO decomposition) in supernatants was determined using a standard Griess reagent. Briefly, 50 μl of supernatants collected 22 hours after incubation of PM with test and control compounds were mixed with 50 μl of 1% sulfonamide in 2.5% H_3PO_4 and 50 μl of 0.1% *N*-(1-naphthyl) ethylenediamine in 2.5% H_3PO_4. After incubation at 23°C for 10 min in an orbital shaker, the optical density was determined at a wavelength of 550 nm with Infinite M200 PRO (Tecan, Austria) microplate reader.

S2.3.3 Isolation of rat cardiac fibroblasts

Fibroblasts of non-linear male rats (3 months old, 225-250 g, Rappolovo nursery, Leningrad region) were obtained using proteolytic dissociation and selective adhesion, as described previously [54,55]. Cell viability was assessed before each subsequent passage by staining with 0.4% trypan blue (PanEco, Russia). The purity of the cell culture and the state of the cells during the experiments were monitored using an inverted microscope (Zeiss, Germany). Fibroblasts were cultured in 96-well plates in complete DMEM medium with 10% serum and gentamicin (50 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2. All experiments with fibroblasts were performed on cells from the first two passages [56].

S2.3.4 MTT assay

MTT assay was performed according to a standard procedure. In brief, 24 hours after the addition of studied compounds, 20 μl of 5 mg/ml MTT solution were added, and cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 h. The culture medium was removed, the cells were lysed, and formazan crystals were dissolved in 150 μl of DMSO. The plates were shaken at room temperature for 10 min. The optical density was measured with an Infinite M200 PRO microplate reader (Tecan, Grodig, Austria) at a wavelength of 565 nm. Cells treated with 0.01% Triton X-100 served as a positive control, and 0.5% DMSO-treated as a negative one.

S2.3.5. Neutral red assay

Cells were treated with test compounds as described above. At the end of the exposure, the medium was carefully aspirated. Cells were washed twice with 100 μl/well 37°C PBS (pH 7.2-7.4). To increase cell adhesion, the plates were air dried. Then 40 μl of 100 μg/ml neutral red in PBS were added and incubated for 90 min at 37°C. Neutral red solution was gently but quickly removed and cells were washed twice with 100 μl/well PBS (37°C). Dye was extracted from cells with 100 μl/well 1:1 citrate

buffer-ethanol mixture, followed by shaking at room temperature for 20 min. Optical density was measured at 550 nm using an Infinite M200 PRO microplate reader (Tecan, Grodig, Austria). Cells treated with 0.01% Triton X-100 served as a positive control, and 0.5% DMSO-treated as a negative one.

S2.3.6 Phagocyte-dependent neutrophil chemiluminescence

Assay was performed in white 96-well polypropylene plates (Thermo Fischer, Denmark) according to a modification of the described method [57]. Neutrophils were isoladet from peritoneal cavity of mice and seeded 1.0×10^6 cells per well. Shortly after, 100 μl of test compounds solution in RPMI-1640 medium (final DMSO <0.3%) and 50 μl of a 0.56 mM of luminol solution in deionized water were added to the wells. Cells were incubated in at 37°C and 300 rpm for 15 min to allow permeation of compounds and luminol into cells. Then, 100 μl of opsonized zymosan solution was added to experimental samples to induce chemiluminescence. Signal was recorded with 1000 ms integration time every 2 min for 1 hour with an Infinite M200 Pro microplate reader (Tecan, Grodig, Austria) at a constant temperature of 37 °C.

S2.4 Animals

All procedures with animals were carried out under ethical standards adopted by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986) and taking into account the International Recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental research (1997). All sections of this study adhere to the ARRIVE Guidelines for reporting animal research [58]. Male Wistar rats aged 9-12 weeks were procured from "Pushchino" facility and housed 4 per cage in a ventilated room with 12/12-hour light/dark cycle and an ambient temperature of 25°C. Animals had free access to food and water before the study.

S2.4.1 Oral maltose tolerance test

Animals were randomly assigned to the experimental and control groups (n = 5) and fasted overnight for 16 h. Animals of the experimental groups received 5 mg/kg of the test substance or 5 mg/kg acarbose in 1% DMSO solution *p.o*. Control group received the equal volume of vehicle (10 ml/kg). After 30 min, all animals were administered with 2.0 g/kg maltose (Sigma, USA) in 0.9% sodium chloride solution with intragastrial gavage. Blood samples were taken from the t ail vein at –30, 0, 30, 60, 90, and 120 min. with an "end-to-end" capillary of 20 μl volume and hemolyzed with 1 ml of commercial glucose/lactate hemolytic solution. Concentration of glucose in the samples was determined using Biosen C_Line biochemical analyzer (EKF Diagnostics, Germany).

References

49. Elya, B.; Basah, K.; Mun'im, A.; Yuliastuti, W.; Bangun, A.; Septiana, E.K. Screening of α-Glucosidase Inhibitory Activity from Some Plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae, *J Biomed Biotechnol.* **2012**, *2012*, 1–6. https://doi.org/10.1155/2012/281078
50. Spasov, A.A.; Babkov, D.A.; Prokhorova, T.Y.; Sturova, E.A.; Muleeva, D.R.; Demidov, M.R.; Osipov, D.V.; Osyanin, V.A.; Klimochkin, Y.N. Synthesis and biological evaluation of 2-acylbenzofuranes as novel α-glucosidase inhibitors with hypoglycemic activity. *Chem Biol Drug Des*. **2017**, *90*, 1184–1189. https://doi.org/10.1111/cbdd.13038.
51. Fujisawa, T.; Ikegami, H.; Inoue, K.; Kawabata, Y.; Ogihara, T. Effect of two α-glucosidase inhibitors, voglibose and acarbose, on postprandial hyperglycemia correlates with subjective abdominal symptoms. *Metabolism* **2005**, *54*, 387–390. https://doi.org/10.1016/j.metabol.2004.10.004
52. Lubben, T.; Clampit, J.; Stashko, M.; Trevillyan, J.; Jirousek, M.R. In vitro enzymatic assays of protein tyrosine phosphatase 1B., Current Protocols in Pharmacology / Editorial Board, S.J. Enna (Editor-in-Chief) [et Al.]. Chapter 3 (2001) Unit3.8. https://doi.org/10.1002/0471141755.ph0308s13
53. Song, M.; Park, J.E.; Park, S.G.; Lee, D.H.; Choi, H.K.; Park, B.C.; Ryu, S.E.; Kim, J.H.; Cho, S. NSC-87877, inhibitor of SHP-1/2 PTPs, inhibits dual-specificity phosphatase 26 (DUSP26). *Biophys Res Commun*. **2009**, *381*, 491–495. https://doi.org/10.1016/j.bbrc.2009.02.069
54. Villarreal, F.J.; Kim, G. N.N.; Ungab, D.; Printz, M.P.; Dillmann, W.H. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation* **1993**, *88*, 2849–2861. https://doi.org/10.1161/01.CIR.88.6.2849
55. Dubey, R.K.; Gillespie, D.G.; Mi, Z.; Jackson, E.K. Exogenous and Endogenous Adenosine Inhibits Fetal Calf Serum–Induced Growth of Rat Cardiac Fibroblasts: Role of A 2B Receptors. *Circulation* **1997**, *96*, 2656–2666. https://doi.org/10.1161/01.CIR.96.8.2656
56. Chen, Y.; Fan, J.; Cao, L.; Han, T.; Zeng, M.; Xu Y., Ling, Z.; Yin, Y. Unique mechanistic insights into the beneficial effects of angiotensin-(1-7) on the prevention of cardiac fibrosis: A metabolomic analysis of primary cardiac fibroblasts. *Exp Cell Res.* **2019**, *378*, 158–170. https://doi.org/10.1016/j.yexcr.2019.03.006
57. Kopprasch, S.; Pietzsch, J.; Graessler, J. Validation of different chemilumigenic substrates for detecting extracellular generation of reactive oxygen species by phagocytes and endothelial cells. *Luminescence* **2003**, *18*, 268–273. https://doi.org/10.1002/bio.737
58. Percie du Sert, N.; Hurst, V.; Ahluwalia, A.; Alam, S.; Avey, M.T.; Baker, M.; Browne, W.J.; Clark, A.; Cuthill, I.C.; Dirnagl, U.; Emerson, M.; Garner, P.; Holgate, S.T.; Howells, D.W.; Karp, N.A.; Lazic, S.E.; Lidster, K.; MacCallum, C.J.; Macleod, M.; Pearl, E.J.; Petersen, O.H.; Rawle, F.; Reynolds, P.; Rooney, K.; Sena, E.S.; Silberberg, S.D.; Steckler, T.; Würbel, H. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLOS Biology* **2020**, *18*, e3000410. https://doi.org/10.1371/journal.pbio.3000410