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Tubuloclustin analogues with ether moiety: synthesis and evaluation of tubulin clustering and antimitotic activity in cancer cells

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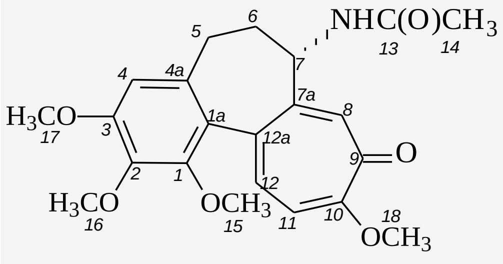
Molecular modeling

3D Model of the colchicine-binding site (PDB ID:4O2B) was used for computer molecular modeling. All compounds used for X-ray of the protein and all water molecules were previously excluded from the model (other molecules and ions at the interface of α- and β-subunits of the protein were maintained). Atomic charges of protein amino acids were assigned by standard Kollman method using AutoDock Tools 1.5.6. Three-dimensional structures of the compounds were submitted to a conformational MMFF Amber ff14SB optimization using Gasteiger charges in USCF Chimera 1.13.1 program [1]. Molecular docking was carried out with AutoDock Vina 1.1.2 software [2] (grid box 23.25Å×25.5Å ×24.0Å, grid center size x=17.51 Å, y=69.503 Å, z=43.258 Å, exhaustiveness = 16). Complexes with the best values of scoring functions were selected and visualized using CLC Drug Discovery Workbench (Limited mode, Version 4).

Chemistry

Solvents for extraction and chromatography were technical grade and were purified by standard procedures prior to use. Flash and column chromatography were performed on silica gel Acros (40-60 μm). Reaction control was carried out by thin-layer chromatography on Silufol silica gel plates. 1D and 2D (gHSQC, gHMBC) NMR spectra were recorded on spectrometer Agilent 400-MR (400.0 MHz for ^{1}H; 100.6 MHz for ^{13}C) at room temperature; chemical shifts were measured with reference to the solvent ($CDCl_3$, δ_H=7.24 ppm, δ_C=77.0 ppm). Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), m (multiplet). Assignment of NMR signals of colchicine fragment in the target conjugates was made using data from [3] with the atomic numbering of colchicine presented on the figure at right. Electron impact mass spectra were obtained on a Bruker Autoflex II mass spectrometer with accelerating voltage of 20 kV. Elemental analysis of synthesized compounds was performed

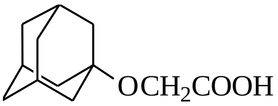


on CNH analyser “Carlo-Erba” ER-20. Initial alcohols: 2-(adamantan-1-yloxy)ethanol (**2a**), 4-(adamantan-1-yloxy)butan-1-ol (**2b**), 5-(adamantan-1-yloxy)pentan-1-ol (**2c**) were synthesized via reaction of 1,3-dehydroadamantane and ethane-1,2-diol, butane-1,2-diol or pentane-1,2-diol correspondingly using procedure described in [4].

General procedure A for the oxidation of ω-(adamantan-1-yloxy)alkan-1-ols 2a-c.

To a stirred solution of ω-(adamantan-1-yloxy)alkan-1-ol in THF (5 ml) was added dropwise 0.50 ml solution of Jones reagent (2.67 g CrO_3 in 2.3 ml H_2SO_4 and diluted to 10 ml with water) at room temperature. After five minutes the precipitate was filtered and washed with water. The filtrate was extracted with methylene chloride (3×10 ml), dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography [ethyl acetate/petroleum ether (40–70°C)] in a gradient mixture 1:8–1:5.

(Adamantan-1-yloxy)ethanoic acid (3a) was synthesized according to general procedure A from 100 mg (0.510 mmol) 2-(adamantan-1-yloxy)ethanol (**2a**) to give 0.055 g of acid **3a** as white waxy solid 0.065 (yield 61%).

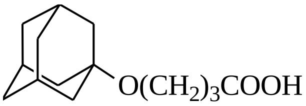


^1H NMR ($CDCl_3$, δ, *J*/Hz): 1.59–1.69 (6H, m, Ad), 1.77–1.78 (6H, m, Ad), 2.19 (3H, m, Ad), 4.09 (2H, s, CH_2), 10.80 (1H, br s, COOH).

^{13}C NMR: ($CDCl_3$, δ): 30.42, 36.06, 41.18, 58.39 (OCH_2), 74.87 (C-O-CH2), 172.97 (COOH).

Anal. calcd. for $C_{12}H_{18}O_3$, %: C, 68.54; H, 8.63. Found: C, 68.57; H, 8.66.

4-(Adamantan-1-yloxy)butan-1-oic acid (3b) was synthesized according to general procedure A from 100 mg (0.446 mmol) 4-(adamantan-1-yloxy)butan-1-ol (**2b**) to give 0.055 g of acid **3b** as white waxy solid (yield 52%).



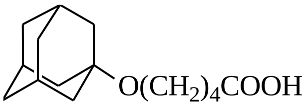
^1H NMR ($CDCl_3$, δ, *J*/Hz): 1.65–1.69 (7H, m, Ad+H), 1.69–1.75 (7H, m, Ad+H), 2.14 (3H, m, Ad), 2.40 (2H, t, $CH_2C=O$), 3.50 (2H, t, OCH_2), 10.70 (1H, br s, COOH).

^{13}C NMR: ($CDCl_3$, δ): 21.75, 29.66, 30.46, 33.85, 36.44, 41.48, 59.18 (OCH_2), 72.12 (C-O-CH2), 179.20 (COOH).

MS (MALDI-TOF), *m/z*: 238 $[M]^+$, 261 $[M+Na]^+$

Anal. calcd. for $C_{14}H_{22}O_3$, %: C, 70.56; H, 9.30. Found: C, 70.60; H, 9.32.

5-(Adamantan-1-yloxy)pentan-1-oic acid (3c) was synthesized according to general procedure A from 100 mg (0.419 mmol) 5-(adamantan-1-yloxy)pentan-1-ol (**2c**) to give 0.060 g of acid **3c** as white waxy solid (yield 57%).



¹H NMR (CDCl$_3$, δ, *J*/Hz): 1.57–1.63 (8H, m, Ad+CH$_2$), 1.69–1.75 (8H, m, Ad+CH$_2$), 2.14 (3H, m, Ad), 2.39 (2H, t, *J*=7.43, CH$_2$C=O), 3.44 (2H, t, *J*=6.24, OCH$_2$), 10.73 (1H, br s, COOH).

^{13}C NMR: (CDCl$_3$, δ): 21.75, 29.66, 30.46, 33.86, 36.45, 41.48, 59.19 (O$\underline{C}$H$_2$), 72.13 ($\underline{C}$-O-CH2), 179.20 (COOH).

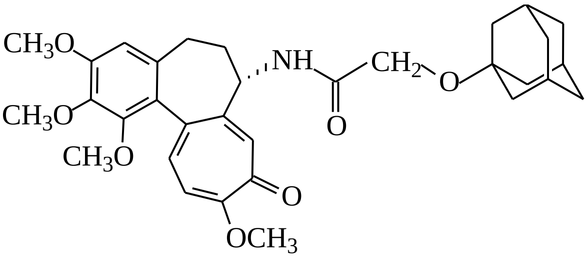
MS (MALDI-TOF), *m/z*: 252 [M]$^+$, 275 [M+Na]$^+$, 291 [M+K]$^+$.

Anal. calcd. for $C_{15}H_{24}O_3$, %: C, 71.39; H, 9.59. Found: C, 71.43; H, 9.63.

General procedure B for the amidation of acids with N-deacetylcolchicine.

To a solution of acids **3a–c** or **5** in CH_2Cl_2 (5 ml) was added N-deacetylcolchicine and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) at room temperature. After stirring for 24 h, the solvent was removed under reduced pressure, and the residue was first purified by column chromatography (ethyl acetate/petroleum ether (40–70°C) 1:2, then CH_2Cl_2/methanol in a gradient ratio 99:1 – 98:2) and then (to remove the small remaining admixtures of initial compounds) – by thin layer chromatography (3.5% methanol in CH_2Cl_2).

N-(Adamantan-1-yloxy)ethanoyl-N-deacetylcolchicine (**4a**) was synthesized according to general procedure B from acid **3a** (0.038 g, 0.167 mmol), N-deacetylcolchicine (0.050 g, 0.140 mmol), EEDQ (0.050 g, 0.202 mmol) to give 0.022 g of conjugate **4a** (yield 29%) as slightly yellowish waxy solid.



¹H NMR (CDCl$_3$, δ, *J*/Hz): 1.64–1.65 (6H, m), 1.76–1.78 (7H, m, H^{Ad}+ H^{5colch}), 1.80–1.91 (1H, m), 2.14 (1H, m), 2.24–2.34 (2H, m), 2.33–2.43 (2H, m), 2.53 (1H, dd, *J*=6.2, 13.2 Hz), 3.66 (3H, s, OCH$_3$), 3.90 (3H, s, OCH$_3$), 3.93 (3H, s, OCH$_3$), 4.01 (3H, s, OCH$_3$), 4.04 (2H, s, CH$_2$), 4.64 (1H, m, H^{7colch}), 6.55 (1H, s, H^{4colch}), 6.83 (1H, d, *J* = 10.9 Hz, H^{11colch}), 7.00 (1H, br. s, NH), 7.34 (1H, d, *J* = 10.9 Hz, H^{12colch}), 7.54 (1H, s, H^{8colch}).

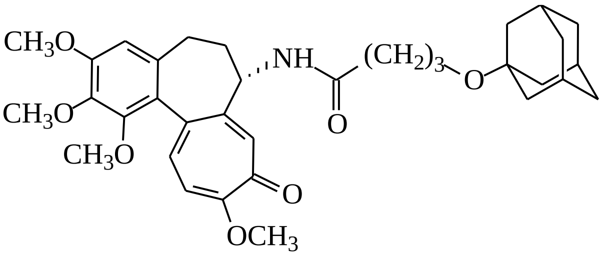
^{13}C NMR: (CDCl$_3$, δ): 29.95, 30.48, 37.10, 36.45, 41.52, 52.13 (C^{7}HNH), 56.14 (OCH$_3$), 56.50 (OCH$_3$), 59.50, 61.44 (OCH$_3$), 61.69 (OCH$_3$), 71.78, 107.39, 113.31, 125.45, 130.59, 134.28, 136.12, 137.32, 141.66, 151.16, 152.60, 153.60, 164.00, 172.74 (C=O), 178.54 (C=O).

MS (MALDI-TOF), *m/z*: 549 [M]$^+$, 572 [M+Na]$^+$.

Anal. calcd. for $C_{32}H_{39}NO_7$, %: C, 69.92; H, 7.15. Found: C, 69.94; H, 7.17.

N-(4-(Adamantan-1-yloxy)-4-butanoyl)-N-deacetylcolchicine (**4b**) was synthesized according to general procedure B from acid **3b** (0.033 g, 0.141 mmol), N-deacetylcolchicine (0.060 g, 0.168 mmol), EEDQ (0.050 g, 0.202 mmol) to give 0.029 g of conjugate **4b** (yield 36% g) as slightly yellowish waxy solid.

^{1}H NMR ($CDCl_3$, δ, *J*/Hz): 1.45–1.52 (2H, m), 1.56–1.64 (6H, m), 1.70–1.73 (6H, m, H^{Ad}), 1.82–1.92 (1H, m), 2.11 (3H, m), 2.21–2.31 (3H, m), 2.33–2.43 (1H, m), 2.53 (1H, dd, *J*=6.2, 13.2 Hz), 3.35 (2H, t, *J*=6.6 Hz), 3.65 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.02 (3H, s, OCH_3), 4.68 (1H, m, H^{7colch}), 6.54 (1H, s, H^{4colch}), 6.85 (1H, br. s, NH), 6.91 (1H, d, *J*=10.8 Hz, $H^{11colch}$), 7.39 (1H, d, *J*=10.8 Hz, $H^{12colch}$), 7.60 (1H, s, H^{8colch}).



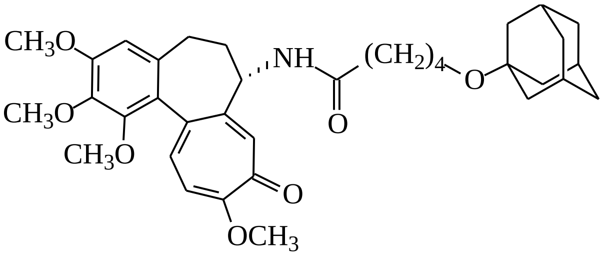
^{13}C NMR: ($CDCl_3$, δ): 25.97, 29.91, 30.46, 36.26, 36.48, 37.04, 41.54, 52.16 (C^7HNH), 56.09 (OCH_3), 56.46 (OCH_3), 59.44, 61.37 (OCH_3), 61.63 (OCH_3), 71.77, 107.37, 113.31, 125.46, 130.58, 134.24, 136.02, 137.26, 141.64, 151.16, 152.59, 153.59, 163.97, 172.71 (C=O), 178.55 (C=O).

MS (MALDI-TOF), *m/z*: 577 $[M]^+$, 600 $[M+Na]^+$ 616 $[M+K]^+$.

Anal. calcd. for $C_{34}H_{43}NO_7$, %: C, 70.69; H, 7.50. Found: C, 70.73; H, 7.54.

N-(5-(Adamantan-1-yloxy)-5-pentanoyl)-N-deacetylcolchicine (**4c**) was synthesized according to general procedure B from acid **3c** (0.045 g, 0.178 mmol), N-deacetylcolchicine (0.053 g, 0.149 mmol), EEDQ (0.050 g, 0.202 mmol) to give 0.025 g of conjugate **4c** (yield 28% g) as slightly yellowish waxy solid.

^{1}H NMR ($CDCl_3$, δ, *J*/Hz): 1.20 (2H, t, *J*=7.15 Hz), 1.63–1.64 (6H, m), 1.75–1.76 (7H, m, H^{Ad}+ H^{5colch}), 1.82–1.92 (1H, m), 2.15 (3H, m), 2.22–2.32 (2H, m), 2.35–2.45 (4H, m, *J*=7.0 Hz,), 3.46 (2H, t, *J*=6.4 Hz), 3.66 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.00 (3H, s, OCH_3), 4.63 (1H, m, H^{7colch}), 6.54 (1H, s, H^{4colch}), 6.83 (1H, d, *J*=10.8 Hz, $H^{11colch}$), 7.02 (1H, br. s, NH), 7.32 (1H, d, *J*=10.8 Hz, $H^{12colch}$), 7.53 (1H, s, H^{8colch}).

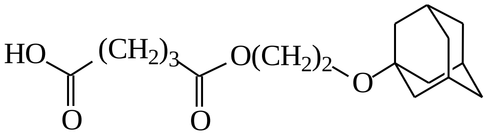


^{13}C NMR: ($CDCl_3$, δ): 26.12, 29.93, 30.45, 33.99, 36.38, 36.88, 41.57, 52.10 (C^7HNH), 56.07 (OCH_3), 56.29, 56.34 (OCH_3), 59.34, 61.37 (OCH_3), 61.55 (OCH_3), 72.51, 107.31, 112.59, 125.66 130.75, 134.19, 135.38, 136.69, 141.61, 151.18, 151.78, 153.44, 163.96, 172.89 (C=O), 178.93 (C=O).

MS (MALDI-TOF), *m/z*: 591 $[M]^+$, 614 $[M+Na]^+$ 630 $[M+K]^+$.

Anal. calcd. for $C_{35}H_{45}NO_7$, %: C, 71.04; H, 7.67. Found: C, 71.09; H, 7.70.

5-(2-(Adamantan-1-yloxy)ethan-1-oxy)-5-oxopentanoic acid (**5**). To a solution of 2-(adamantan-1-ylamino)ethan-1-ol (**2**) (0.100 g, 0.509 mmol) in CH_2Cl_2 (5 ml) was added glutaric anhydride (0.500 g, 4.38 mmol) and catalytic amount (0.01 g) of 4-dimethylaminopyridine (DMAP). The mixture was stirred at room temperature for 48 h and then the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (ethyl acetate/petroleum ether (40–70°C) in a gradient mixture 1:3 – 1:5). Yield 0.104 g (66%), colorless oily liquid.

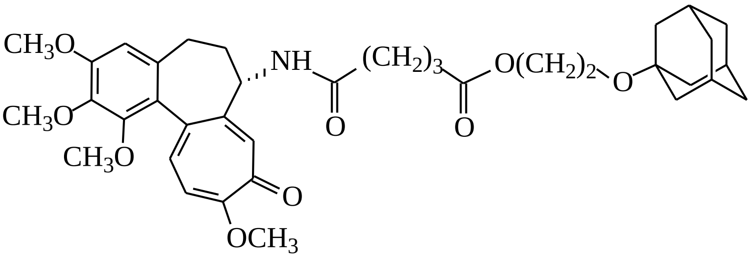


^{1}H NMR ($CDCl_3$, δ, *J*/Hz): 1.53–1.62 (6H, m, Ad), 1.71 (6H, m, Ad), 1.88–1.95 (2H, m), 2.11 (3H, m, Ad), 2.35–2.43 (4H, m), 3.59 (2H, t, *J*=5.0 Hz), 4.15 (2H, t, *J*=5.0 Hz), 10.81 (1H, br s, COOH).

^{13}C NMR: ($CDCl_3$, δ): 20.03, 30.42, 33.20, 36.33, 41.35, 57.99, 64.40, 72.50, 173.01 ($COOCH_2$), 174.53 (COOH).

Anal. calcd. for $C_{17}H_{26}O_5$, %: C, 65.78; H, 8.44. Found: C, 65.84; H, 8.49.

N-(2-(Adamantan-1-yloxy)ethan-1-oxy-5-oxopentanoyl)-N-deacetylcolchicine (6) was synthesized according to general procedure B from acid **5** (0.052 g, 0.167 mmol), N-deacetylcolchicine (0.050 g, 0.140 mmol), EEDQ (0.050 g, 0.202 mmol) to give 0.024 g of conjugate **6** (yield 26% g) as slightly yellowish waxy solid.



^{1}H NMR ($CDCl_3$, δ, *J*/Hz): 1.21 (2H, t, *J*=7.4 Hz, CH_2), 1.55–1.64 (6H, m, CH_2, Ad), 1.72–1.73 (6H, m, CH_2, Ad), 1.85–1.91 (3H, m), 2.13 (3H, m, Ad), 2.20–2.26 (1H, m), 2.28–2.34 (2H, m), 2.40 (1H, m, H^6), 2.52 (1H, m, H^5), 3.61 (2H, t, *J*=5.1 Hz), 3.65 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.99 (3H, s, OCH_3), 4.16 (2H, t, *J*=5.1 Hz, OCH_2), 4.63 (1H, m, $C^7\underline{H}NH$), 6.53 (1H, s, H^4), 6.83 (1H, d, H^{11}, *J*=10.6 Hz), 7.21 (1H, br.s., NH), 7.34 (1H, d, H^{12}, *J*=10.6 Hz), 7.52 (1H, s, H^8).

^{13}C NMR: ($CDCl_3$, δ): 20.77, 29.87, 30.39, 33.22, 34.83, 36.30 (CH_2, Ad), 36.80, 41.42 (CH_2, Ad), 52.16 (C^7), 56.04, 56.34, 57.96, 61.34, 61.55, 64.50, 72.74 (Ad-O), 107.29, 112.66, 125.55, 130.64, 134.16, 135.43, 136.66, 141.58, 151.14, 151.88, 153.45, 163.95, 171.88 (C=O), 173.24 (C=O), 179.10 (C=O).

MS (MALDI-TOF), *m/z*: 650 $[M]^+$, 673 $[M+Na]^+$ 689 $[M+K]^+$.

Anal. calcd. for $C_{37}H_{47}NO_9$, %: C, 68.39; H, 7.29. Found: C, 68.43; H, 7.36.

Biological testing

MTT Cytotoxicity Assay

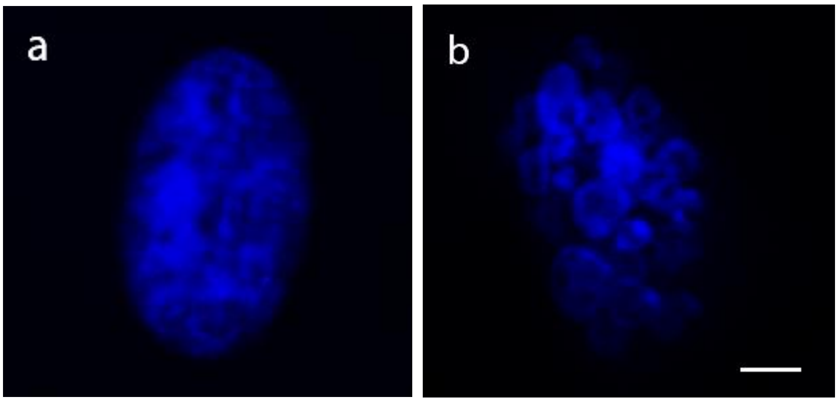
3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT, Roth GmbH, Karlsruhe, Germany) was used in a standard quantitative colorimetric assay to measure the cytotoxicity, viability and metabolic activity [5]. A549 human lung epithelial carcinoma cells (CCL-185™) were cultured under a 5% CO_2 humidified atmosphere with Dulbecco´s Modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C. The cells were seeded in 96-well plates (~3000 cells per well). Stock solutions of tested compounds (20 mM) were prepared in DMSO (0.5% served as a negative control). Cells were treated for 24 h with selected compounds at 1–120 nM (8 wells for each concentration). Optical density was measured at 550 nm with 690 nm reference filter using a plate reader SpectraMax iD3 (Molecular devices, San Jose, USA). Experiments were repeated 3–6 times and EC_{50} values were determined by sigmoid curve fitting using Excel-based software.

Proliferation assay

Lung carcinoma A549 cells were incubated with 1–55 nM of each tested compound during 24 h (0.5% DMSO served as a negative control). After culturing the cells were resuspened in phosphate buffer saline (PBS) fixed with 4% PFA and counted directly by phase-contract microscopy using hemocytometer. Experiments were repeated 3 times and IC_{50} values were determined by sigmoid curve fitting using Excel-based software.

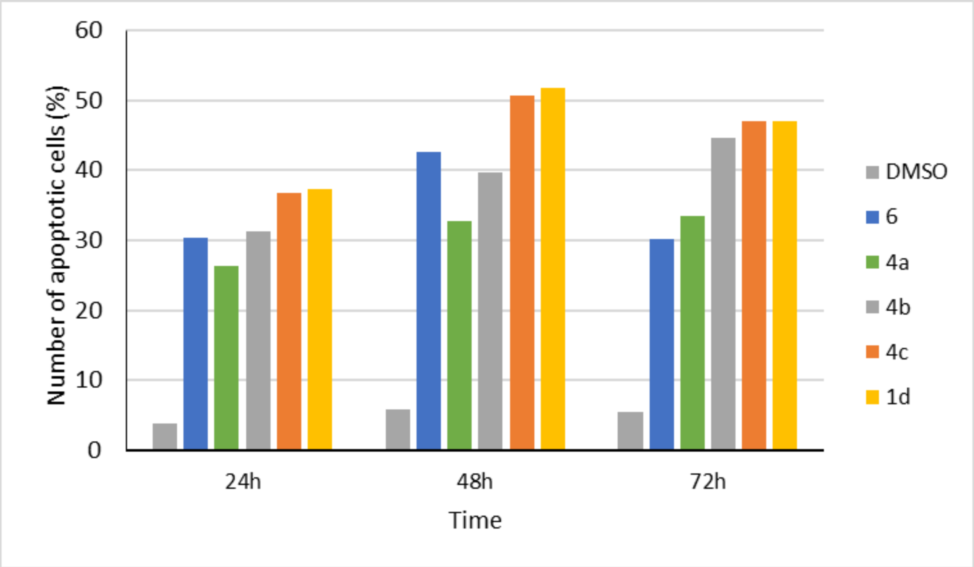
Immunofluorescence staining of cellular microtubules and nuclei

Lung carcinoma A549 cells were cultured in 12-well plates on 11 mm glass coverslips (~20000 cells per coverslip). Cells were incubated with tested compounds at 100–2400 nM for 24 h (0.5 % DMSO served as a negative control). The cells were fixed and stained as described in [6]. Fixed cells were labeled with mouse monoclonal antibody against α-tubulin (Sigma, St. Louis, USA) at a dilution of 1:300, followed by incubation of Alexa Fluor488 labeled goat anti-mouse IgG (Invitrogen, Germany) at a dilution of 1:300. To analyze the effect of compounds on the apoptosis induction, the cell nuclei were stained with the Hoechst Nr. 33258 (Sigma, St. Louis. USA) at 5 μg/ml. Images of all samples were acquired with a Nikon Diaphot 300 inverted microscope (Nikon GmbH, Düsseldorf, Germany) equipped with a cooled charge-couple device camera system (SenSys; Photometrics, Munich, Germany). The number of cells, containing fragmented nuclei (see the figure) related the total 200 cells, was counted using fluorescent microscopy.



Nuclear fragmentation in the cells undergoing apoptosis:
a) intact nucleus; b) nucleus after fragmentation. Bar 10 µm.

Apoptotic index was defined as a percentage that refers to the indicated cell deaths in comparison to all cells [7]. The results are presented at the diagram:



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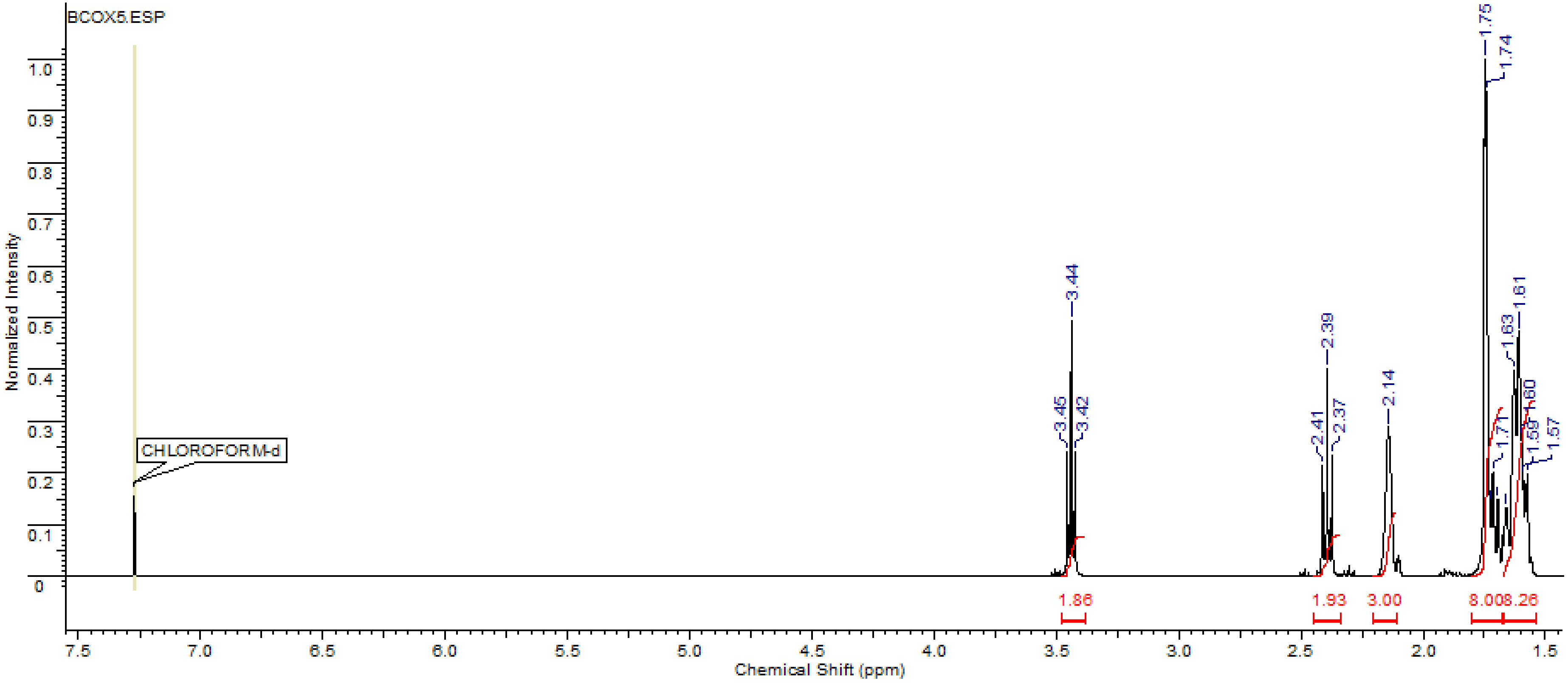
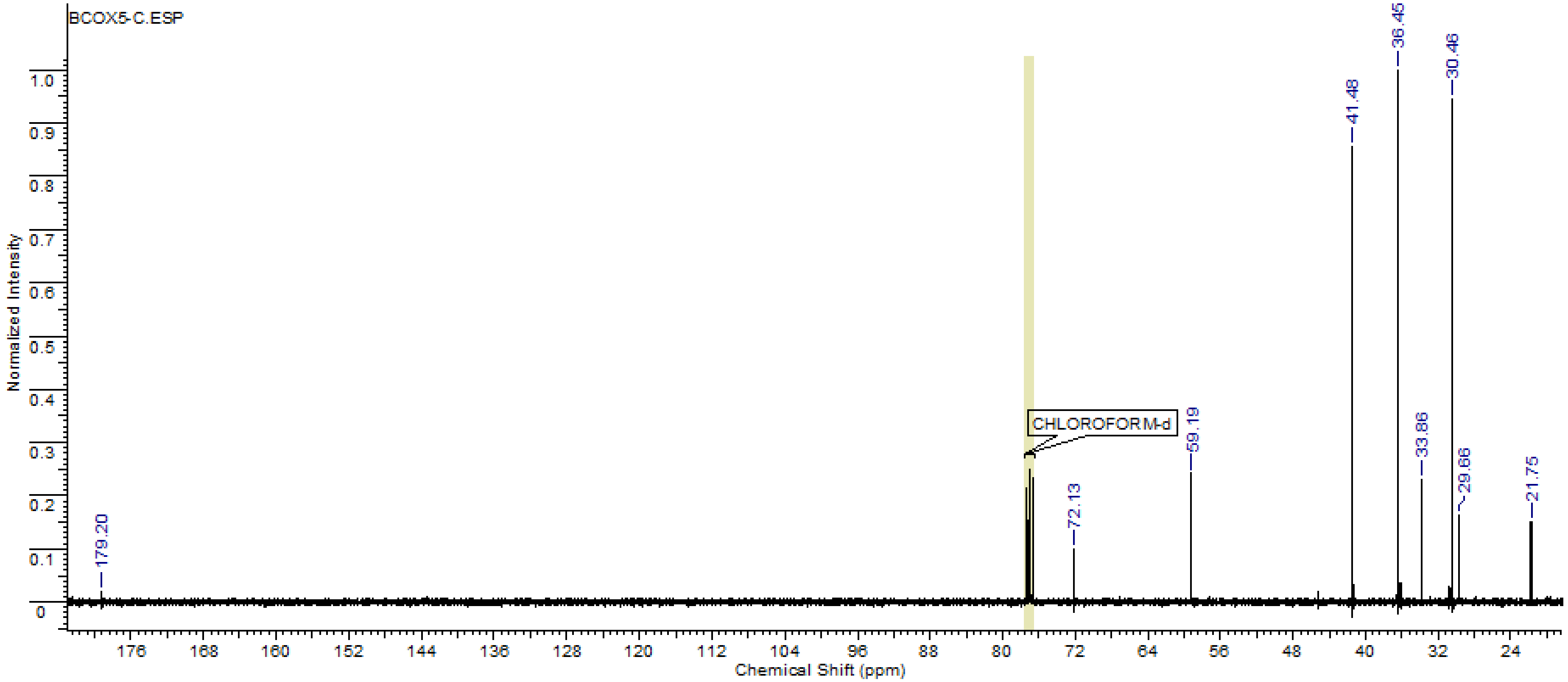
^{1}H and ^{13}C NMR spectra of representative compounds

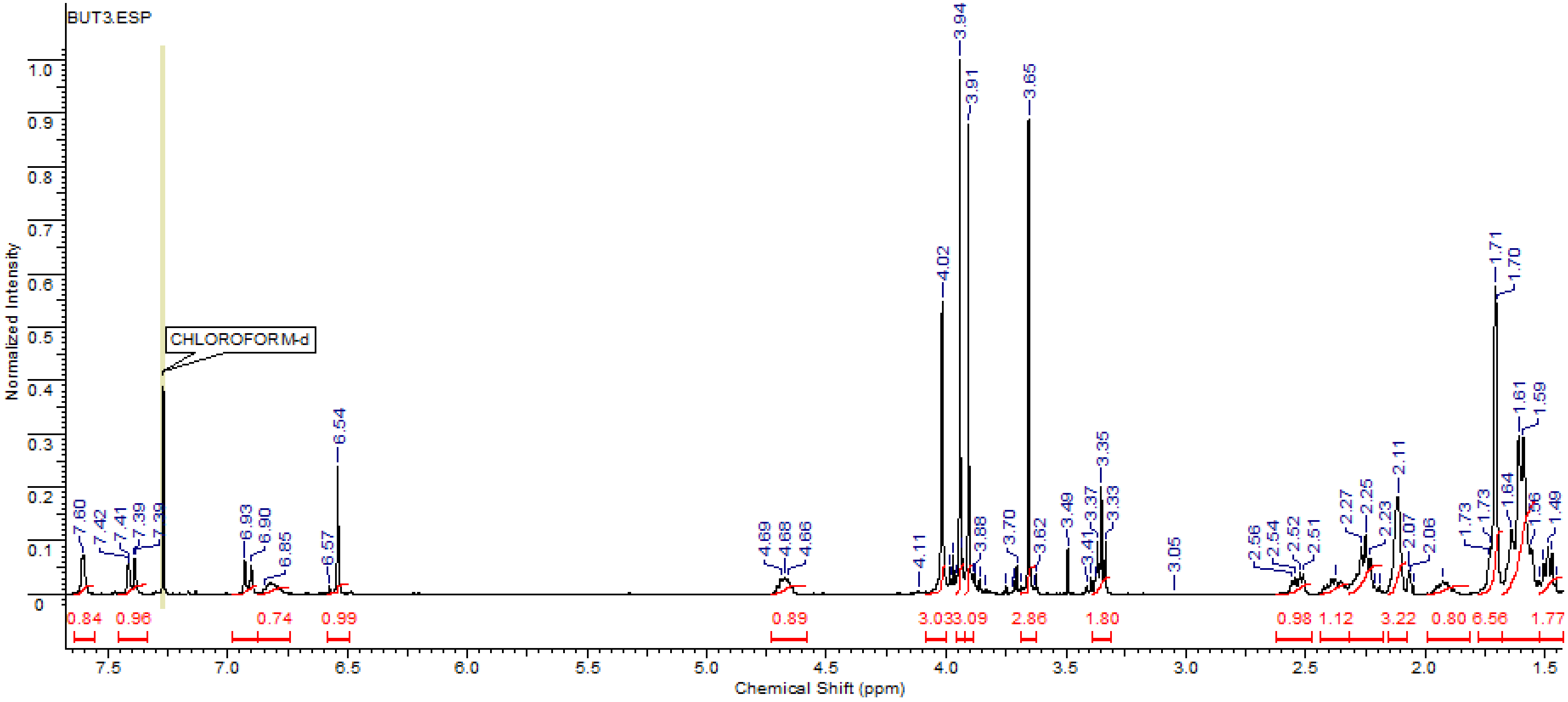
*^{1}H NMR spectrum of 5-(adamantan-1-yloxy)pentan-1-oic acid (**3c**)*



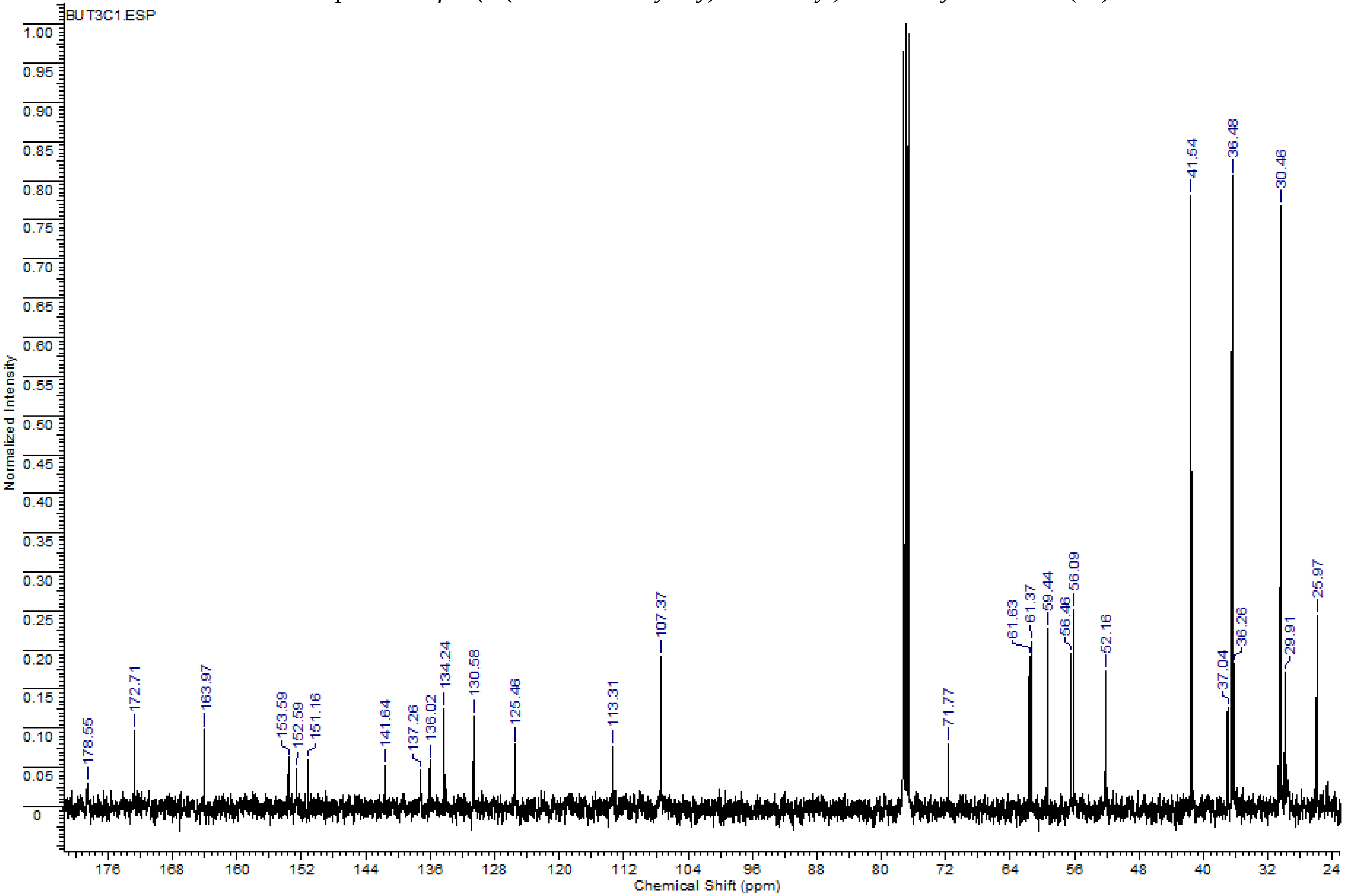
*^{1}H NMR spectrum of 5-(adamantan-1-yloxy)pentan-1-oic acid (**3c**)*



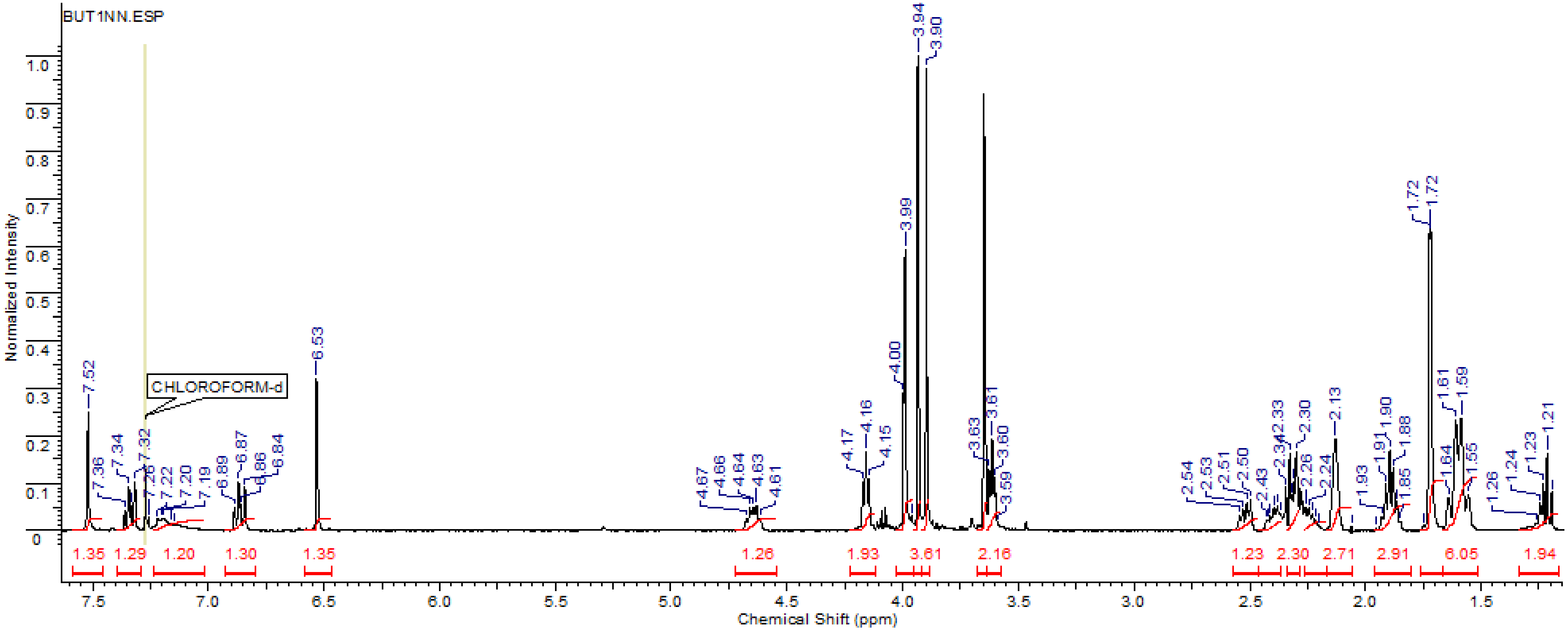
*^{1}H NMR spectrum of N-(4-(adamantan-1-yloxy)-4-butanoyl)-N-deacetylcolchicine (**4b**)*



^{13}C NMR spectrum of *N*-(4-(adamantan-1-yloxy)-4-butanoyl)-*N*-deacetylcolchicine (**4b**)



^{1}H NMR spectrum of N-(2-(Adamantan-1-yloxy)ethan-1-oxy-5-oxopentanoyl)-N-deacetylcolchicine (**6**)



^{13}*C NMR spectrum of N-(2-(Adamantan-1-yloxy)ethan-1-oxy-5-oxopentanoyl)-N-deacetylcolchicine* (**6**)