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for

Subtype Selective Tetracycline Agonists and their Application for a Two-Stage Regulatory System

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Experimental Details

General: All reactions were carried out in air unless otherwise indicated. Doxycycline hydrate, tetracycline hydrochloride and tetracycline hydrate for chemical synthesis were aquired from Heumann Pharma, Synopharm GmbH and Fluka, respectively. All chemicals were used as purchased. Doxycycline and anhydrotetrycycline for biological testing were purchased as hydrochlorides from Sigma and Acros Organics (Geel, Belgium), respectively. D-luciferin and coelenterazine were purchased from P.J.K. (Kleinblittersdorf, Germany). ^{1}H and ^{13}C NMR spectra were recorded at 300 K on a Bruker Avance 360 (360 MHz) or a Bruker Avance 600 (600 MHz). LC-MS analyses were performed with an Agilent 1100 HPLC combined with a Bruker Esquire 2000 mass spectrometer. HR-EIMS spectra were recorded on a Jeol GCmateII. Preparative HPLC was performed on an Agilent 1100 system using RP-18 colums (Agilent Zorbax 300SB, 7µm or CS-Chromatographie Eurospher C-18, 7µm) and CH_3CN/ 0.1% aq. TFA or MeOH/0,1% aq. TFA as solvent systems. MPLC separation was performed on a Büchi Chromatography System (binary pump B-688, gradient former B-687 and glass columns B-685) with UV detection at 254 nm using Europrep 60-30 C18 (Eurochrom®, Knauer) RP silica gel and CH_3CN / 0.1 % aq. TFA as a solvent system. In all cases HPLC grade solvents were used.

Bacterial strains and human cell lines: The bacterial strain DH5α[1] was used for general cloning procedures and is derived from *Escherichia coli* K12. The cell lines HeLa (ATCC #CCL-2), X1/5[2] and HR2^{S}M2L,[3] the latter two derived from HeLa cells, have been described. All cell lines were cultured in high glucose Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% foetal bovine serum (Gibco-BRL), 120 µg/mL penicillin, 120 µg/mL streptomycin and 2 mM L-glutamine in a humidified incubator at 37°C under 7.5% CO_2.

Plasmid constructions: pWHE120(BD)r2 was constructed by amplifying the *tetR*-(B/D) gene containing the mutations E15A/L17G/L25V[4] in a standard polymerase chain reaction[5] reaction with the primers "qac-bwd" and "tetR(D)-Cterm-NgoMIV" using the respective pWH1925 derivative as template. The resulting DNA-fragment was incubated with the restriction enzymes XbaI/NgoMIV and cloned into likewise-restricted pWHE120[6]. The Flp-In integration vector pWHE336 was constructed by restricting both pWHE330 (encoding rtTA2^{S}-M2; A. Schmidt and C. Berens, unpublished) and pWHE120(BD)r2 containing rtTA2^{D}-2 with XcmI/BamHI and ligating the transactivator-encoding fragment with the pWHE330 vector backbone. The correct insertion of the respective fragments was verified by chain-terminating dideoxy sequencing[7]. pWHE120(sM2+M2)6C encoding rtTA-2 was a kind gift from C. Danke. All plasmid and primer sequences are available upon request.

Table 2: Nomenclature, mutations and source of the transactivators used in this study.

Transregulator	official designation	Mutations	Tet class[8]	Source
rtTA-1	rtTA2^{S}-M2	S12G, E19G, A56P, D148E, H179R	B	[10]
rtTA-2	scrtTA2-M2$_{6C}$	S12G, E19G, E37S, W43R, H44N, A56P, D148E, H179R	B	C. Danke and C. Berens, unpublished
rtTA-D	rtTA2^{D}-2;	E15A, L17G,	D	[4]

	derived from revTetR-r1.7	L25V		

Generation of HeLa-derived stable cell lines

Flp-In host cell line HF1-1: pFRT/lacZeo (Invitrogen) was linearized with ScaI and transfected into HeLa cells in 2 wells of a 6-well plate using Lipofectamine (Invitrogen) as recommended by the manufacturer. 24 h after transfection, cells from one 6-well were transferred onto one 15 cm tissue culture dish each and kept in selective medium [100 or 150 µg/mL Zeocin (Cayla, Toulouse, France)]. After 10–14 days, visible single-cell clones were picked, transferred into individual 6-wells and expanded. Zeocin-resistant clones were screened for ß-galactosidase activity[9] to determine the transcriptional activity of the integration site and Southern blot analysis (DIG DNA labeling and detection kit, Roche Diagnostics) was performed to identify single integrants. With 400 units according to Miller[9], the cell line HF1-1 had the highest ß-galactosidase activity of all cell lines obtained and was chosen for the further experiments.

Flp-In reporter cell line HLF33: pUHC13-3[2] carrying a luciferase reporter gene under control of P_{tet-1} and pWHE260 carrying a neomycin resistance gene under control of a simian virus 40 promoter/enhancer were both linearized with ScaI and cotransfected in a molar ratio of 20:1 into HF1-1 cells in 6-well plates using Lipofectamine. After 24 h, the transfected cells from one well were transferred to a 15 cm tissue culture dish and kept in selective medium [800 µg/mL geneticin (Gibco)]. After approximately 10 days, visible single-cell clones were picked, transferred into individual 6-wells and expanded. Cell lines containing an insertion of pUHC13-3 into a silent, but activatable locus were identified by transient transfection of pUHrT62-1[10] encoding $rtTA2^S$-M2 and determination of the luciferase activity 24 h after transfection in the presence or absence of 1 µg/mL dox. The cell clone termed HLF33 displayed no detectable luciferase activity in the absence of dox and a 10^4-fold higher activity in the presence of dox and was used for further experiments.

Flp-In assay cell line HLF33-336: pWHE336 encoding $rtTA2^D$-2 was linearized with ScaI and co-transfected in a 9:1 molar ratio with pOG44 (Invitrogen) encoding Flp recombinase in a 6-well plate into the reporter cell line HLF33 using Lipofectamine. After 3 days at 30°C, the transfected cells from one well were transferred to one 15 cm

tissue culture dish and kept in selective medium [250 µg/mL hygromycin B (Invitrogen)]. Visible cell clones were picked after 10–14 days, transferred into individual 6-wells and expanded. The correct insertion of pWHE336 into the Flp recombinase target site of HLF33 was verified by testing for negative LacZ-staining, sensitivity towards Zeocin and resistance to hygromycin B. Eight of ten clones met these requirements and one was designated HLF33-336.

96-well assay for doxycycline or anhydrotetracycline activity titrations in human cell lines: White, flat-bottom 96-well plates (Greiner Lumitrac 200, #655075) were seeded with 100 µl D-MEM containing approximately 10^4 cells per well and incubated o/n. Then, 100 µl D-MEM containing twice the respective final concentration of the derivative to be tested was added to each well. After 24 h incubation, the medium was sucked off and the cells were lysed by a 10 min incubation in lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM EDTA (pH 8.0), 5% glycerol, 1% Triton X-100, 20 mM DTT]. 25 µl 100 mM K^+-phosphate (pH 7.8) were added to the extract, 5 µL withdrawn to determine the protein content with the Bio-Rad protein assay kit and the remaining 45 µl were used to determine the firefly luciferase activity (see below).

Transient transfections of HeLa cells: Transfections were performed at 50–60% confluency in 24-well-plates with 1 µL Perfectin (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. The DNA mixtures consisted of 2.5 ng for pWHE120(sM2+M2)6C (C. Danke and C. Berens, unpublished) and 20 ng for pWHE120(B/D)r2, 50 ng each of the reporter plasmids pUHC13-9[11] and pWHE192[12], and 100 ng of LacZ expression vector pUHD16-1[13]. Mixtures were adjusted to a total of 250 ng DNA by adding unspecific pWHE121 plasmid DNA[6]. A higher amount of rtTA2^D-2 expressing plasmid DNA was transfected to counterbalance the high protein amounts obtained from plasmids encoding sc TetR variants containing a mammalian codon-usage optimized *tetR* sequence[6]. The plasmid amounts transfected here led to approximately equivalent luciferase activity levels in the HeLa cells. Three wells were transfected in parallel per experiment, and experiments were performed at least twice. After 4 h, doxycycline- or anhydrotetracycline-derivatives were added at twice their final concentration in 1 volume of D-MEM/20% foetal bovine serum to the transfected cells. Cells were harvested 24 h after induction by incubation with 100 µL 100 mM K^+-phosphate (pH 7.8), 2 mM DTT and scraping from the dishes. Cells were lysed by three freeze-thaw cycles because Triton X-100 enhances the autolumines-cence

of the *Renilla* luciferase substrate coelenterazine. Experiments were repeated at least twice.

Luciferase assays: Firefly luciferase activity was determined with 100 mM K^+-phosphate (pH 7.8), 15 mM $MgSO_4$, 5 mM ATP and 0.125 mM D-luciferin in a Berthold Orion microplate luminimeter (P.J.K., Kleinblittersdorf, Germany). *Renilla* luciferase activity was quantified with 250 mM K^+-phosphate (pH 7.8), 250 mM NaCl, 100 mM EDTA and 1.43 µM coelenterazine. 5–10 µl the respective of HeLa cell lysates were added to 100 µl of the respective K^+-phosphate buffer. If signal intensities were too high, lysates were diluted in 100 mM K^+-phosphate (pH 7.8). Light units were normalized for protein amount which was determined by the Bio-Rad protein assay kit and corrected for ß-galactosidase activity[9] to account for different transfection efficiencies.

Determination of minimal inhibitory concentrations by broth microdilution: An 18 h stationary phase culture of *E. coli* WH207(λ*tet*50)[14] was diluted in Luria broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) to a cell density of 2×10^5 cells/mL. 50 µL were then pipetted into a well of a 96-well plate and 50 µl containng a defined concentration (256/128/32/16/8/4/2/1 µg/mL) of the compound to be tested were added. The mixture was incubated for another 18 h in the dark under constant agitation. The lowest concentration exhibiting no turbidity by visual inspection was defined as the minimal inhibitory concentration.

Representative Chemical Syntheses

Anhydrotetracycline-10-triflate (2a): To a stirred solution of **1a** (519.9 mg, 1.22 mmol) in CH_2Cl_2 (80 mL) under N_2 at -20 °C were added NEt_3 (340 µL, 2.44 mmol) and Tf_2O (202 µL, 1.22 mmol). The reaction mixture was warmed to RT within 2.5 h and stirred for 1.5 h before the solvent was removed in vacuo. Unreacted starting material was removed by reversed-phase MPLC. After lyophilization the resulting product was dissolved in MeOH (5 mL), upon addition of Et_2O (70 mL) a precipitate formed, that was removed by filtering the mixture through Celite™ after 3 days at -30 °C. Removal of the solvent in vacuo yielded 460 mg of **2a** · TFA (56 %), purity (LC, 254 nm): 90 %.

4-(Dedimethylamino)anhydrotetracycline-10-triflate (2b): To a solution of **1b** (556.0 mg, 1.45 mmol) in CH_2Cl_2 (75 mL) under N_2 at -20 °C were added NEt_3 (0.2 mL, 1.44 mmol) and Tf_2O (0.36 mL, 2.17 mmol). The reaction mixture was stirred at

-20 °C for 30 min, then at room temperature for 2.5 h. The solution was washed with brine (3 x 50 mL), dried over Na_2SO_4, before the solvent was removed in vacuo. The crude residue was then slurried in CH_2Cl_2 (20 mL), stirred at -20 °C and NEt_3 (0.2 mL, 1.44 mmol) and Tf_2O (0.2 mL, 1.21 mmol) were added. The mixture was stirred at room temperature for 1 h, before it was washed with brine (3 x 50 mL) and dried over Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by reversed phase MPLC to yield 654 mg of **2b** (87.5 %), purity (LC, 254 nm): 98 %.

10-Deoxyanhydrotetracycline (3a): To a solution of triflate **2a** · TFA (40.2 mg, 0.060 mmol), PPh_3 (8.3 mg, 0.032 mmol) and $Pd(OAc)_2$ (1.6 mg, 0.007 mmol) in dry THF under N_2, NEt_3 (33 µl, 0.237 mmol) and formic acid (9.5 µl, 0.248 mmol) were added. The reaction mixture was stirred at 65 °C for 9 hours, then more NEt_3 (33 µL, 0.237 mmol) and formic acid (9.5 µL 0.248 mmol) were added. After 12 h at 65 °C the mixture was filtered through Celite™ and purified by reversed-phase MPLC to yield 18.3 mg of **3a**·TFA (58 %), purity (LC, 254 nm): 97 %.

4-Dedimethylamino-10-deoxyanhydrotetracycline (3b): The compound was prepared with only 2.5 h reaction time according to the procedure for compound **3a**. Yield: 77 %, purity (LC, 254 nm): >99 %.

10-Acetyl-10-deoxyanhydrotetracycline (4a): To a solution of **2a** (63.8 mg, 0.114 mmol), CuI (3.7 mg, 0.019 mmol) and $PdCl_2(PPh_3)_2$ (8.9 mg, 0.013 mmol) in dry THF under N_2 were added TMS-acetylene (65 µL, 0.457 mmol) and piperidine (56 µL, 0.567 mmol). The reaction mixture was stirred at room temperature for 6 h, filtered through a pad of Celite™, then purified by reversed-phase MPLC. After lyophilization, the crude product was dissolved in 2.5 mL of CH_3CN, $pTsOH \cdot H_2O$ (5 equiv) was added and the mixture was refluxed for 50 h. Preparative HPLC yielded 13.6 mg of **4a**·TFA (21 %), purity (LC, 254 nm): 94 %.

10-Acetyl-4-dedimethylamino-10-deoxyanhydrotetracycline (4b): The compound was prepared according to the procedure for compound **4a** (reaction time: 12 h). For the hydrolysis only 45 °C and 2 h were required. Yield: 16.5 %, purity (LC, 254 nm): 98 %.

Carbamate 5c: To a solution of **5a** (32.9 mg, 0.075 mmol) in CH_2Cl_2 were added CDI (12.3 mg, 0.076 mmol) and NEt_3 (21 µl, 0.151 mmol). After the mixture had been stirred at RT for 1 h, the solvent was removed in vacuo. Preparative reversed-phase HPLC yielded 9.0 mg of **5c**·TFA (21 %), purity (LC, 254 nm): 99 %.

General procedure for acylation of 9-aminoanhydrotetracycline (6a-d): To a solution of **5a** in CH_2Cl_2 (20 mg/mL) were added NEt_3 (1 equiv) and carboxylic acid anhydride (1 equiv). The reaction mixture was stirred at room temperature for 1.5 to 2.5 h before the solvent was removed in vacuo. The product was purified by reversed-phase MPLC or HPLC to yield the 9-acylaminoanhydrotetracyclines **6a-d**·TFA in 43–61 %, purity (LC, 254 nm): 92–99 %.

General procedure for acylation of 9-aminodoxycycline (13a-d): To a solution of **11a** hydrochloride in DMF was added carboxylic acid anhydride (2-5 eq.) and $NaHCO_3$ (2 eq.). The mixture was stirred at ambient temperature for 2 h, diluted with MeOH (2 mL) and H_2O (2 mL) and purified by reversed-phase HPLC to yield the 9-acylaminodoxycyclines **13a-d**·TFA in 35–57 % yield, purity (LC, 254 nm): 96–99 %.

General procedure for reductive amination (5b, 11b, 12b): To a solution of the amine **5a, 11a** or **12a** in aqueous MeOH (50 %) propionaldehyde (10 equiv), $NaCNBH_3$ (1 equiv) and HCl (1 equiv) were added. The reaction mixture was stirred at room temperature for 1.5 h, then the product was isolated by reversed-phase MPLC or HPLC as di-trifluoracetate in 43–65 % yield, purity (LC, 254 nm): 91–97 %.

9-(p-Carboxyphenyl)anhydrotetracycline (8a): **8a** was prepared from **7a**·TFA according to the procedure for **8b**. The reaction mixture was either heated in an oil bath or by microwave irradiation, when the temperature was ramped from rt to 150 °C with irradiation of 100 W. The desired temperature was held for 5 min. Reversed phase MPLC followed by preparative HPLC yielded 12–17 % of **8a.**

9-(p-Carboxyphenyl)-4-(dedimethylamino)anhydrotetracycline (8b): In a 10 mL glass tube were placed **7c** (68.7 mg, 0.149 mmol), 4-carboxyphenylboronic acid (99.0 mg, 0.597 mmol), tetrabutylammonium bromide (61.9 mg, 0.192 mmol), $Pd(OAc)_2$ (2.6 mg, 0.012 mmol) and K_2CO_3 (119.5 mg, 0.918 mmol), 1 mL H_2O and a magnetic stirring bar. The vessel was sealed, purged with N_2, and stirred in an oilbath heated to 125 °C for 16 h. After cooling, 1 mL CH_3CN was added and the mixture was filtered through a pad of Celite™, then purified by reversed-phase MPLC to yield 27 % of **8b,** purity (LC, 254 nm): 98 %.

General procedure for Suzuki coupling of 9-iododoxycyclines (15a,b): A solution of the respective 9-iododoxycycline **14a,b** (50 mg), $PhB(OH)_2$ (2equiv), $Pd(OAc)_2$ (0.1 equiv) and Na_2CO_3 in a mixture of DMF, MeOH and H_2O was heated to 70°C, whereas the reaction progress was monitored by LC-MS. After consumption of the iodo de-

rivative the mixture was filtered through a pad of Celite™ and purified by preparative HPLC to give pure **15a,b** in 63–72 % yield, purity (LC, 254 nm): 90–99 %.

9-Acetylanhydrotetracycline (9): To a solution of **7b** · HCl (88.0 mg, 0.149 mmol), CuI (8.0 mg, 0.042 mmol) and $PdCl_2(PPh_3)_2$ in 2 mL dry THF under N_2 were added TMS-acetylene (85 µl, 0.597 mmol) and piperidine (75 µl, 0.759 mmol). The reaction mixture was stirred 40 °C for 18 h, filtered through celite, then the solvent was removed in vacuo. To the crude residue were added 2 mL CH_3CN and 2 mL of aqueous TFA (5 %). After stirring at 80 °C for 4 h, the solvent was removed by lyophilization. Preparative reversed-phase HPLC yielded 13.1 mg of **9** TFA (14 %), purity (LC, 254 nm): 99 %.

General procedure for the preparation of furotetracyclines 16a,b: The solution of **7b/d**, CuI (0.15 equiv), $PdCl_2(PPh_3)_2$ (0.1 equiv) and piperidine (5equiv) in dry THF under N_2 was cooled to -20 °C and purged with propyne for 20 s. The mixture was kept under a propyne atmosphere and stirred at -20 °C for 1.5 h, before it was slowly warmed to 40 °C. After 24 h at 40 °C, the mixture was filtered through Celite™ and the solvent removed in vacuo. Reversed phase MPLC and preparative HPLC yielded **16a**·TFA (4 %) and **16b** (49 %), purity (LC, 254 nm): 83 %.

Analytical data of selected compounds

*Anhydrotetracycline-10-triflate (**2a**)·TFA*: ^{1}H NMR (600 MHz, C_5D_5N): δ (ppm) = 2.42 (3 H, s), 2.60 (6 H, s), 3.17 (1 H, ddd, J = 11.9, 5.0, 1.8 Hz), 3.59 (1 H, dd, J = 16.8, 5.0 Hz), 3.70 (1 H, d, J = 11.9 Hz), 3.77 (1 H, dd, J = 16.8, 1.8 Hz), 7.48 (1 H, d, J = 7.7 Hz), 7.64 (1 H, dd, J = 8.5, 7.7 Hz), 7.94 (1 H, d, J = 8.5 Hz), 10.16 (1 H, br s), 10.39 (1 H, br s); ^{13}C NMR (150 MHz, C_5D_5N): δ (ppm) = 14.8, 25.8, 42.1, 42.4, 65.7, 78.4, 101.5, 112.2, 117.4, 118.5 (q, J = 292.2 Hz), 119.7 (q, J = 321.2 Hz), 119.8, 123.3, 125.9, 130.7, 135.5, 140.5, 148.1, 162.2, 162.6 (q, J = 34.3 Hz), 175.0, 193.7, 198.1, 201.7; HR-EIMS: calcd for $C_{23}H_{21}F_3N_2O_9S$ $[M]^+$ 558.0920, found: 558.0921.

*4-(Dedimethylamino)anhydrotetracycline-10-triflate (**2b**)*: ^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) = 2.53 (3 H, s), 2.69 (1 H, dd, J = 18.5, 8.0 Hz), 2.91-2.99 (1H, m), 3.06 (1H, dd, J = 17.2, 6.6 Hz), 3.09 (1 H, dd, J = 18.5, 5.4 Hz), 3.51 (1 H, dd, J = 17.2, 4.8 Hz), 4.88 (1 H, br s), 5.90 (1 H, br s), 7.38 (1 H, br d, J = 7.7 Hz), 7.70 (1 H, dd, J = 8.7, 7.7 Hz), 8.4 (1 H, dd, J = 8.7, 0.7 Hz), 9.12 (1 H, br s), 14.14 (1 H, s), 18.07 (1 H, s); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) = 14.7, 31.1, 34.7, 35.8, 76.9, 100.6, 110.6, 117.4, 119.0 (q, J = 321 Hz), 119.6, 122.4, 124.8, 130.4, 132.8, 140.0, 147.8, 162.2,

173.3, 191.5, 194.9, 199.3; HR-EIMS: calcd for $C_{21}H_{16}F_3NO_9S$ $[M]^+$ 515.0498, found: 515.0494.

*10-Deoxyanhydrotetracycline (**3a**)·TFA*: ^{1}H NMR (600 MHz, C_5D_5N): *d* (ppm) = 2.46 (3 H, s), 2.58 (6 H, s), 3.14 (1 H, ddd, *J* = 11.7, 4.8, 2.0 Hz), 3.62 (1 H, dd, *J* = 16.6, 4.8 Hz), 3.72 (1 H, d, *J* = 11.7 Hz), 3.78 (1 H, dd, *J* = 16.6, 2.0 Hz), 7.49 (1 H, dd, *J* = 8.1, 6.9 Hz), 7.66 (1 H, ddd, *J* = 8.5, 6.9, 1.2 Hz), 7.95 (1 H, d, *J* = 8.5 Hz), 8.06 (1 H, d, *J* = 8.1 Hz), 10.15 (1 H, br s), 10.34 (1 H, br s), 14.07 (1 H, br s); ^{13}C NMR (90 MHz, C_5D_5N): *d* (ppm) = 14.1, 25.5, 42.5, 42.6, 65.7, 78.4, 101.7, 110.8, 118.5 (q, *J* = 295.0 Hz), 122.8, 124.4, 124.8, 125.3, 125.7, 131.5, 132.9, 138.4, 162.6 (q, *J* = 34.3 Hz), 163.5, 175.0, 194.2, 198.1, 200.9; HR-EIMS: calcd for $C_{22}H_{22}N_2O_6$ $[M]^+$ 410.1478, found: 410.1478.

*4-Dedimethylamino-10-deoxyanhydrotetracycline (**3b**)*: ^{1}H NMR (360 MHz, $CDCl_3$): *d* (ppm) = 2.49 (3 H, s), 2.67 (1 H, dd, *J* = 18.5, 7.7 Hz), 2.85-2.93 (1 H, m), 2.97-3.10(2 H, 2 x dd, *J* = 17.3, 6.8 Hz and 18.4, 5.1 Hz), 3.45 (1 H, dd, *J* = 17.3, 5.1 Hz), 4.83 (1 H, br s), 5.80 (1 H, br s), 7.50 (1 H, ddd, *J* = 8.3, 6.9, 1.1 Hz), 7.69 (1 H, ddd, *J* = 8.5, 6.9, 1.3 Hz), 7.94 (1 H, br d, *J* = 8.5 Hz), 8.47 (1 H, dd, *J* = 8.3, 1.3 Hz), 9.1 (1 H, br s), 13.47 (1 H, br s), 17.99 (1 H, br s); ^{13}C NMR (90 MHz, $CDCl_3$): *d* (ppm) = 13.9, 28.7, 34.8, 36.3, 77.0, 100.7, 109.2, 122.2, 123.9, 124.0, 125.2, 125.5, 130.3, 131.4, 137.9, 163.3, 173.4, 192.1, 195.1, 198.8; HR-EIMS: calcd for $C_{20}H_{17}NO_6$ $[M]^+$ 367.1056, found: 367.1055.

*10-Acetyl-10-deoxyanhydrotetracycline (**4a**)·TFA*: ^{1}H NMR (600 MHz, C_5D_5N): *d* (ppm) = 2.47 (3 H, s), 2.56 (3 H, s), 2.60 (6 H, s), 3.15 (1 H, ddd, *J* = 1.7, 4.6, 11.7 Hz), 3.63 (1 H, d, *J* = 4.6, 16.6 Hz), 3.73 (1 H, d, *J* = 11.7 Hz), 3.79 (1 H, br d, *J* = 16.6 Hz), 7.38 (1 H, d, *J* = 7.2 Hz), 7.65 (1 H, dd, *J* = 7.2, 8.7 Hz), 7.97 (1 H, d, *J* = 8.7 Hz), 10.14 (1 H, br s), 10.36 (1 H, br s); ^{13}C NMR (90 MHz, C_5D_5N): *d* (ppm) = 14.5, 25.7, 32.3, 42.5, 42.6, 65.7, 78.5, 101.7, 111.6, 118.6 (q, *J* = 294.2 Hz), 120.5, 123.1, 123.5, 125. 7, 130.9, 134.2, 138.6, 143.4, 162.6, 162.7 (q, *J* = 34.4 Hz), 175.1, 194.1, 198.3, 200.9, 200.9; HR-EIMS: calcd for $C_{24}H_{24}N_2O_7$ $[M]^+$ 452.1584, found: 452.1584.

*10-Acetyl-4-dedimethylamino-10-deoxyanhydrotetracycline (**4b**)*: ^{1}H NMR (360 MHz, $CDCl_3$): *d* (ppm) = 2.50 (3 H, s), 2.59 (3 H, s), 2.66 (1 H, dd, *J* = 18.9, 7.9 Hz), 2.88–2.95 (1 H, m), 2.99-3.09 (2 H, m), 3.48 (1 H, dd, *J* = 17.2, 4.3 Hz), 5.85 (1 H, br s), 7.25-7.27 (1 H, m), 7.68 (1 H, dd, *J* = 8.3, 7.2 Hz), 8.00 (1 H, d, *J* = 8.3 Hz), 9.10 (1

H, br s), 13.84 (1 H, br s), 18.00 (1 H, br s); ^{13}C NMR (150 MHz, $CDCl_3$): *d* (ppm) = 14.3, 28.5, 32.2, 34.7, 36.1, 77.0, 100.6, 109.9, 119.7, 122.8, 122.9, 125.0, 130.7, 131.5, 138.2, 142.5, 162.1, 173.3, 191.7, 195.0, 198.0, 205.9; HR-EIMS: calcd for $C_{22}H_{19}NO_7$ $[M]^+$ 409.1161, found: 409.1161.

Carbamate derivative ***5c**·TFA*: ^{1}H NMR (600 MHz, CD_3OD): *d* (ppm) = 2.30 (3 H, s), 3.02-3.09 (1 H, m), 3.16 (6 H, s), 3.49 (1 H, dd, *J* = 16.6, 4.2 Hz), 3.54-3.59 (1 H, m), 4.10-4.25 (1 H, m), 7.26 (1 H, d, *J* = 8.7 Hz), 7.50 (1 H, d, *J* = 8.7 Hz); ^{13}C NMR (90 MHz, CD_3OD): *d* (ppm) = 14.3, 30.3, 37.7, 43.9, 69.8*, 78.1, 99.0, 111.2, 111.4, 115.4, 118.3 (q, *J* = 293.1 Hz), 121.3, 123.4, 128.2, 129.9, 134.4, 140.6, 157.3, 161.0, 163.1 (q, *J* = 34.1 Hz), 174.6, 193.9, 199.7, 200.8. (*weak signal due to peak broadening); HR-EIMS: calcd for $C_{23}H_{21}N_3O_8$ $[M]^+$ 467.1328, found: 467.1333.

*9-Butyrylaminoanhydrotetracycline (**6c**)*: ^{1}H NMR (600 MHz, $CDCl_3$): *d* (ppm) = 1.04 (3 H, t, *J* = 7.4 Hz), 1.81 (2H, tq, *J* = 7.4, 7.4 Hz), 2.44 (2H, t, *J* = 7.4 Hz), 2.45 (3H, s), 2.46 (6H, s), 2.69 (1H, ddd, *J* = 10.6, 4.3, 3.3 Hz), 3.22 (1H, dd, *J* = 16.7, 4.3 Hz), 3.32 (1H, d, *J* = 10.6 Hz), 3.51 (1H, br d, *J* = 16.7 Hz), 5.91 (1H, d, *J* = 3.6 Hz), 7.45 (1H, d, *J* = 9.1 Hz), 7.82 (1H, s), 8.77 (1H, d, *J* = 9.1 Hz), 9.20 (1H, d, *J* = 3.6 Hz), 10.15 (1H, s), 15.61 (1H, br s), 18.18 (1H, br s); ^{13}C NMR (90 MHz, $CDCl_3$): *d* (ppm) = 13.8, 14.2, 19.1, 24.3, 40.0, 41.2, 42.4, 64.7, 76.3, 101.0, 108.5, 111.7, 115.4, 123.5, 122.9, 125.5, 128.1, 128.7, 145.2, 165.8, 171.4, 173.1, 171.4, 191.7, 197.1, 197.1, 197.6; HR-EIMS: calcd for $C_{26}H_{29}N_3O_8$ $[M]^+$ 511.1954, found: 511.1956.

*9-(p-Carboxyphenyl)-4-(dedimethylamino)anhydrotetracycline (**8b**)*: ^{1}H NMR (600 MHz, $(CD_3)_2SO$, H/D-exchange): *d* (ppm) = 2.34 (3 H, s), 2.48-2.54 (1 H, m), 2.87 (1 H, dd, *J* = 17.2, 8.9 Hz), 2.78-2.83 (1 H, m), 2.84-2.91 (1 H, m), 3.33 (1 H, dd, *J* = 17.2, 4.7 Hz), 7.54 (1 H, d, *J* = 8.7 Hz), 7.69 (1 H, d, *J* = 8.7 Hz), 7.74 (2 H, m), 7.99 (2 H, m); ^{13}C NMR (90 MHz, $(CD_3)_2SO$): *d* (ppm) = 13.8, 28.9, 34.8, 36.3, 77.5, 99.2, 109.2, 112.0, 115.4, 121.4, 121.9, 129.0, 129.1, 129.5, 132.6, 133.8, 138.4, 142.0, 154.6, 163.4, 167.2, 173.1, 191.7, 195.5, 201.5; HR-EIMS: calcd for $C_{27}H_{21}NO_9$ $[M]^+$ 503.1216, found: 503.1217.

*9-Acetylanhydrotetracycline (**9**)·TFA*: ^{1}H NMR (600 MHz, C_5D_5N): *d* (ppm) = 2.40 (3 H, s), 2.61 (6 H, s), 2.71 (3 H, s), 3.14 (1 H, ddd, *J* = 11.7, 4.9, 1.8 Hz), 3.61 (1 H, dd, *J* = 17.0, 4.9 Hz), 3.76 (1 H, d, *J* = 11.7 Hz), 3.77 (1 H, dd, *J* = 17.0, 1.8 Hz), 7.35 (1 H, d, *J* = 9.1 Hz), 8.08 (1 H, d, *J* = 9.1 Hz), 10.16 (1 H, s), 10.35 (1 H, s); ^{13}C NMR (90 MHz, C_5D_5N): *d* (ppm) = 14.7, 26.2, 29.1, 42.5, 42.6, 65.9, 78.7, 101.8, 111.7,

114.7, 115.1, 116.3, 118.6 (q, *J* = 289.3 Hz), 121.8, 132.2, 138.8, 144.0, 162.7 (q, *J* = 34.6 Hz), 167.4, 169.1, 175.1, 194.6, 198.2, 199.7, 202.7; HR-EIMS: calcd for $C_{24}H_{24}N_2O_8$ $[M]^+$ 468.1533, found: 468.1533.

*9-Dipropylaminodoxycycline (**11b**)·2 TFA*: ^{1}H NMR (360 MHz, CD_3OD): *d* (ppm) = 0.98 (6 H, t, *J* = 7.4 Hz), 1.48-1.60 (4 H, m), 1.63 (3 H, d, *J* = 6.8 Hz), 2.70 (1 H, dd, *J* = 12.4, 8.3 Hz), 2.85-2.96 (2 H, m), 2.99 (6 H, s), 3.33-3.36 (1H, m), 3.55-3.67 (1 H, m), 4.45 (1H, br s), 7.28 (1 H, d, *J* = 8.6 Hz), 7.86 (1 H, d, *J* = 8.6 Hz); HR-EIMS: calcd for $C_{28}H_{37}N_3O_8$ $[M]^+$ 543.2580, found: 543.2581.

*4-Dedimethylamino-9-dipropylaminodoxycycline (**12b**)·TFA*: ^{1}H NMR (360 MHz, CD_3OD): *d* (ppm) = 0.94 (6 H, t, *J* = 7.4 Hz), 1.44-1.54 (4 H, m), 1.57 (3 H, d, *J* = 6.8 Hz), 2.37 (1 H, ddd, *J* = 10.7, 5.6, 2.4 Hz), 2.47 (1 H, dd, *J* = 13.0, 8.1 Hz), 2.82 (1 H, dq, *J* = 13.0, 6.8 Hz), 2.92 (1 H, dd, *J* = 18.6, 2.4 Hz), 3.05 (1H, dd, *J* = 18.6, 5.6 Hz), 3.53-3.62 (4 H, m), 3.68 (1 H, dd, *J* = 10.7, 8.1 Hz), 7.22 (1 H, d, *J* = 8.7 Hz) 7.81 (1 H, d, *J* = 8.7 Hz); ^{13}C NMR (90 MHz, CD_3OD): *d* (ppm) = 10.8, 16.2, 19.5, 31.0, 40.1, 44.9, 47.5, 61.2, 69.9, 75.9, 99.6, 108.0, 125.0, 129.5, 151.8, 155.6, 175.1, 178.2, 194.4, 196.5; APCI-MS: *m*/*z* = 501 $[M+1]^+$.

*9-Acetylaminodoxycycline (**13b**)·TFA*: ^{1}H NMR (360 MHz, CD_3OD): *d* (ppm) = 1.58 (3 H, d, *J* = 6.7 Hz), 2.23 (3 H, s), 2.60 (1 H, dd, *J* = 12.3, 8.3 Hz), 2.75-2.88 (2 H, m), 2.99 (6 H, s), 3.60 (1 H, dd, *J* = 11.5, 8.3 Hz), 4.44 (1H, br s), 6.98 (1 H, d, *J* = 8.4 Hz), 8.20 (1 H, d, *J* = 8.4 Hz); HR-EIMS: calcd for $C_{24}H_{27}N_3O_9$ $[M]^+$ 501.1747, found: 501.1748.

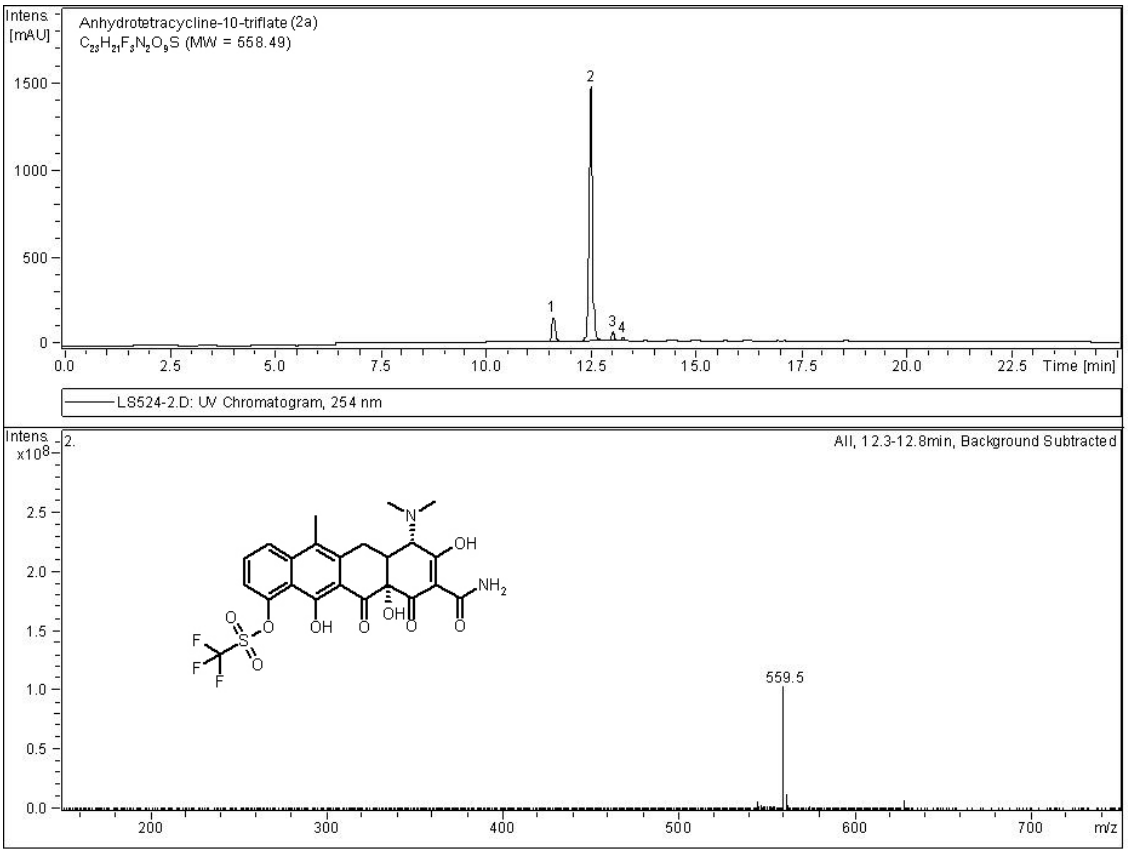
*9-Benzoylaminodoxycycline (**13d**)·TFA*: ^{1}H NMR (360 MHz, CD_3OD): *d* (ppm) = 1.59 (3 H, d, *J* = 7.0 Hz), 2.63 (1 H, dd, *J* = 12.1, 8.3 Hz), 2.78-2.87 (2 H, m), 2.97 (6 H, s), 3.60 (1 H, dd, *J* = 11.5, 8.4 Hz), 4.43 (1H, br s), 7.04 (1 H, d, *J* = 8.4 Hz), 7.55 (2 H, t, *J* = 7.6 Hz), 7.62 (1 H, t, *J* = 7.6 Hz), 7.97 (2 H, *J* = 7.6 Hz), 8.25 (1 H, d, *J* = 8.4 Hz); HR-EIMS: calcd for $C_{29}H_{29}N_3O_9$ $[M]^+$ 563.1903, found: 563.1904.

9-Phenyl-4-dedimethylaminodoxycycline (**15b**): ^{1}H NMR (360 MHz, CD_3OD): *d* (ppm) = 1.55 (3 H, d, *J* = 6.6 Hz), 2.36 (1 H, ddd, *J* = 10.9, 5.4, 2.2 Hz), 2.42 (1 H, dd, *J* = 12.5, 7.9 Hz), 2.74 (1 H, dq, *J* = 13.22, 6.4 Hz), 2.94 (1 H, dd, *J* = 18.8, 2.4 Hz), 3.66 (1 H, dd, *J* = 10.8, 8.1 Hz), 7.02 (1 H, d, *J* = 7.9 Hz), 7.31 (1 H, m), 7.39 (2 H, m), 7.55 (3 H, m); APCI-MS: *m*/*z* = 478 $[M+1]^+$.

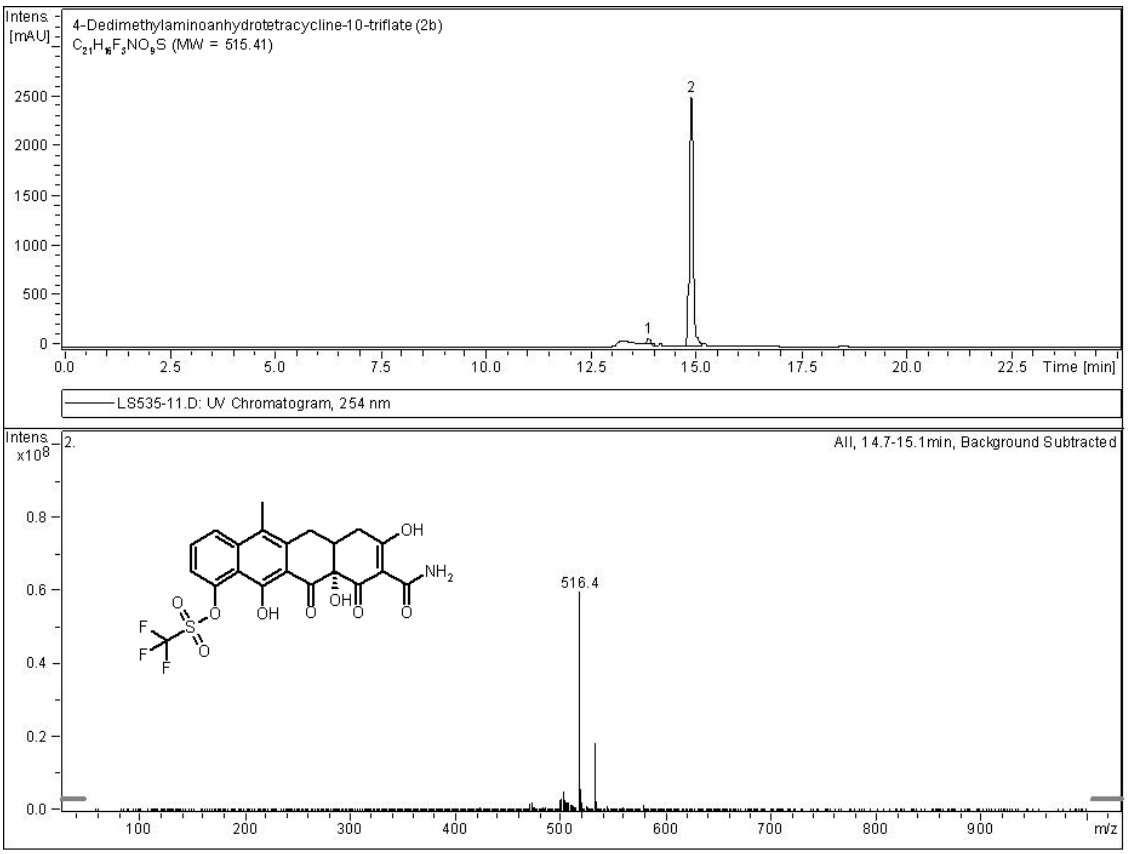
Furotetracycline derivative ***16b***: ^{1}H NMR (600 MHz, $CDCl_3$): *d* (ppm) = 2.50 (3 H, s), 2.63 (3 H, d, *J* = 1.0 Hz), 2.68 (1 H, dd, *J* = 18.8, 7.5 Hz), 2.91 (1 H, dddd, *J* = 7.3, 7.2, 5.4, 4.6 Hz), 2.99-3.12 (2 H, m), 3.46 (1 H, dd, *J* = 17.0, 4.6 Hz), 5.88 (1 H, br s), 6.52 (1 H, d, *J* = 1.0 Hz), 7.74 (1 H, d, *J* = 8.7 Hz), 7.79 (1 H, d, *J* = 8.7 Hz), 9.11 (1 H, br s), 13.88 (1 H, br s), 18.01 (1 H, br s); ^{13}C NMR (90 MHz, $CDCl_3$): *d* (ppm) = 14.5, 14.8, 29.0, 34.7, 36.2, 77.0, 100.7, 103.0, 109.7, 111.7, 119.0, 122.6, 124.7, 126.2, 129.4, 135.7, 151.2, 156.1, 161.8, 173.3, 192.0, 195.1, 198.8; HR-EIMS: calcd for $C_{23}H_{19}NO_7$ $[M]^+$ 421.1161, found: 421.1162.

LC-MS chromatograms of representative anhydrotetracycline derivatives: Analyses were performed with an Agilent 1100 Binary Gradient (MeOH/0.1N aq. HCOOH 10/90-90/10) HPLC system and UV detection at 254 nm using a Zorbax SB-C8 (4.6 mm x 150 mm, 5 µm) column in combination with a Bruker Esquire 2000 mass spectrometer using APC ionization.

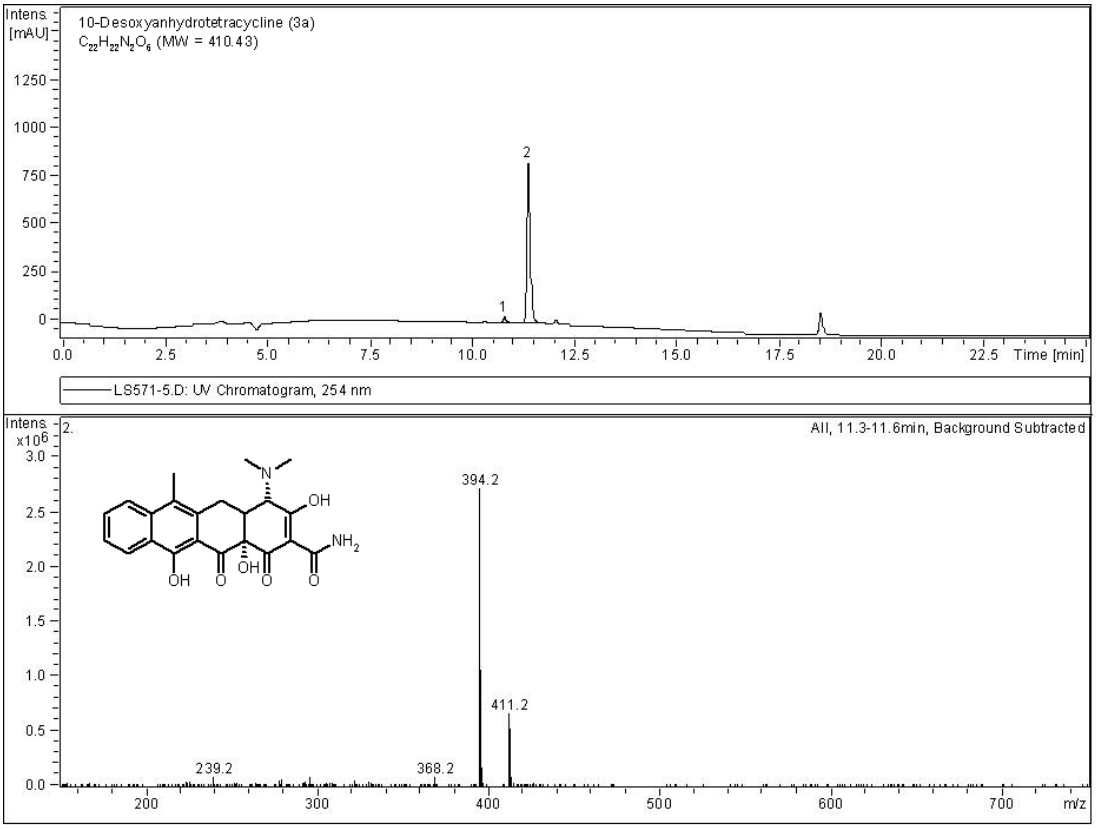
HPLC purities are given below the depicted spectra. Anhydrotetracycline derivatives tended to show partial epimerization within the HPLC run. In these cases the amount of the minor diasteromer is also given.



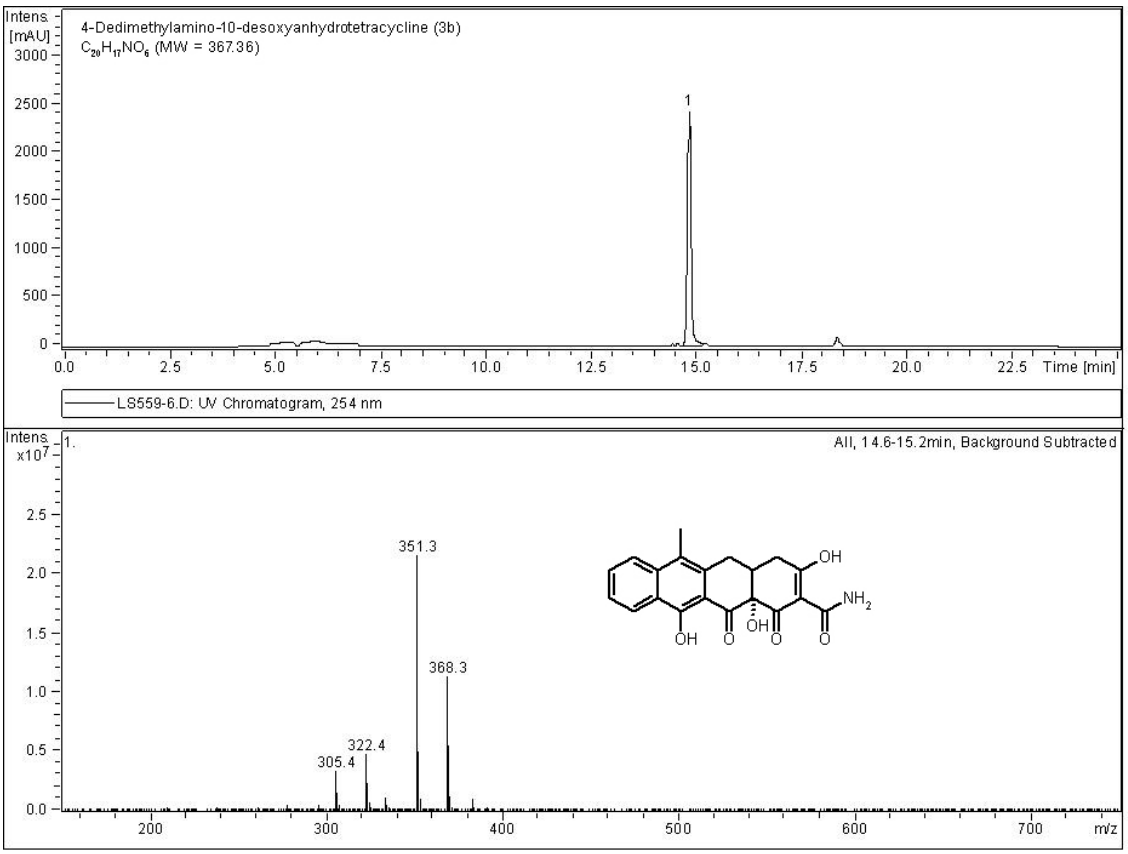
Purity of **2a** (peak 2): 90%, amount of minor diastereomer (peak 1): 7%



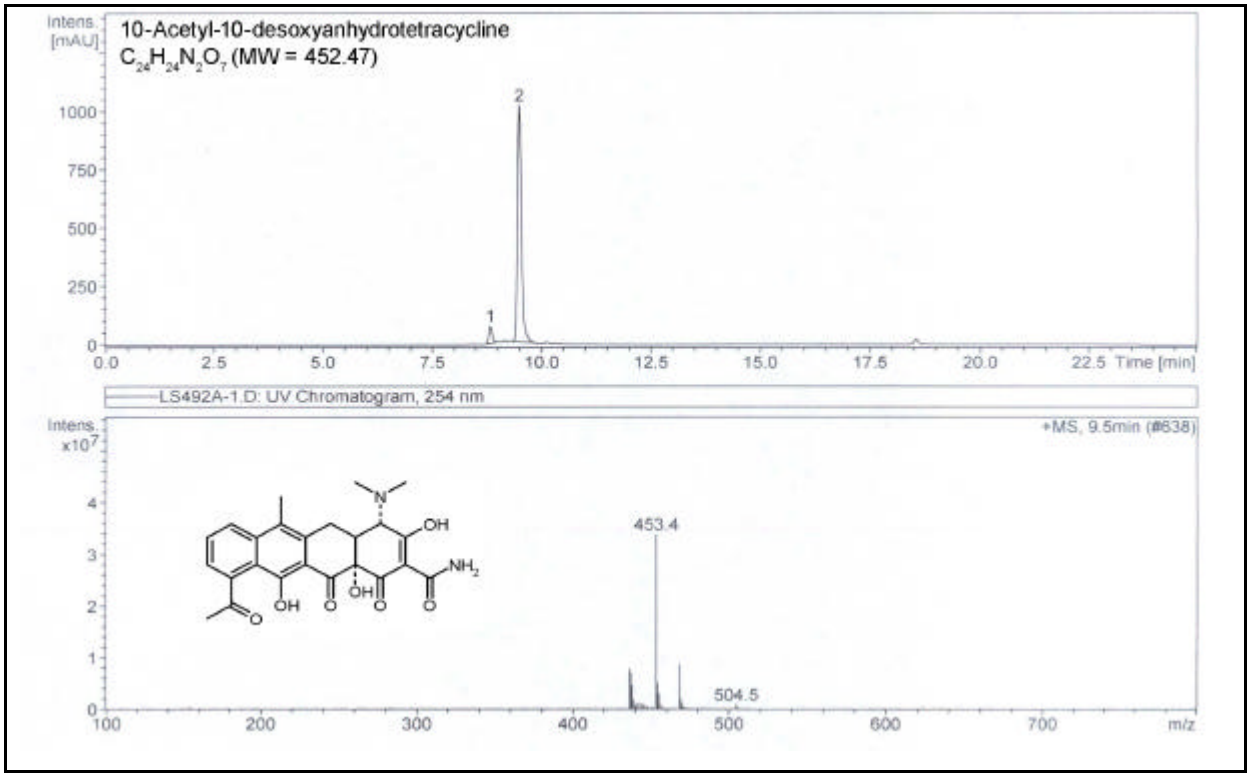
Purity of **2b** (peak 2): 98%



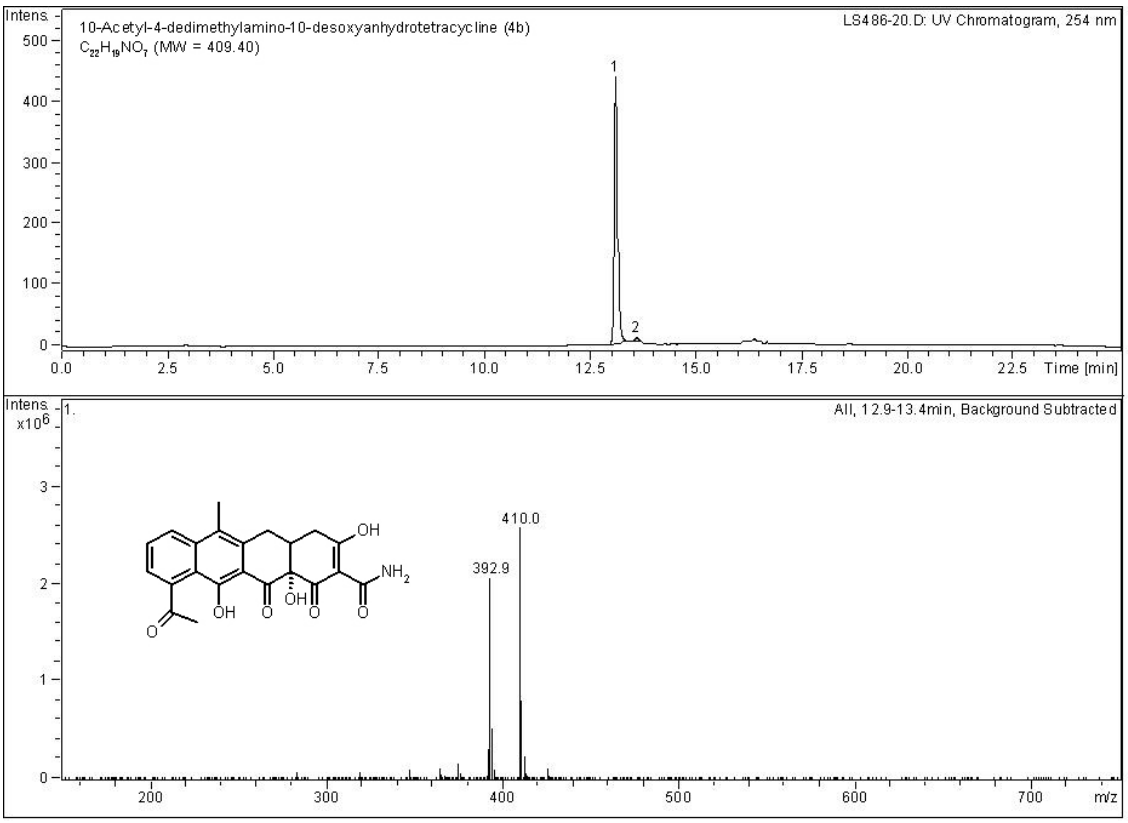
Purity of **3a** (peak 2): 97% (peak at 18.5 min retention time is due to impurities in the injection system)



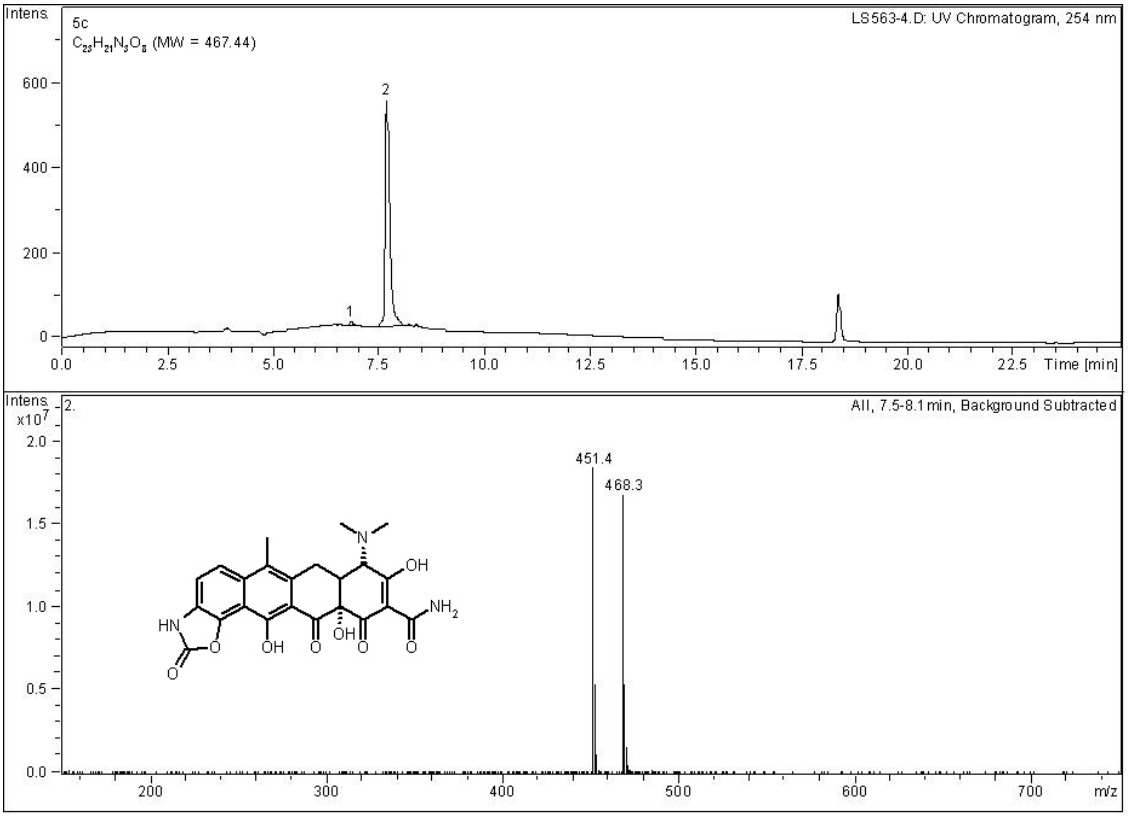
Purity of **3b** (peak 1): 99% (peak at 18.5 min retention time is due to impurities in the injection system)



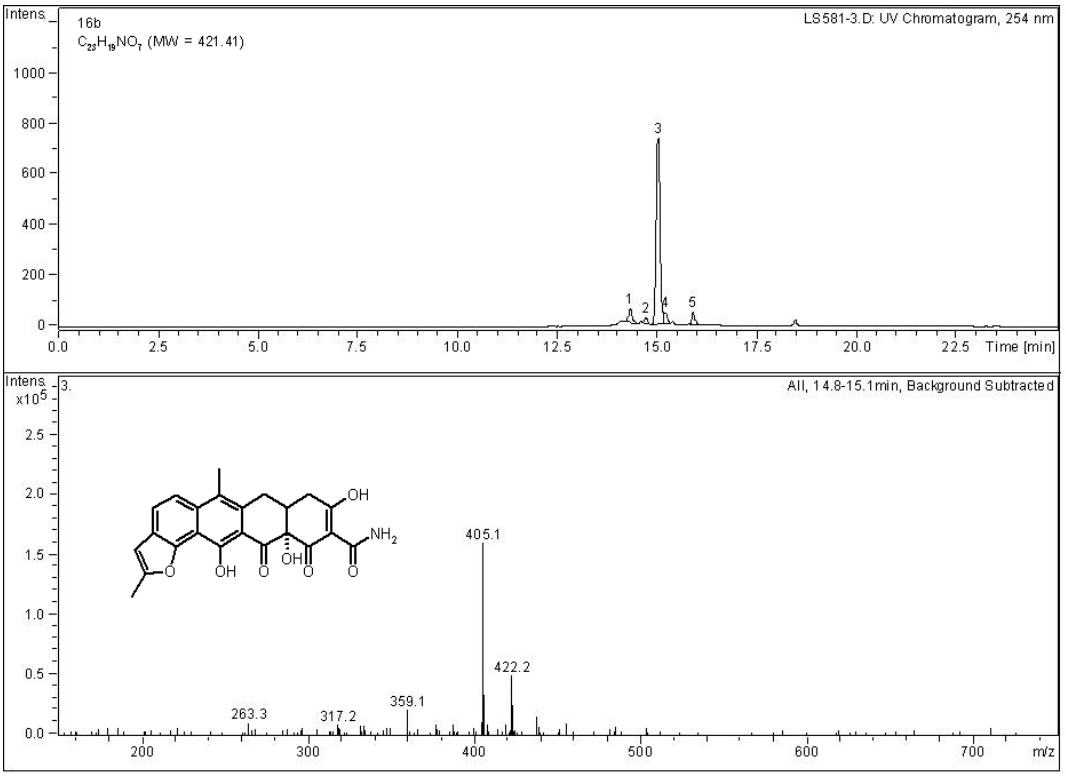
Purity of **4a** (peak 2): 94 %, amount of minor diastereomer (peak 1): 6 %



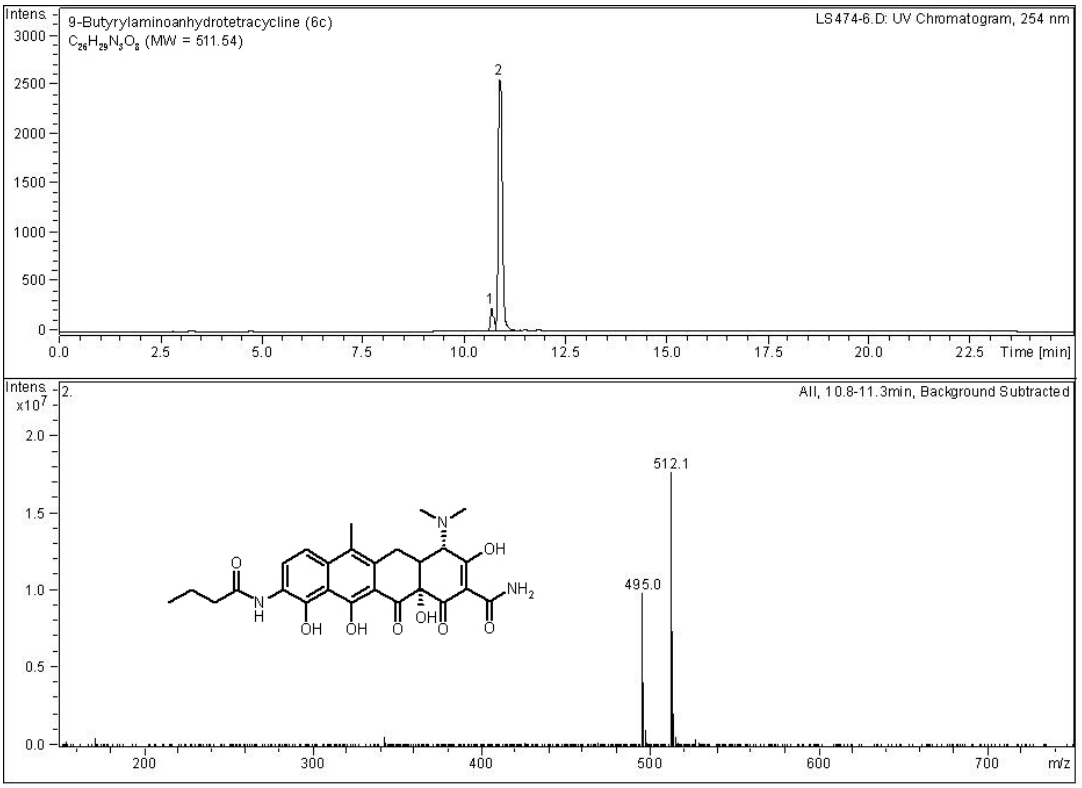
Purity of **4b** (peak 1): 98%



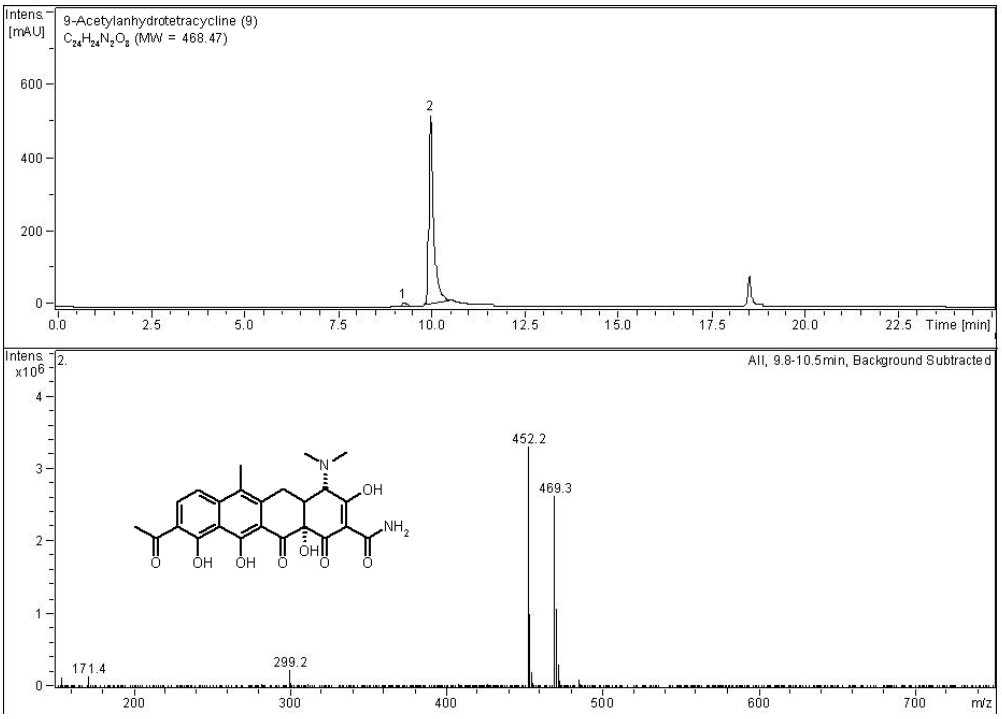
Purity of **5c** (peak 2): 99% (peak at 18.5 min retention time is due to impurities in the injection system)



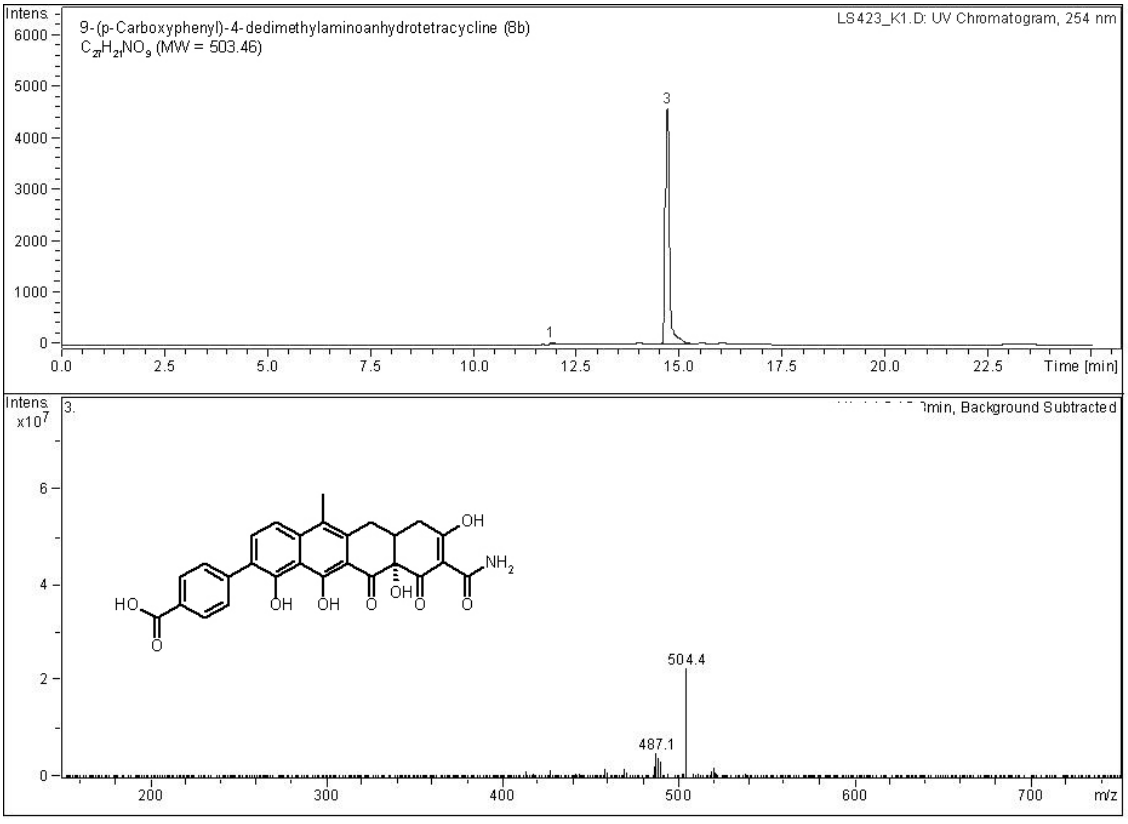
Purity of **16b** (peak 3): 83%



Purity of **6c** (peak 2): 93%, amount of minor diastereomer (peak 1): 7%



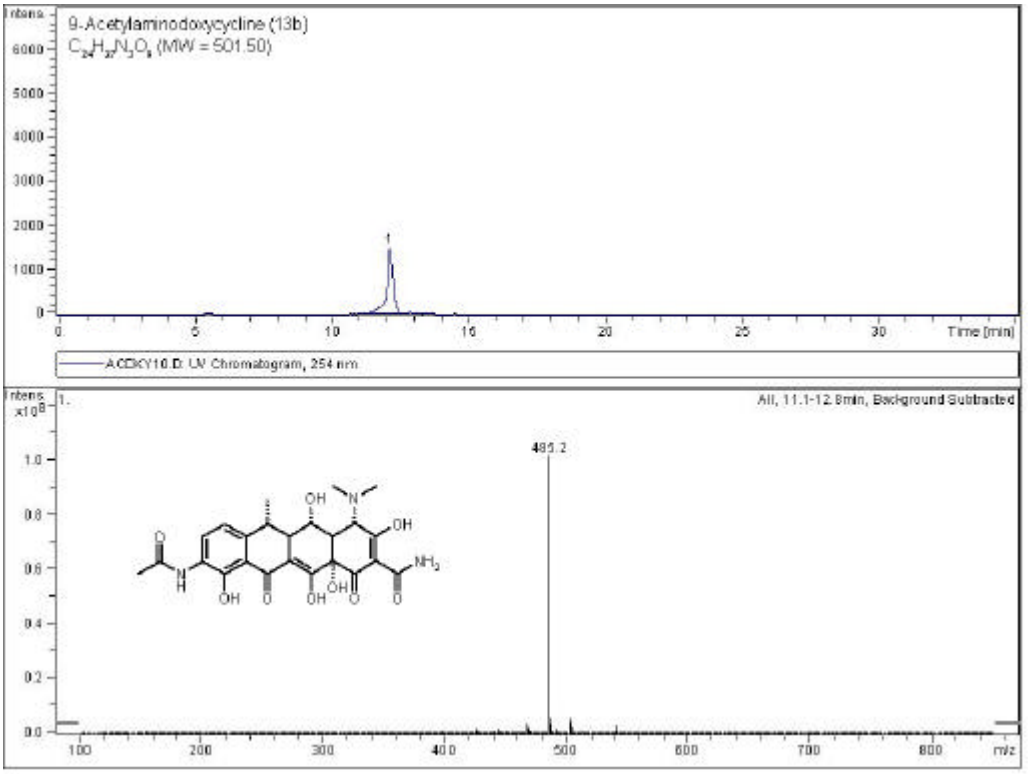
Purity of **8b** (peak 3): 98 %



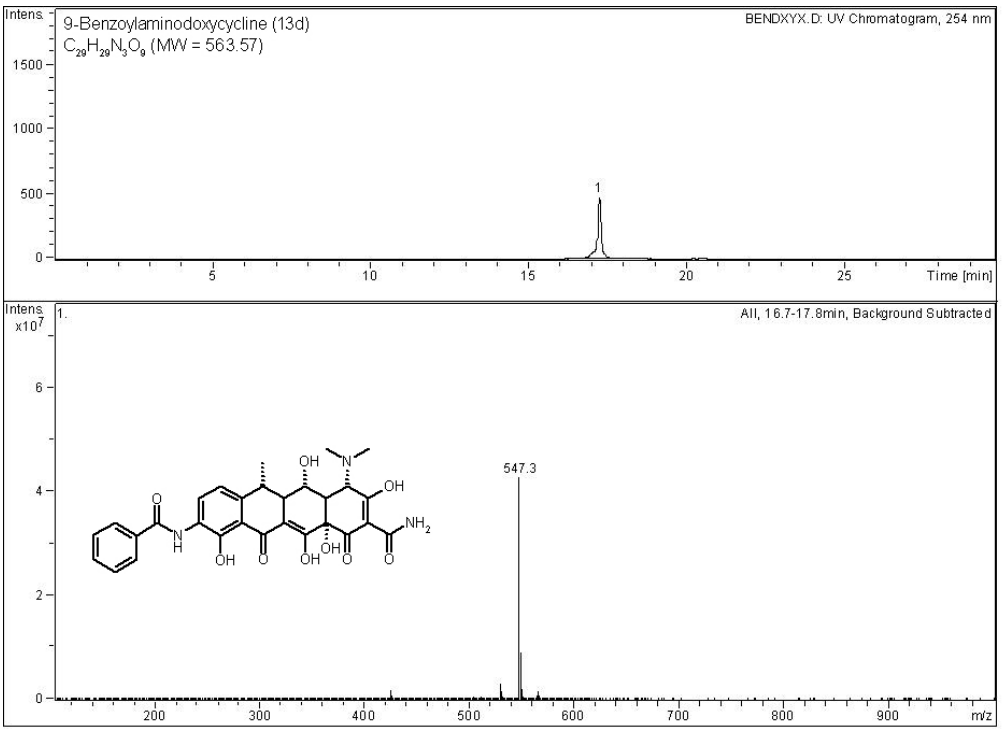
Purity of **9** (peak 2): 98% peak at 18.5 min retention time is due to impurities in the injection system)

LC-MS chromatograms of representative dox derivatives: Analyses were performed with an Agilent 1100 Binary Gradient (MeOH/0.1N aq. HCOOH 10/90-90/10) HPLC system and UV detection at 254 nm using a Zorbax SB-C8 (4.6 mm x 150 mm, 5 µm) column in combination with a Bruker Esquire 2000 mass spectrometer using APC ionization.

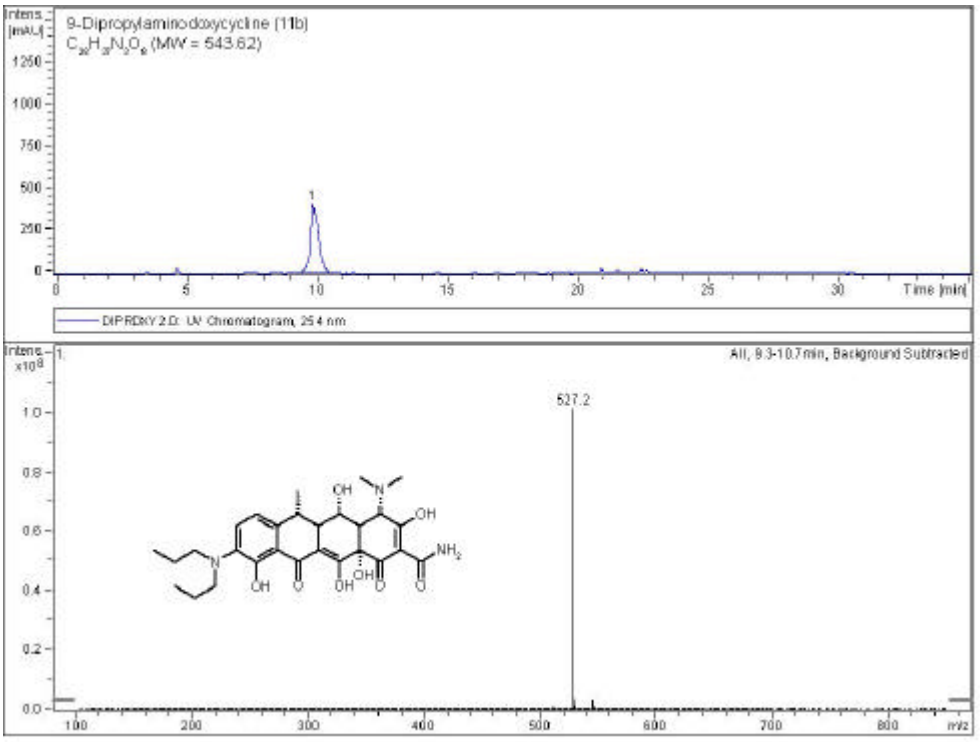
The applied APC ionization conditions in all cases led to $[M+1]^+$-17 signals for the investigated dox derivatives.



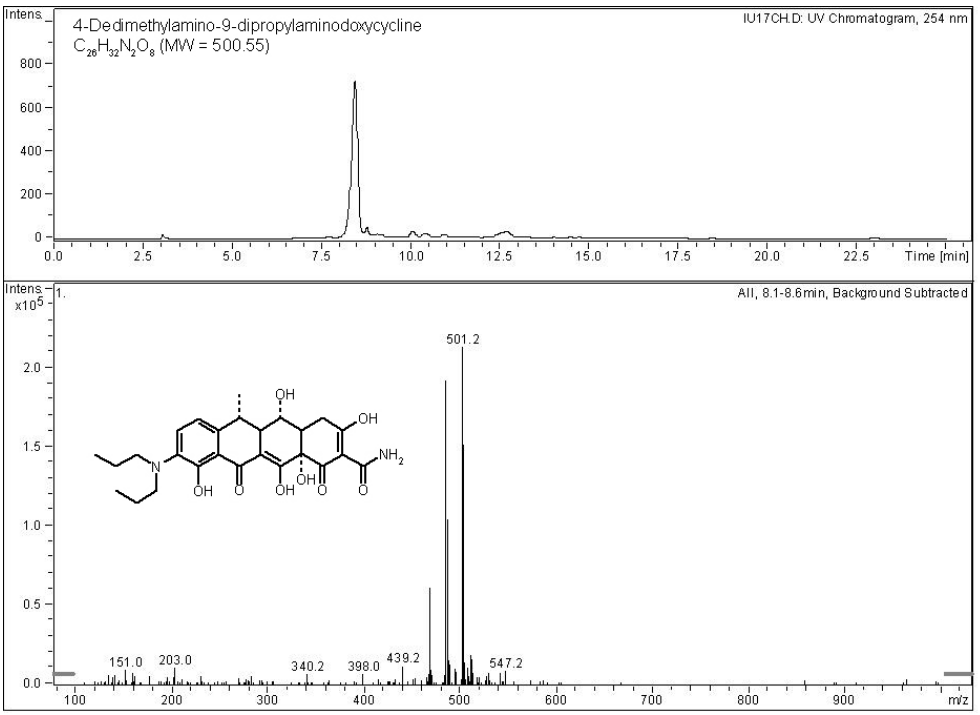
Purity of **13b** (peak 1): 98%



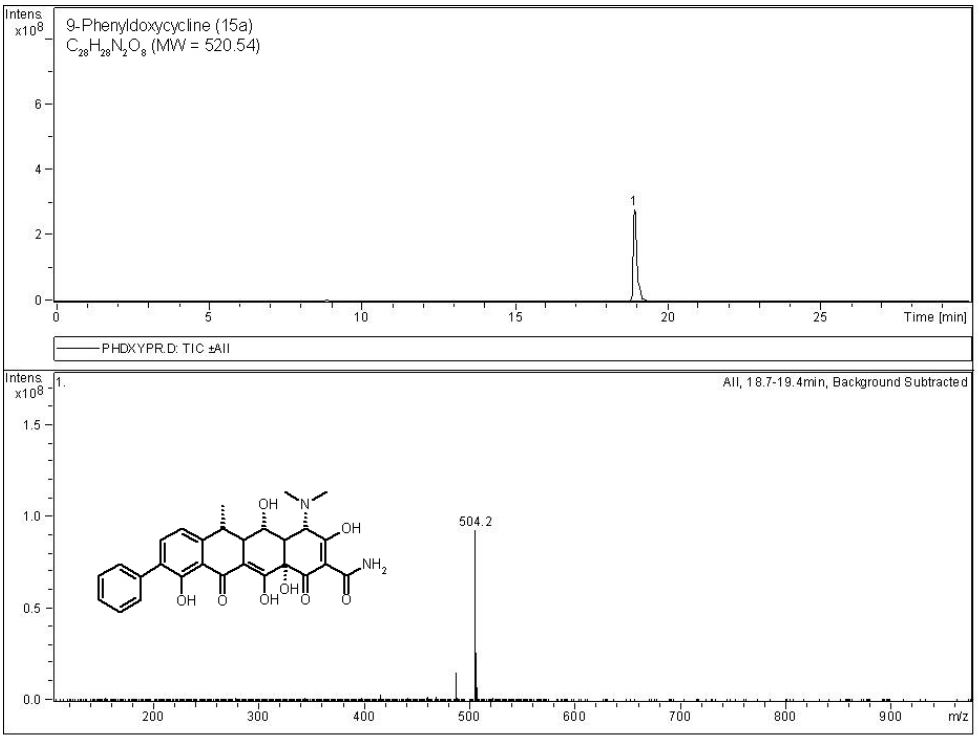
Purity of **13d** (peak 1): 98%



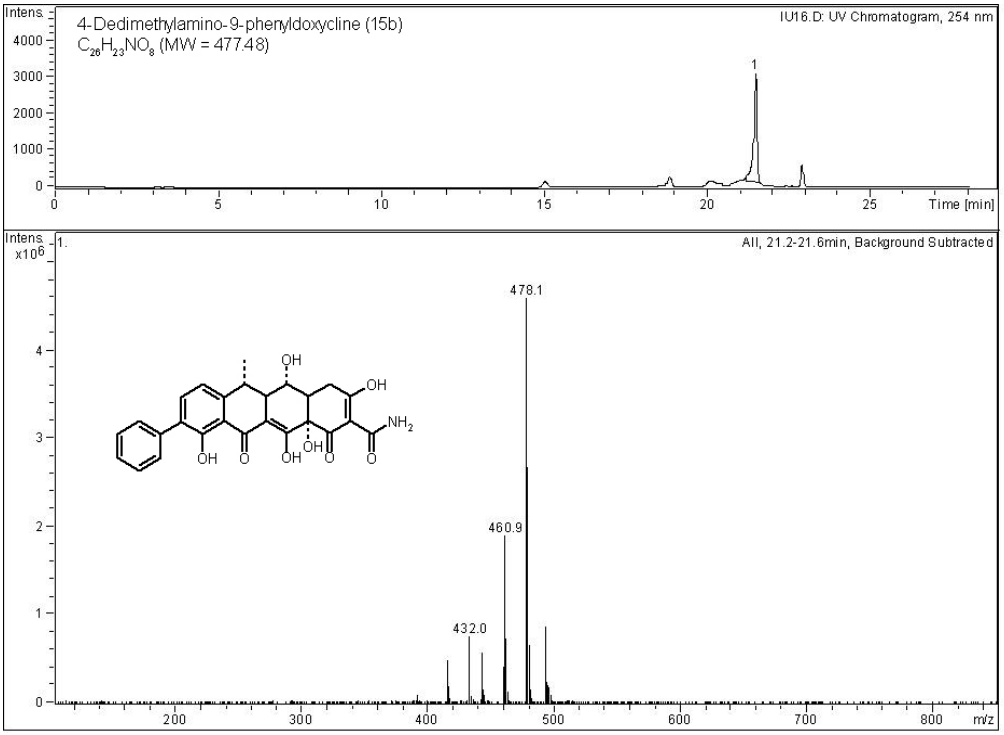
Purity of **11b** (peak 1): 97%



Purity of **12b** (peak 1): 91%



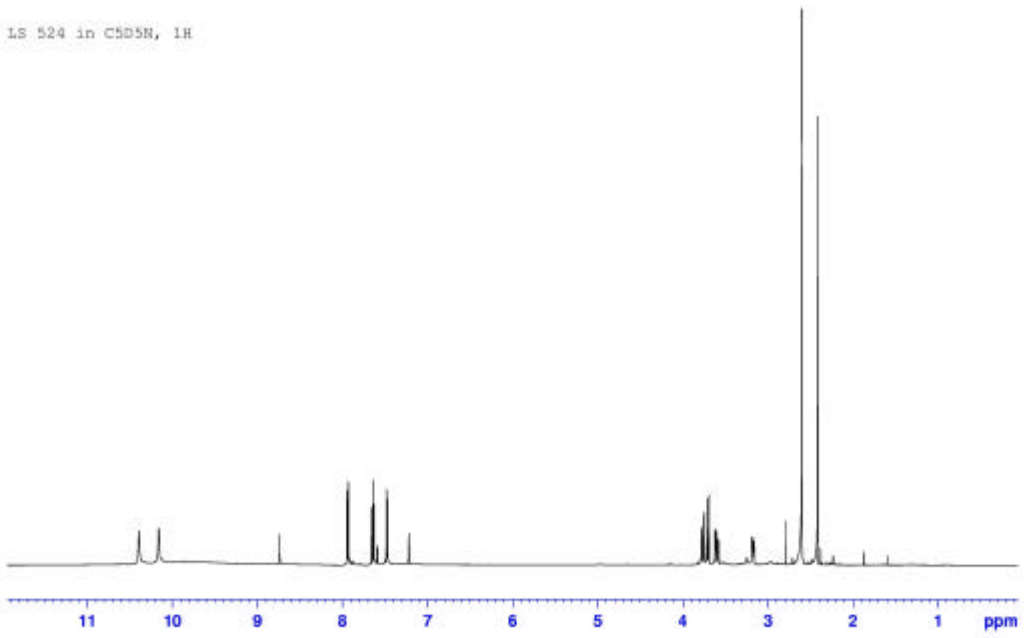
Purity of **15a** (peak 1): 99%



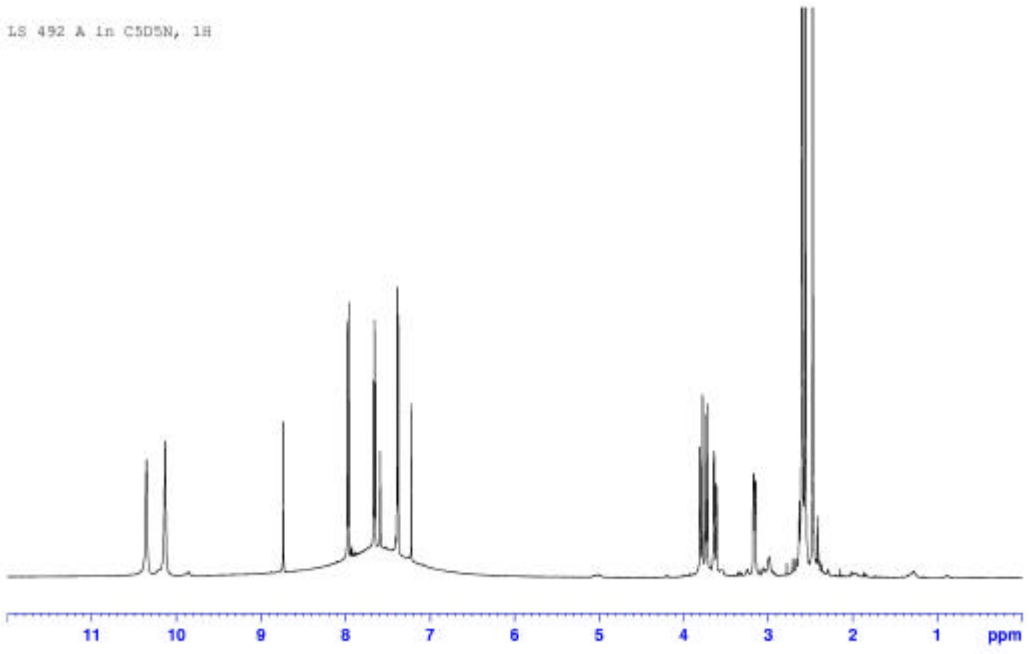
Purity of **15b** (peak 1): 90%

^{1}H NMR spectra of representative anhydrotetracycline derivatives

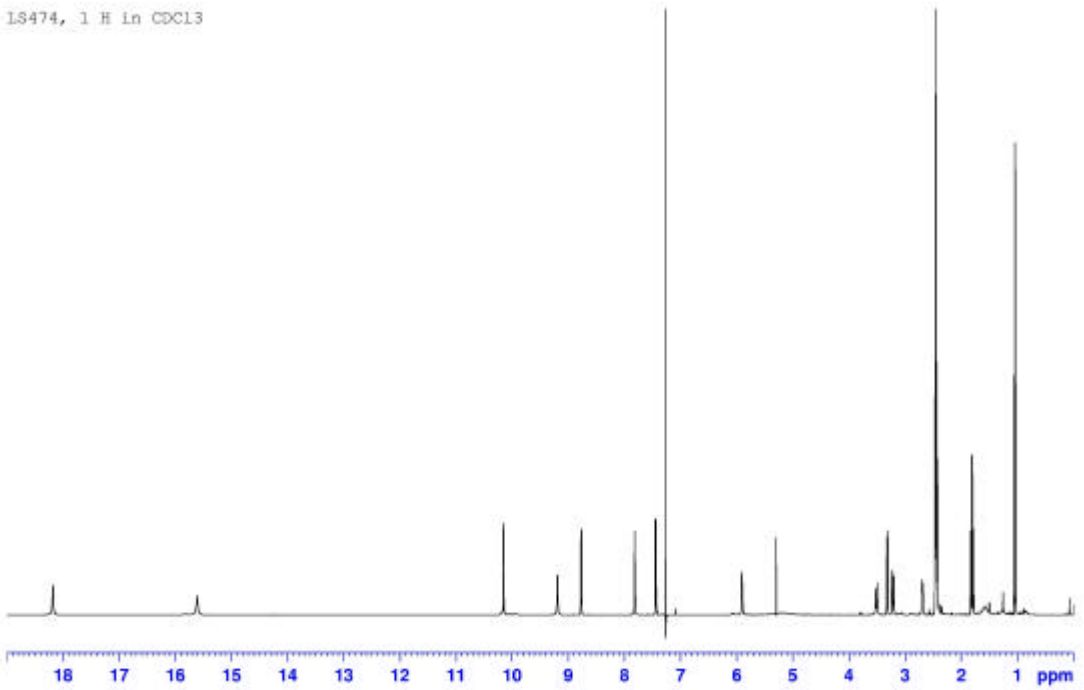
Anhydrotetracycline-10-triflate **2a**



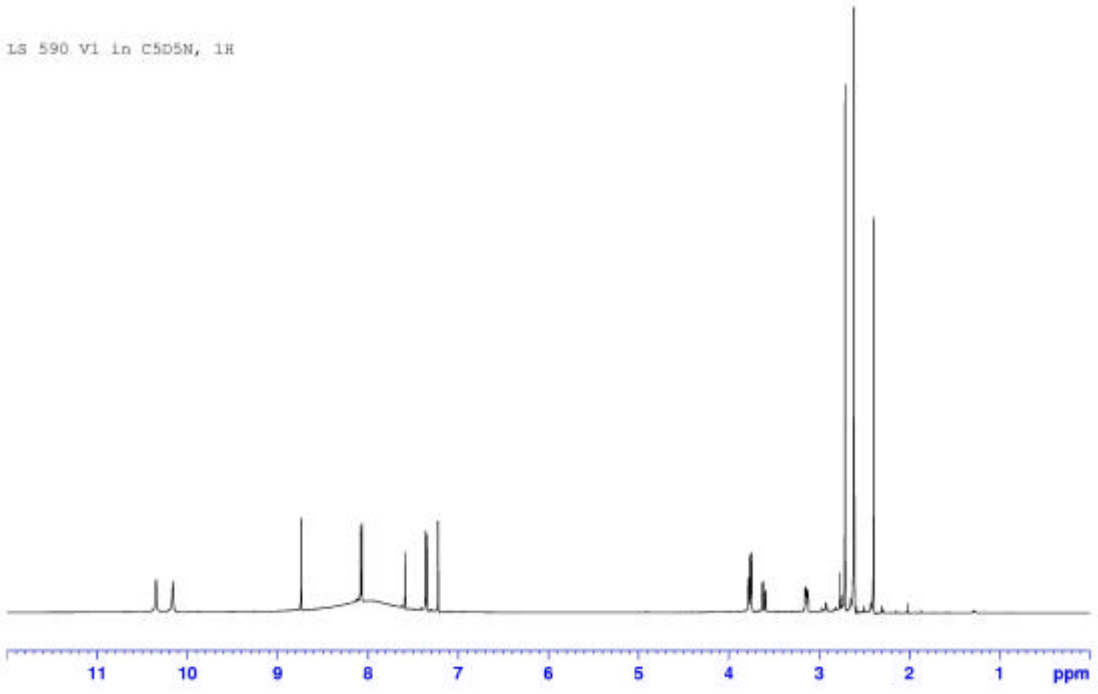
10-Acetyl-10-deoxyanhydrotetracycline **4a**:



9-Butyrylaminoanhydrotetracycline **6c**:

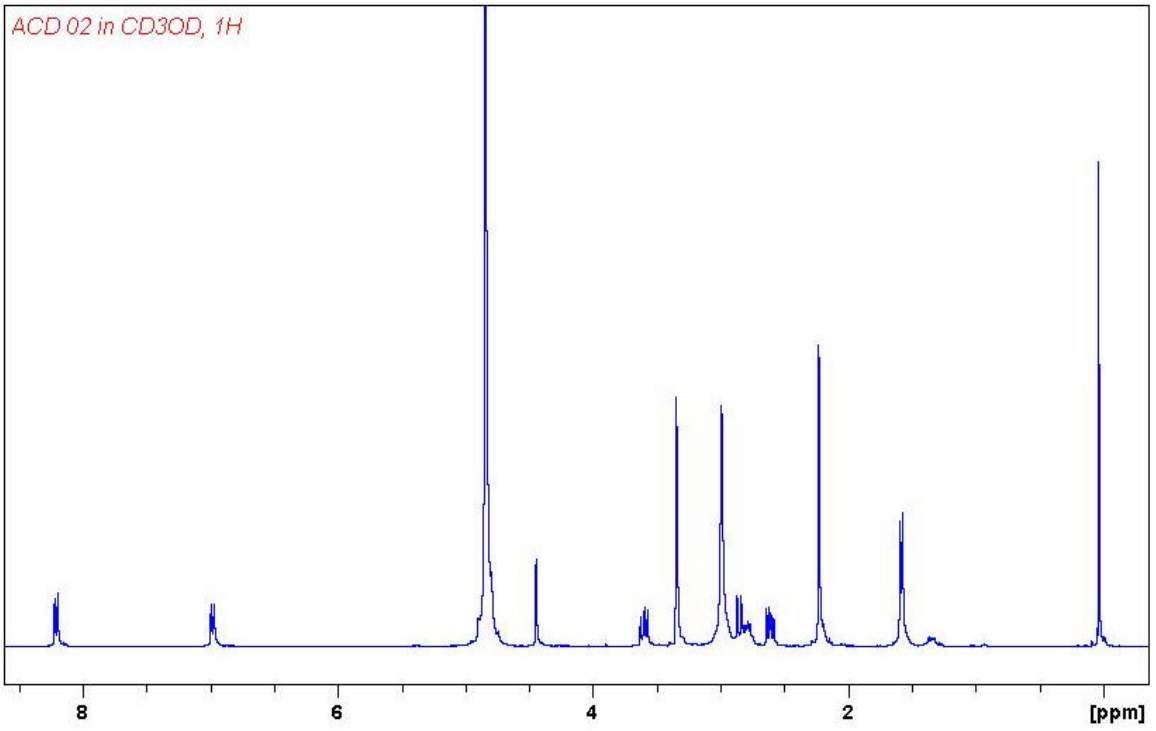


9-Acetylanhydrotetracycline **9**:

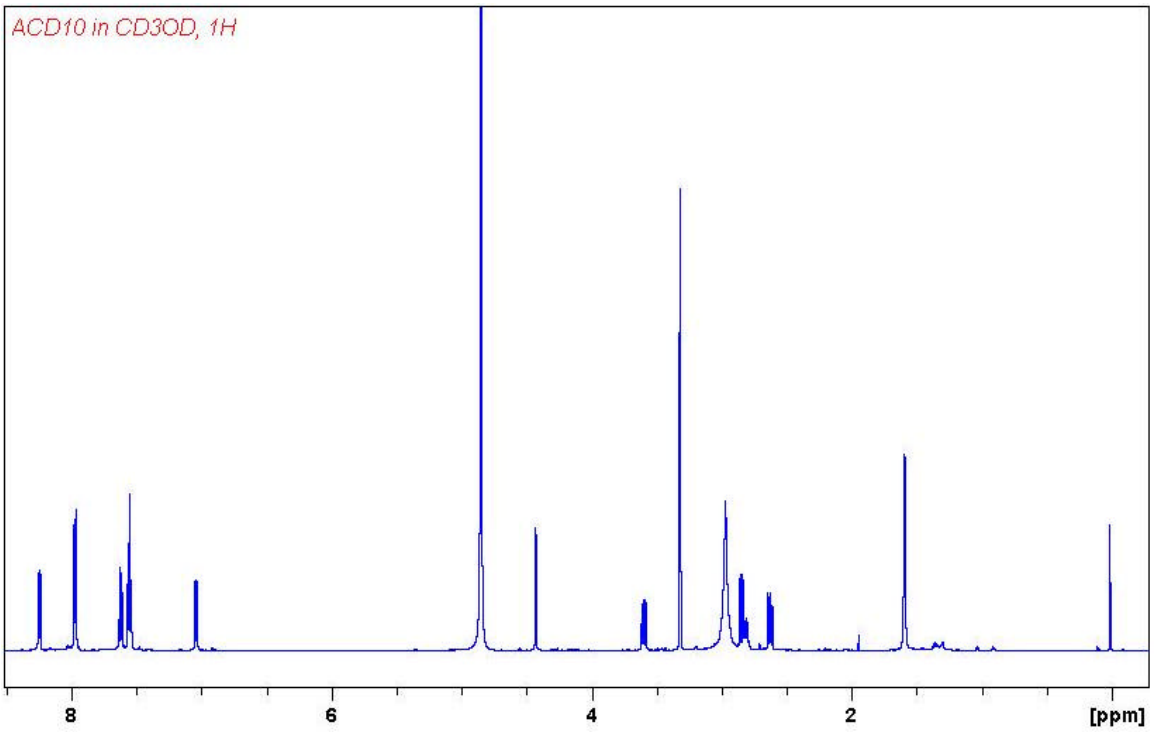


^{1}H NMR spectra of representative doxycycline derivatives

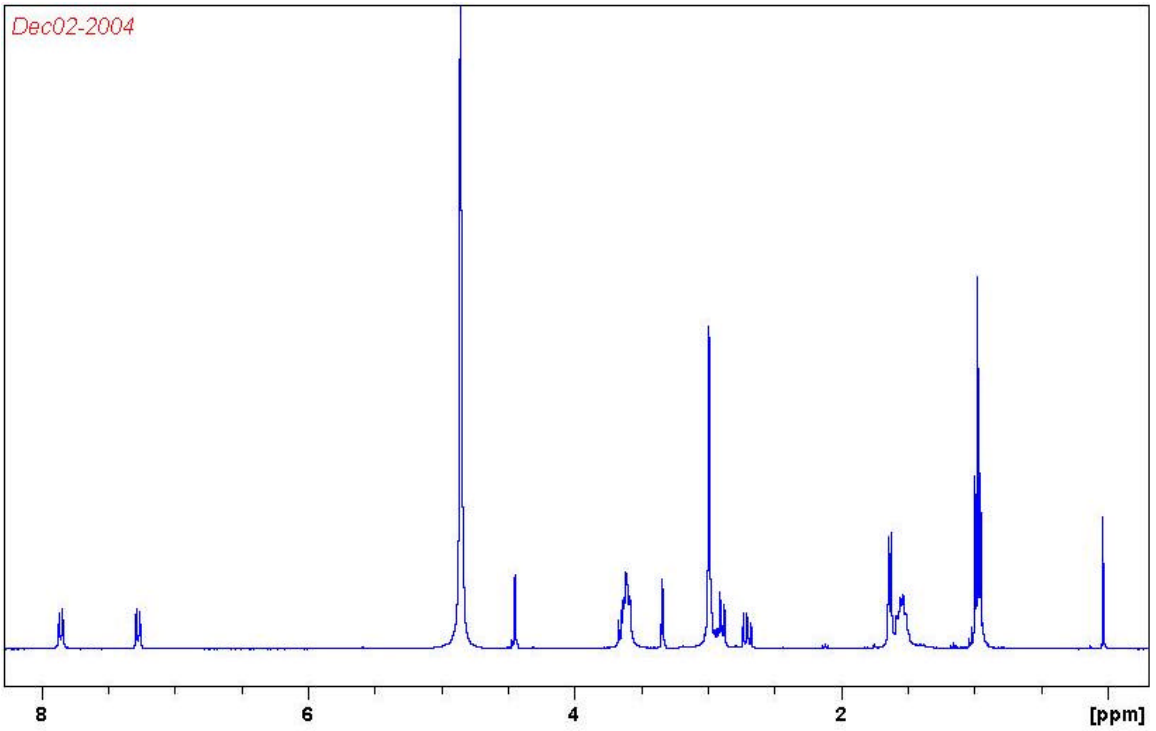
9-Acetylaminodoxycycline **13b**



9-Benzoylaminodoxycycline **13d:**



9-(*N,N*-Dipropylamino)doxycycline **11b:**



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[2] M. Gossen, H. Bujard, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5547-5551.

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