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SUPPORTING INFORMATION

Streamlined synthesis of the neurosteroid 3β-methoxypregnenolone assisted by statistical experimental design and automation

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General methods

Unless otherwise noted, chemicals were obtained from commercial suppliers and used without further purification. Melting points were determined using a electrothermal apparatus (Buchi 535, B-535, Büchi, Switzerland) and are uncorrected. NMR spectra were recorded on a Bruker AC 400 MHz spectrometer (Bruker, Madison, WI – USA) in the indicated solvent. Chemical shifts are reported in parts per million (ppm) and are relative to $CDCl_3$ (7.26 ppm and 77.0 ppm) or to d^6-DMSO (2.49 ppm and 39.7 ppm). The abbreviations used are as follows: s, singlet; brs, broad singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; brm, broad multiplet. Thin-layer chromatography was performed on aluminum backed silica plates (silica gel 60 F-254, Merck, Darmstadt, Germany). Spots were visualized by UV detector (λ: 254 nm) and/or by staining and worming with phosphomolibdic acid (5% wt in EtOH). When required, flash chromatographic purifications were performed using Biotage Isolera™ Prime (Biotage, Uppsala, Sweden). Synthesised compounds were previously reported and spectroscopic data were consistent with the literature.[1,2]

The continuous flow synthesis of 16-DPA (**8**) from diosgenin (**3**) was performed using a a Vapourtec R2+/R4 module (Vapourtec Ltd., Bury Saint Edmunds, UK) as previously described.[3] The flow synthesis of MAP4343 (**1**) from 16-DPA (**8**) was performed using a commercially available modular flow system (Asia Syrris, Blacktrace Holdings Ltd, Royston, UK) connected to a laptop and controlled by Asia manager PC software (version 1.6). The modular apparatus consists of an Asia automated reagent injector (AutoRIM) equipped with removable vial racks (8 mL vials), two internal loops (2 mL and 5 mL), two-channels Asia syringe pumps (Asia blue syringes, 0.50 mL/1.0 mL and Asia red syringes 2.5 mL/5 mL), an Asia sampler and dilutor, external HPLC pumps (Jasco PU-980, Jasco, Easton, MD – USA), thermocouple-controlled coil reactors (1/16” OD, 0.8 mm ID, 10 mL, PTFE), residence time coil reactors (9 mL, 1/16’’ OD, 0.8 mm ID, PTFE), glass tubular mesoreactors (Omnifit Labware DIBA HIT™ column, ID × L 6.6 mm x 100 mm and Labware DIBA BenchMark Microbore™ column, ID × L 3 mm x 50 mm), T-shaped mixing elements (0.5 mm ID), back pressure regulators (BPR, 40 psi, 100 psi and 250 psi, PEEK, 1/16" OD, 1/4"-28), 3-way hexagonal valves (PTFE, 1/4"-28, 1-4 mm OD, 1.5 mm ID, DIBA), 6-way valves (PTFE, 1/4"-28, 0.5-4 mm OD, 1.5 mm ID, DIBA), an in-line liquid-liquid separator (SEP-10, Zaiput, Cambridge, MA – USA), an UV detector and a fraction collector (Gilson FC 203B, Gilson Inc., Middleton, WI – USA). Flow hydrogenation was performed on the H-Cube apparatus (Thalesnano Nanotechnology Inc., Budapest, Hungary) using 10% Pd/C cartridge (s-cart, 30 × 4 mm i.d.). Design of experiments and

statistical data analysis was performed using Design-Expert® v. 9 (Stat-Ease Inc., Minneapolis, MN – USA). Calibrated HPLC analyses were performed on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA – USA) with a 100 µL stainless steel loop. A GraceSmart RP18 column (Grace, Sedriano, Italy, 250 x 4.6 mm i.d., 5 mm, 100 Å) was used as the analytical column. The HPLC analyses were performed at 210 nm and 254 nm detection wavelengths and a 1.0 mL min^{-1} eluent flow rate, after previous conditioning by passing through the column the selected mobile phase for at least 30 min at the same eluent velocity. Before use, all the mobile phases were always filtered through a 0.22 mm Millipore filter (Bedford, MA – USA) and then degassed with 20 min sonication. The column temperature was controlled through a heather/chiller thermostat (Grace 7956 R, Grace, Sedriano, Italy). All the analyses were performed at a 25 °C column temperature using analytical grade MeCN and ultrapure water (σ = 18.3 MΩ x cm) obtained through New Human machine Power I Scholar (Human Corporation, Seoul, Korea) purification system.

Preliminary reaction screening and DoE optimization

A solution of pregnenolone (**2**, 108 mg, 0.34 mmol, 0.2 M) in CH_2Cl_2 and a solution of MeOTf (77-154 µL, 0.68-1.36 mmol, 2-4 equiv., 0.7 M) in CH_2Cl_2 were injected by the automated reagent injector, pumped with the appropriate flow rate and mixed in a T-shaped mixing element. Then, the mixture entered in a second T-shaped mixing element and reacted with solution of DMAN (**5**, 109-182 mg, 0.51-0.85 mmol, 1.5-2.5 equiv., 0.34 M) in MeCN pumped with the appropriate flow rate. The resulting mixture was reacted into a thermocouple-controlled coil reactors (10 mL, τ= 45 min) heated at the desired temperature. The system was maintained at constant pressure through a BPR element (40 psi). Upon UV detection and software control (Asia Manage), the reactor outcome passed through a 6-way switching valve of a sampler dilutor device (Syrris, Asia Sampler and Dilutor), which was connected to an analytical HPLC (Shimadzu LC-20A Prominence) for on-line yield determination. At regular intervals corresponding to resident time, an aliquot (5 µL) of the crude mixture was collected, diluted and injected into the HPLC apparatus. Reactions were performed in a sequential, automated flow-through fashion and experiments were collected using a fraction collector. Lines were washed with a solution of CH_2Cl_2/MeCN (1.7:1, v/v) before the next run.

Continuous flow synthesis of MAP4343 (1)

A solution of pregnenolone (**2**, 633 mg, 2 mmol, 0.2 M) in CH_2Cl_2 (10 mL) and a solution of MeOTf (792 µL, 7 mmol, 3.5 equiv., 0.70 M) in CH_2Cl_2 (10 mL) were pumped with a flow rate of 31.5 µL min^{-1} for each pump and mixed in a T-shaped mixing element. Then, the mixture was directed into a second T-shaped element and mixed with solution of DMAN (**5**, 857 mg, 4 mmol, 2.0 equiv., 0.34 M) in MeCN (12 mL) which was pumped with a flow rate of 37 µL min^{-1}. The resulting mixture was reacted into a reactor coil (10 mL, τ= 100 min) heated at 34 °C. The reactor output was quenched with an in-line stream of H_2O (0.5 mL min^{-1}) and the biphasic mixture was separated by an in-line membrane liquid-liquid separator. The organic phase was pumped with a flow rate of 0.5 mL min^{-1} through a glass tubular mesoreactors (Omnifit Labware DIBA HIT™ column, ID × L 6.6 mm x 100 mm) filled with alternate layers of Amberlyst A21/silica (2:1 w/w, 4 layers, 300 mg for each layer, loading: 1.3 meq mL^{-1} by wetted bed volume) (see Fig. S4). The outcome was flowed through a second glass tubular mesoreactors (Omnifit Labware DIBA HIT™ column, ID × L 6.6 mm x 100 mm) filled with alternate layers of Amberlyst A15/silica (2:1 w/w, 4 layers, 300 mg for each layer, loading: 4.7 meq g^{-1} by dry weight) (see Fig. S5). The output was collected, dried over anhydrous Na_2SO_4, concentrated under reduce pressure (<40 °C) to afford crude MAP4343 (**1**, 86% yield by calibrated RP-HPLC). The crude was purified by automated flash chromatography (Eluent: petroleum ether/EtOAc from 100:0 to 80:20, v/v) affording the desired MAP4343 (**1**, 552 mg, 1.67 mmol, 84% isolated yield, 1.92 g d^{-1} of productivity) as white solid. Trapped DMAN (**5**) was recovered by washing Amberlyst A15 with 3 N aqueous solution of HCl (812 mg, 3.18 mmol, 81% recovery as chlorohydrate).

Flow synthesis of pregnenolone (2) from 16-dehydropregnenolone acetate (16-DPA, 8)

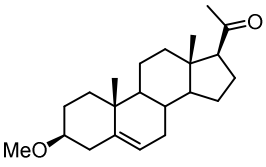
A solution of 16-DPA (**8**, 150 mg, 0.42 mmol, 0.07 M), prepared as previously reported,[3] and HCO_2NH_4 (265 mg, 4.2 mmol, 10.0 equiv., 0.7 M) in MeOH/THF (1:0.6 v/v, 6 mL) was pumped with a flow rate of 27 µL min^{-1} through a thermostatically controlled (100 °C) glass tubular mesoreactors (Labware DIBA BenchMark Microbore™ column, ID × L 3 mm x 50 mm) packed with 5% Pd/Al_2O_3 (22.5 mg, 15% wt with respect to **8**) diluted with Al_2O_3 (220 mg, 1: 9.7 w/w with respect to Pd/Al_2O_3, bed volume: 0.27 mL, τ= 10 min). The system was maintained at constant pressure through a BPR element (100 psi). The flowing mixture was collected in a residence time

reactor (1/16" OD, PTFE, 9 mL) before changing the flow rate for the hydrolysis step. After setting the flow rate at 0.87 mL min^{-1}, the solution was mixed in a T-shaped mixing element with an aqueous solution of KOH (236 mg, 4.2 mmol, 10 equiv., 3.5 M), pumped by an external HPLC pump at 0.13 mL min^{-1}, before entering a coil reactor (10 mL, τ= 10 min) thermostated at 100 °C. The system was maintained at constant pressure through a BPR element (250 psi). The outflowing mixture was collected in a fraction collector and the solvent was evaporated to dryness. The residue was neutralised with 3 N aq. HCl and extracted with EtOAc (3 x 10 mL), washed with H_2O (20 mL) and brine (20 mL), dried on Na_2SO_4 and evaporated to dryness. Crude **2** (126 mg, 0.40 mmol, 95% recovery, 924 mg d^{-1} of productivity from **8**) was analysed by ^{1}H-NMR (400 MHz, $CDCl_3$) and RP-HPLC (97% purity).

As an alternative protocol, a solution of 16-DPA (**8**, 150 mg, 0.42 mmol, 0.07 M) in MeOH (1:0.6 v/v, 6 mL) was pumped with a flow rate of 0.5 mL min^{-1} through a 10% Pd/C cartridge (s-cart, 30 × 4 mm i.d., τ= 4.5 min) thermostated at 25 °C and hydrogenated at 3 bar in a H-Cube apparatus. The output (V= 18 mL) was collected and mixed in a T-shaped mixing element with a methanolic solution of KOH (236 mg, 4.2 mmol, 10 equiv., 0.23 M), pumped by an external HPLC pump at 0.5 mL min^{-1}. The resulting mixture was reacted into a reactor coil (10 mL, τ= 10 min) heated at 50 °C. The system was maintained at constant pressure through a BPR element (100 psi). The outflowing mixture was collected and the solvent was evaporated to dryness. The residue was neutralised with 3 N aq. HCl and extracted with EtOAc (3 x 10 mL), washed with H_2O (20 mL) and brine (20 mL), dried on Na_2SO_4 and evaporated to dryness. Crude **2** (131 mg, 0.41 mmol, 98% recovery, 2 g d^{-1} productivity from **8**) was analysed by ^{1}H-NMR (400 MHz, $CDCl_3$) and RP-HPLC (96% purity).

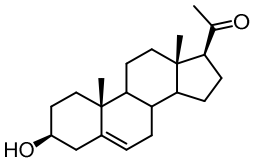
Compounds characterization

3-Methoxypregnenolone (MAP4343) (1)[2]



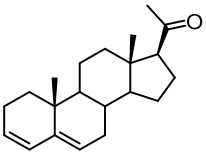
White solid (m.p.: 123–124 °C; lit.:[2] 123–124 °C). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.62 (s, 3H, 18-C*H*$_3$), 0.99 (s, 3H, 19-C*H*$_3$), 2.12 (s, 3H, 21-C*H*$_3$), 2.30-2.45 (m, 1H), 2.52 (t, *J*= 8.87 Hz, 1H), 3.02-3.09 (m, 1H, 3α-C*H*), 3.35 (s, 3H, 3-OC*H*$_3$), 5.35 (d, *J*= 4.56 Hz, 1H, 6-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 13.0, 19.1, 20.8, 22.5, 24.2, 27.7, 31.3, 31.6 (2x), 36.6, 36.9, 38.4, 38.6, 43.7, 49.8, 55.3, 56.6, 63.4, 79.9, 121.0, 140.6, 209.2. Residence time (t_R): 20.754 min. Analytical and spectroscopic data are consistent with literature.[2]

Pregnenolone (2)[1]



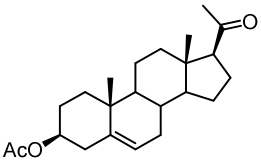
White solid (m.p.: 189–191 °C; lit.:[2] 190–191 °C). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.63 (s, 3H, 18-C*H*$_3$), 1.01 (s, 3H, 19-C*H*$_3$), 2.13 (s, 3H, 21-C*H*$_3$), 3.51-3.56 (m, 1H, 3α-C*H*), 5.37 (d, *J*= 4.85 Hz, 1H, 6-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 13.1, 19.3, 21.0, 22.7, 24.4, 31.4 (2x), 31.6, 31.7, 36.4, 37.1, 38.7, 42.1, 43.9, 49.8, 56.8, 63.6, 71.5, 121.2, 140.7, 209.5. Residence time (t_R): 7.526 min. Analytical and spectroscopic data are consistent with literature.[1]

Pregn-3,5-dien-20-one (6)[2]



Pale yellow solid (m.p.: 136–137 °C; lit.:[2] 135–136 °C). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.66 (s, 3H, 18-C*H*$_3$), 0.94 (s, 3H, 19-C*H*$_3$), 2.14 (s, 3H, 21-C*H*$_3$), 2.56 (t, *J*= 8.87 Hz, 1H), 5.39 (d, *J*= 2.96 Hz, 1H, 6-C*H*), 5.61 (d, *J*= 3.60 Hz, 1H, 3-C*H*), 5.94 (d, *J*= 8.55 Hz, 1H, 4-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 13.5, 18.9, 21.1, 22.9, 23.1, 24.5, 29.9, 31.7, 31.9, 33.9, 35.3, 39.0, 44.3, 48.4, 57.3, 63.9, 122.9, 125.3, 129.0, 141.5, 209.8. Residence time (t_R): 33.911 min. Analytical and spectroscopic data are consistent with literature.[2]

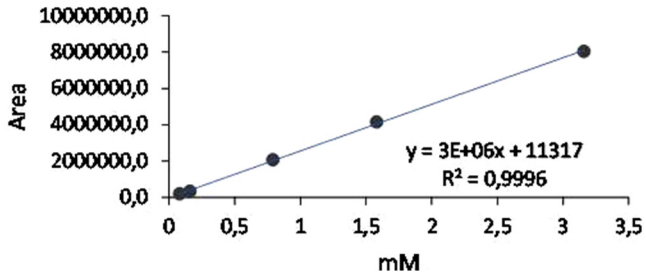
3-Acetylpregnenolone (7)[1]



White solid (m.p.: 149–151 °C, lit.:[4] 148–150 °C). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.61 (s, 3H, 18-C*H*$_3$), 1.00 (s, 3H, 19-C*H*$_3$), 2.02 (s, 3H, 3-CHOCOC*H*$_3$), 2.11 (s, 3H, 21 C*H*$_3$), 2.50-2.52 (m, 1H), 4.57-4.60 (m, 1H, 3α-C*H*OCOCH$_3$), 5.35 (d, *J*= 5.22 Hz, 1H, 6-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 13.0, 19.1, 20.8, 21.2, 22.5, 24.2, 27.5, 31.4, 31.5, 31.6, 36.3, 36.7, 37.8, 38.5, 43.8, 49.6, 56.6, 63.4, 73.6, 122.1, 139.4, 170.3, 209.4. Residence time (t_R): 19.437 min. Analytical and spectroscopic data are consistent with literature.[1]

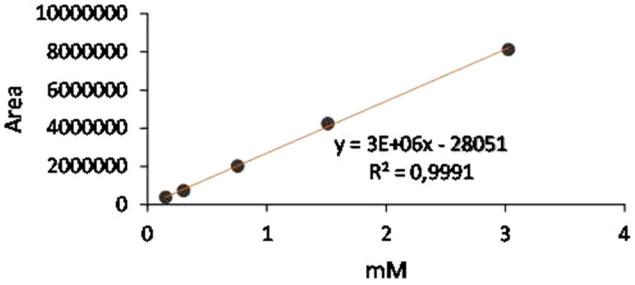
Pregnenolone (2)

Conc. (mg/mL)	Conc. (mM)	Area[a]
0,025	0,078991437	199458,5
0,05	0,157982875	350355,5
0,25	0,789914373	2070413
0,5	1,579828747	4150951,5
1	3,159657493	8045429



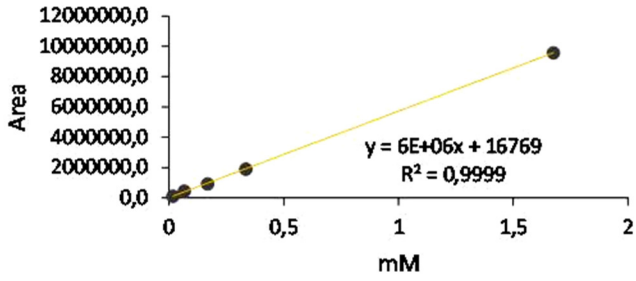
MAP4343 (1)

Conc. (mg/mL)	Conc. (mM)	Area[a]
0,025	0,151281353	378150
0,05	0,302562706	726085
0,25	0,756406765	2016612
0,5	1,512813531	4252602
1	3,025627061	8143738



Pregn-3,5-dien-20-one (6)

Conc. (mg/mL)	Conc. (mM)	Area[a]
0,005	0,016752102	123195,5
0,02	0,06700841	445261,5
0,05	0,167521024	923956
0,1	0,335042048	1914897,5
0,5	1,675210239	9582250



3-Acetylpregnenolone (7)

Conc. (mg/mL)	Conc. (mM)	Area[a]
0,005	0,013946612	60197
0,02	0,055786449	179369
0,05	0,139466124	422518
0,1	0,278932247	927675
0,25	0,697330618	2435923

[a]Average of two measurements

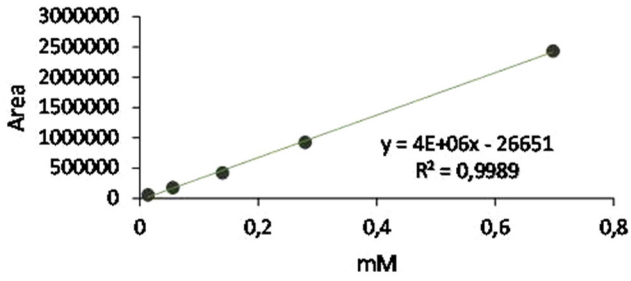


Fig. S1. HPLC calibration curves for the quantitative determination of pregnenolone (**2**), MAP4343 (**1**), pregn-3,5-dien-20-one (**6**) and 3-acetylpregnenolone (**7**). Each solution was analyzed in triplicate. *Conditions*: column: Grace Smart RP18; eluent: H_2O/MeCN (30:70, v/v); flow rate: 1 mL/min; detector: UV-vis (Ch1: 210 nm; Ch2: 254 nm); column T: 25 °C; injection volume: 20 µL.

Pregnenonole (2)

Regression statistics	
R multiple	0,999789451
R squared	0,999578947
Adjusted R squared	0,999438596
Standard error	77234,75628
Number of observations	5

	gdl	*SQ*	*MQ*	*F*	*Significance F*
Regression	1	4,24842E+13	4,2484E+13	7121,998869	3,66732E-06
Residue	3	17895622734	5965207578		
Total	4	4,25021E+13			

	Coefficients	*Standard error*	*Stat t*	*Significance value*	*Inf. 95%*	*Sup. 95%*	*Inf. 95,0%*	*Sup. 95,0%*
Intercept	11316,98499	49159,13804	0,23021122	0,832732417	-145129,3322	167763,302	-145129,332	167763,302
Variable X 1	2559670,983	30330,75319	84,391936	3,66732E-06	2463144,99	2656196,98	2463144,99	2656196,98

MAP4343 (1)

Regression statistics	
R multiple	0,999565468
R squared	0,999131125
Adjusted R squared	0,9988415
Standard error	108951,3581
Number of observations	5

	gdl	*SQ*	*MQ*	*F*	*Significance F*
Regression	1	4,09498E+13	4,095E+13	3449,739601	1,08727E-05
Residue	3	35611195264	1,187E+10		
Total	4	4,09854E+13			

	Coefficients	*Standard error*	*Stat t*	*Significance value*	*Inf. 95%*	*Sup. 95%*	*Inf. 95,0%*	*Sup. 95,0%*
Intercept	-28050,55556	72226,55968	-0,38836898	0,723663806	-257907,7036	201806,592	-257907,704	201806,592
Variable X 1	2723652,853	46372,29565	58,7344839	1,08727E-05	2576075,512	2871230,19	2576075,512	2871230,19

Pregn-3,5-dien-20-one (6)

Regression statistics	
R multiple	0,999961414
R squared	0,99992283
Adjusted R squared	0,999897107
Standard error	40194,74829
Number of observations	5

	gdl	*SQ*	*MQ*	*F*	*Significance F*
Regression	1	6,28029E+13	6,2803E+13	38872,37084	2,87719E-07
Residue	3	4846853371	1615617790		
Total	4	6,28077E+13			

	Coefficients	*Standard error*	*Stat t*	*Significance value*	*Inf. 95%*	*Sup. 95%*	*Inf. 95,0%*	*Sup. 95,0%*
Intercept	16769,39249	22237,63885	0,75409951	0,505581222	-54000,69909	87539,4841	-54000,6991	87539,4841
Variable X 1	5706619,733	28943,99131	197,160774	2,87719E-07	5614507,034	5798732,43	5614507,034	5798732,43

Fig. S2. Statistical analysis of the calibration curves for MAP-4343 (**1**), pregnenolone (**2**), 3-acetylpregnenolone (**7**) and pregn-3,5-dien-20-one (**6**).

	Slope	2559671	Slope	2723653	Slope	3508285	Slope	5706620
	Intercept	11317	Intercept	-28051	Intercept	-26651	Intercept	16769
	R^2	0,9998	R^2	0,9996	R^2	0,9995	R^2	0,99996
	Pregnenolone (2)		**MAP4343 (1)**		**3-Acetylpregnenolone (7)**		**Pregn-3,5-dien-20-one (6)**	
	Area	**Yied (%)**	**Area**	**Yield (%)**	**Area**	**Yield (%)**	**Area**	**Yield (%)**
Entry 1 Table 3	10580991	68,6	4705777	28,9	521087	2,6	0	0,0
Entry 2 Table 3	7965699	54,5	6598823	42,7	454049	2,4	144724	0,4
Entry 3 Table 3	4042375	36,1	7360837	62,2	227131	1,7	0	0,0
Entry 4 Table 3	4714430	62,0	3034266	38,0	0	0,0	0	0,0
Entry 5 Table 3	5155597	33,9	9673091	60,2	1200700	5,9	0	0,0
Entry 6 Table 3	6029357	38,7	9879936	59,9	280045	1,4	0	0,0
Entry 7 Table 3	3952485	37,9	6540492	59,3	374744	2,8	0	0,0
Entry 8 Table 3	764056	16,0	1470964	30,0	1073509	17,1	3891113	37,0
Run 1 DoE Table 4	34950371	79,7	9276527	20,0	172517	0,3	0	0,0
Run 2 DoE Table 4	11236054	17,5	54702186	80,2	1435560	1,7	908417	0,6
Run 3 DoE Table 4	18629891	65,2	9595043	31,7	77195	0,3	1850800	2,9
Run 4 DoE Table 4	1451851	18,3	12780184	78,0	391233	2,2	496604	1,5
Run 5 DoE Table 4	30894619	28,3	80873764	69,7	2380425	1,6	763394	0,3
Run 6 DoE Table 4	4199648	19,9	17124692	76,7	827196	3,0	213363	0,4
Run 7 DoE Table 4	1233884	12,6	7914316	78,9	179255	1,6	1947854	6,9
Run 8 DoE Table 4	3498741	26,8	9469207	69,7	648884	2,9	186882	0,6
Run 9 DoE Table 4	582721	6,0	8753219	86,6	216382	1,9	1196933	5,6
Run 10 DoE Table 4	1484125	17,5	6712085	75,3	110394	1,2	1144554	6,0
Run 11 DoE Table 4	5159651	33,0	5991956	65,9	142683	1,1	46091	0,0
Check point 1 Table 6	2601843	14,3	16189488	83,9	287643	1,3	249503	0,6
Check point 2 Table 6	857383	5,2	15126795	88,0	257560	1,3	1987331	5,5
Scale-up + downstrem	120723	7,9	1246021	86,2	56595	1,5	64136	4,4
Intergated from 16-DPA	2105039	5,1	40611744	92,7	1197268	2,2	100570	0,1

Fig. S3. Overview of the results based on calibration curves.

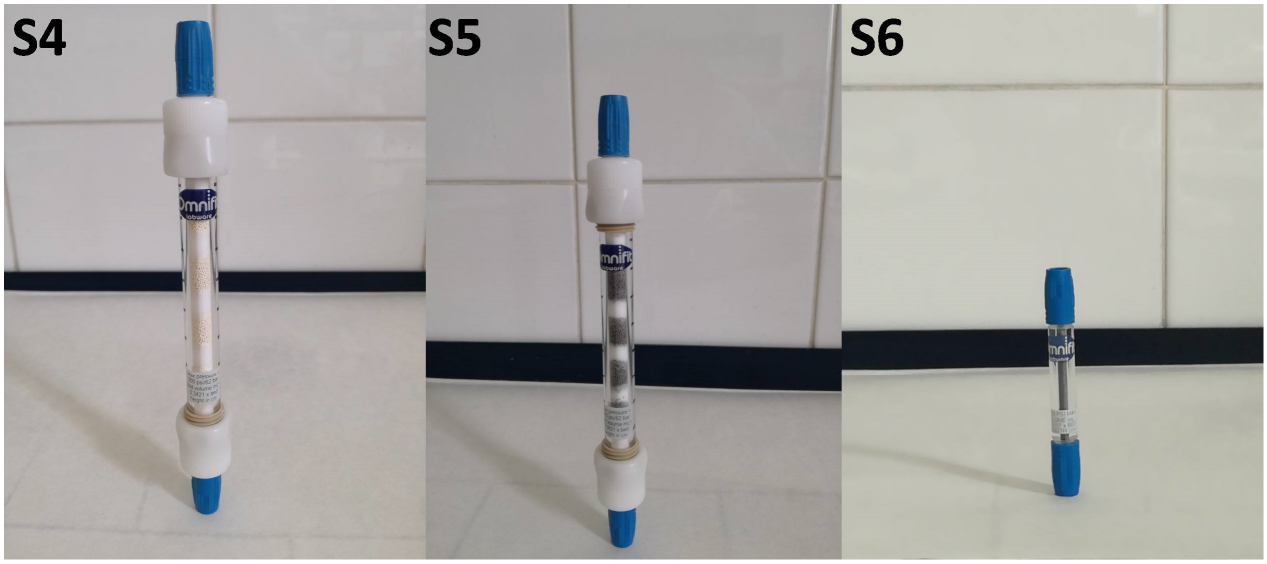


Fig. S4-S6. Omnifit Labware DIBA HIT™ column (ID × L 6.6 mm x 100 mm) filled with alternate layers of Amberlyst A21/silica (2:1 w/w, 4 layers, 300 mg for each layer) (**S4**); Omnifit Labware DIBA HIT™ column (ID × L 6.6 mm x 100 mm) filled with alternate layers of Amberlyst A15/silica (2:1 w/w, 4 layers, 300 mg for each layer) (**S5**); Labware DIBA BenchMark Microbore™ column (ID × L 3 mm x 50 mm) packed with 5% Pd/Al_2O_3 diluted with Al_2O_3 (22.5 mg/220 mg) (**S6**).

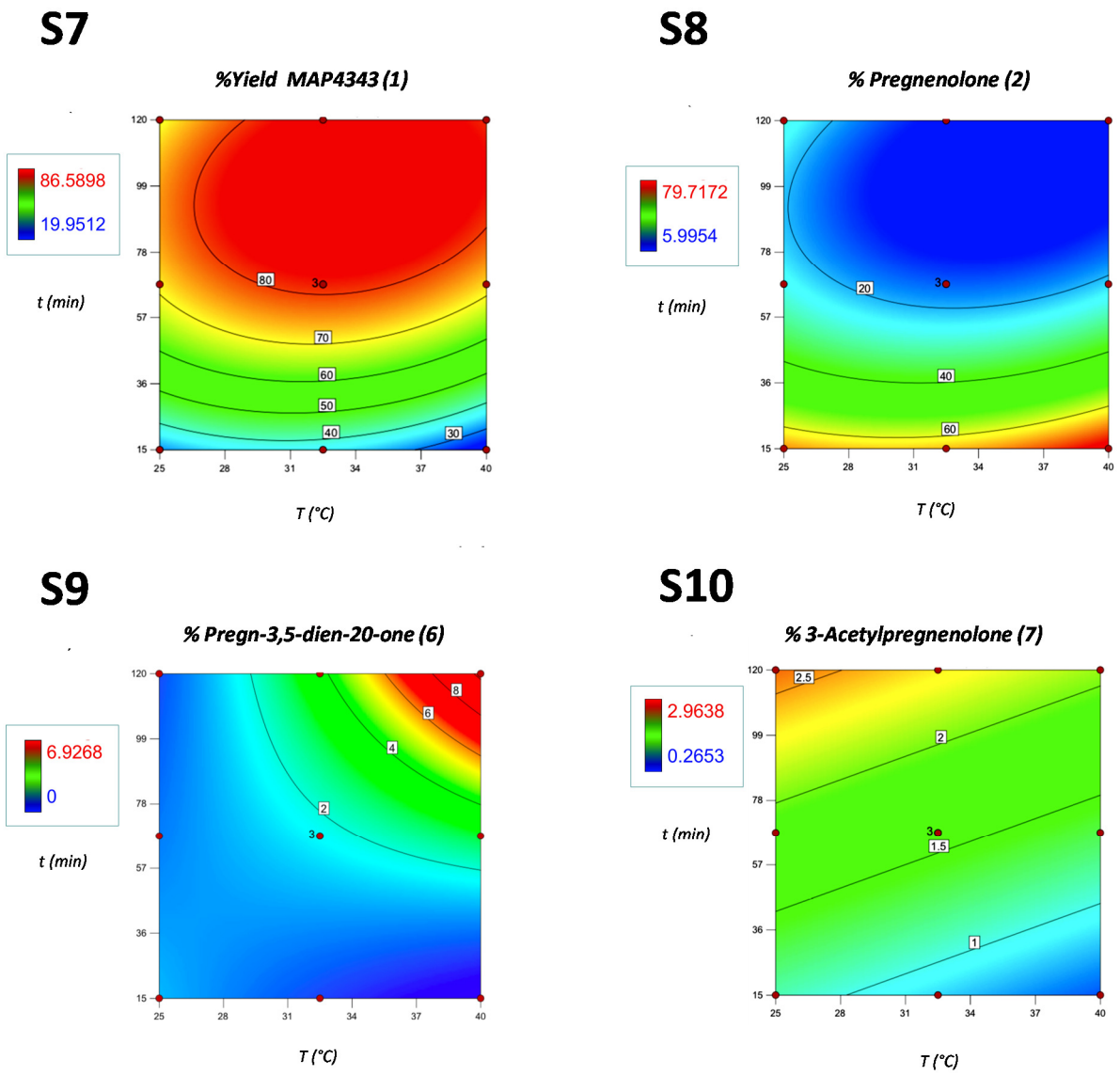


Fig. S7-S10. Contour plots for observable responses as relative percentages of MAP-4343 (**1**, **S7**), pregnenolone (**2**, **S8**), pregn-3,5-dien-20-one (**6**, **S9**) and 3-acetylpregnenolone (**7**, **S10**).

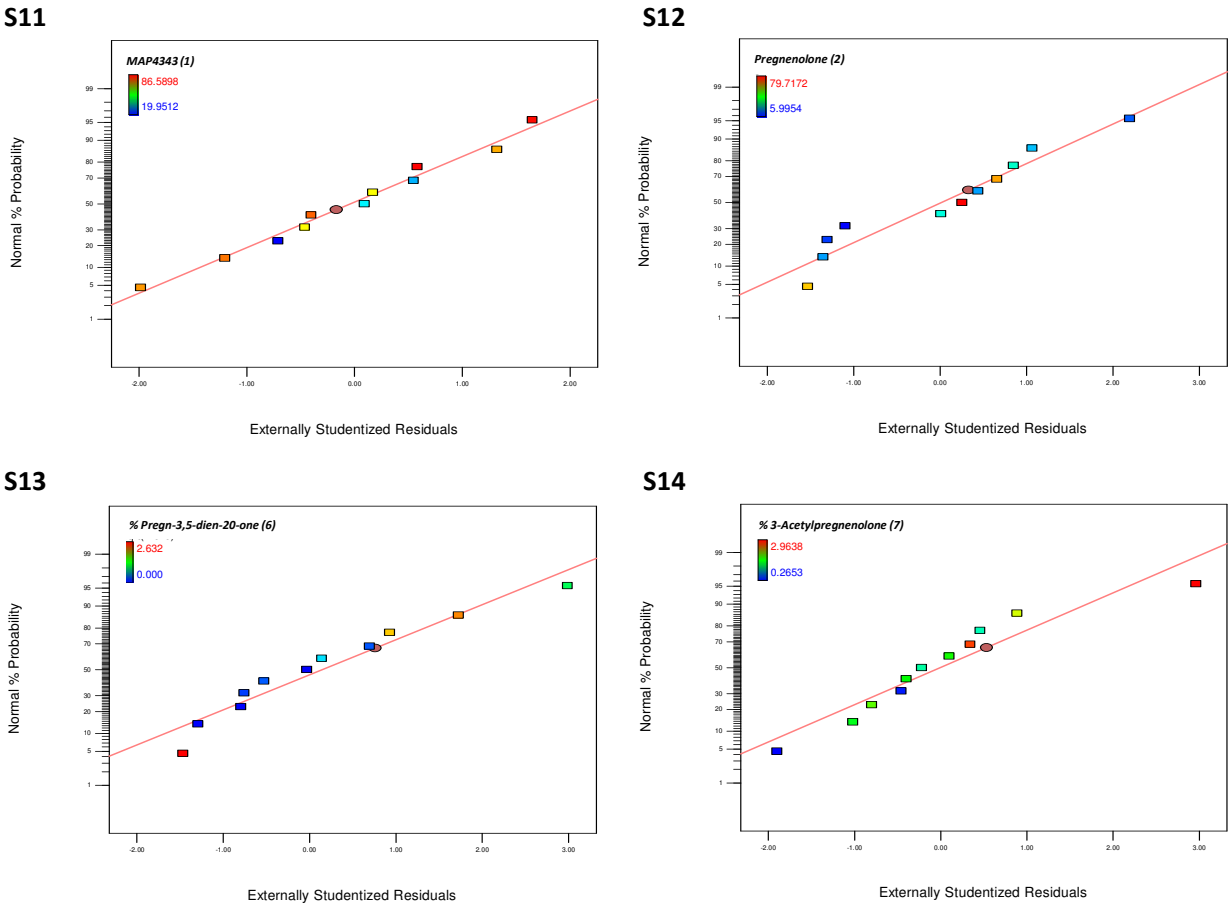
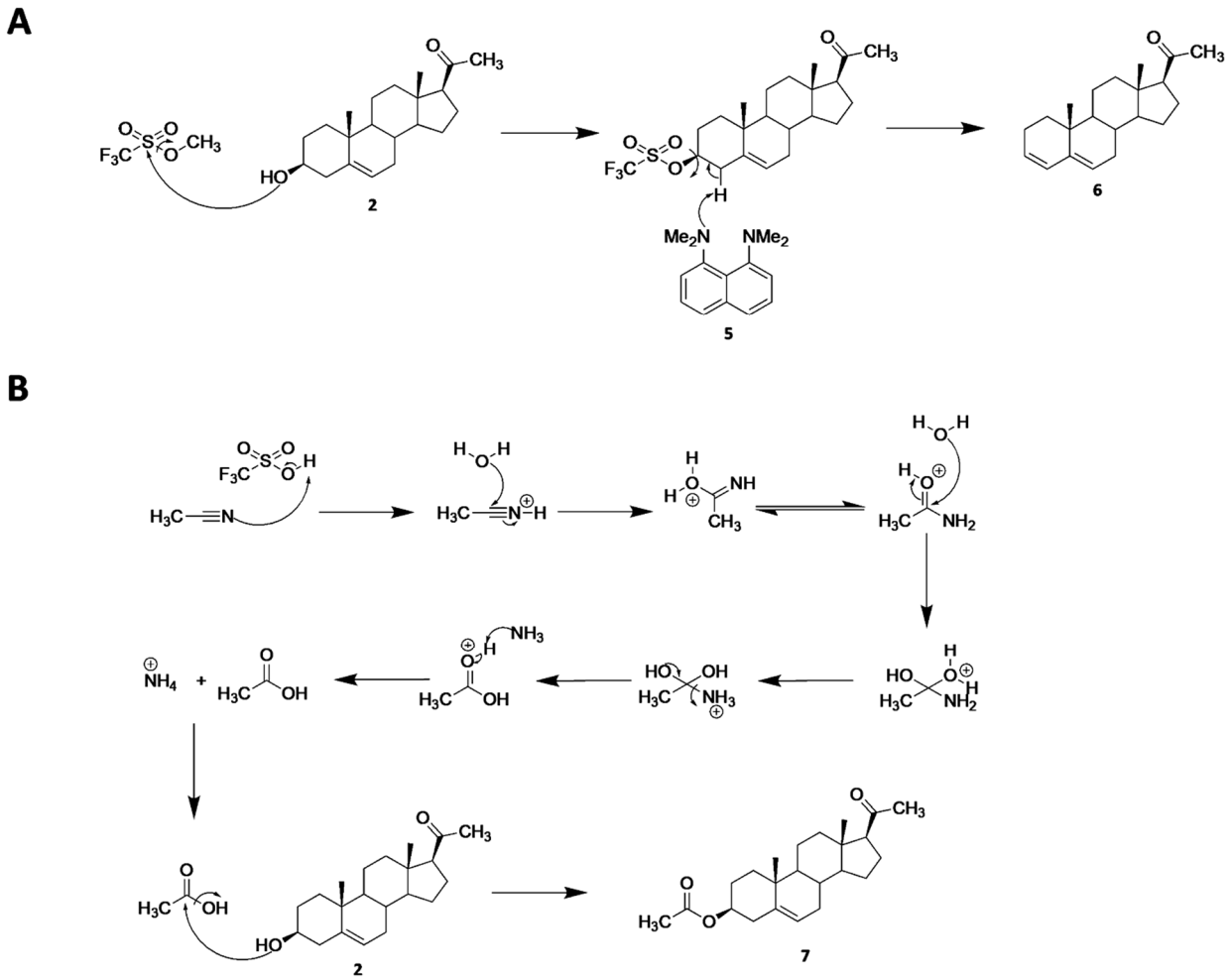
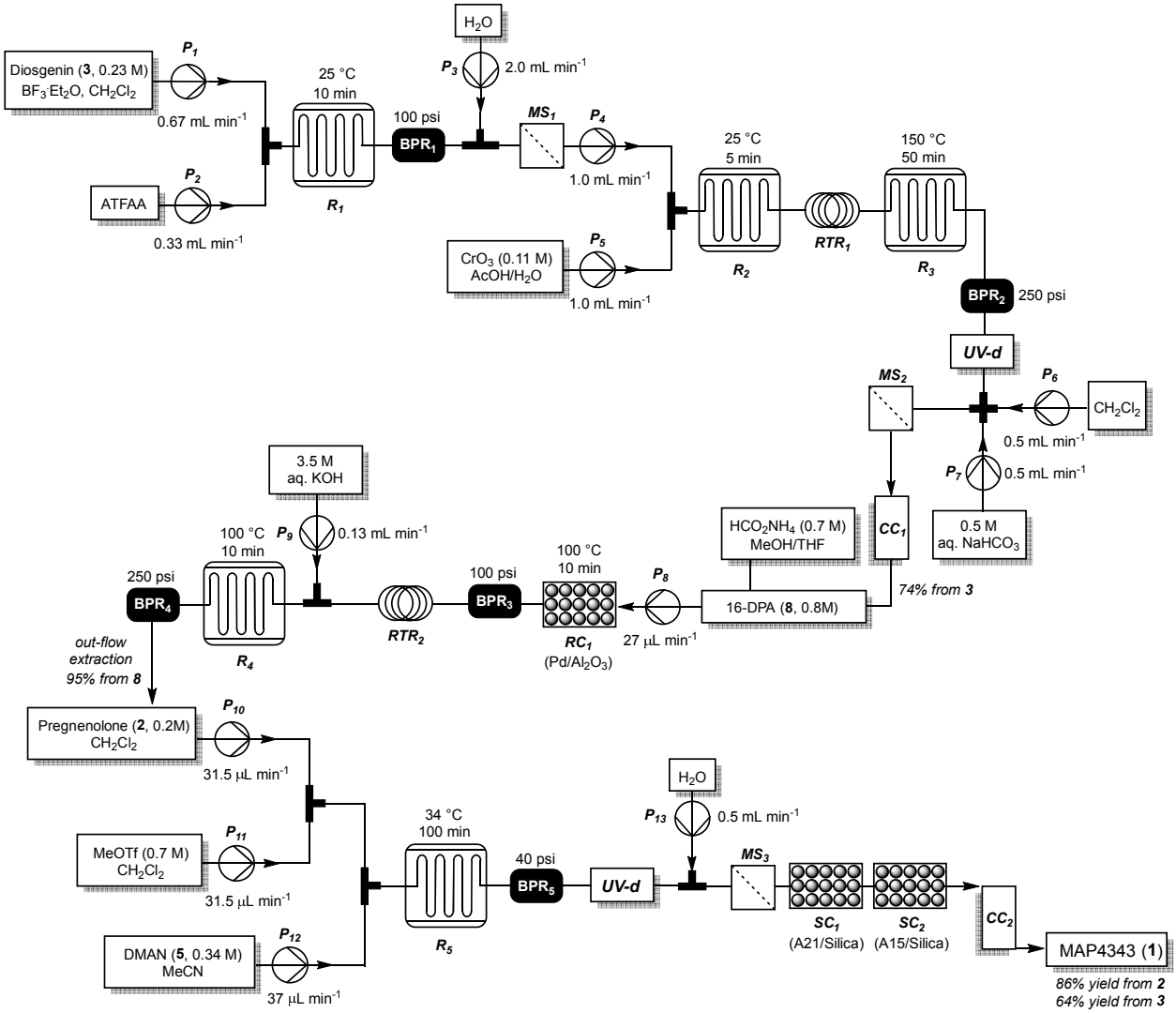


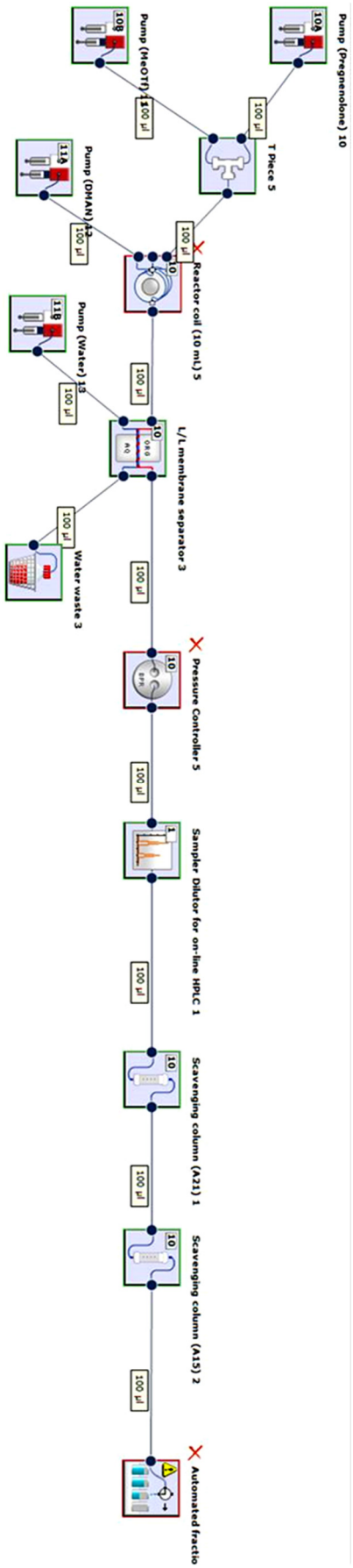
Fig. S11-S14. Normal plots of residuals relative to MAP-4343 (**1**, **S11**), pregnenolone (**2**, **S12**), pregn-3,5-dien-20-one (**6**, **S13**) and 3-acetylpregnenolone (**7**, **S14**).



Scheme S1. Hypothesized mechanism for the formation of the side-products pregn-3,5-dien-20-one (**6**) (**A**) and 3-acetylpregnenolone (**7**) (**B**).



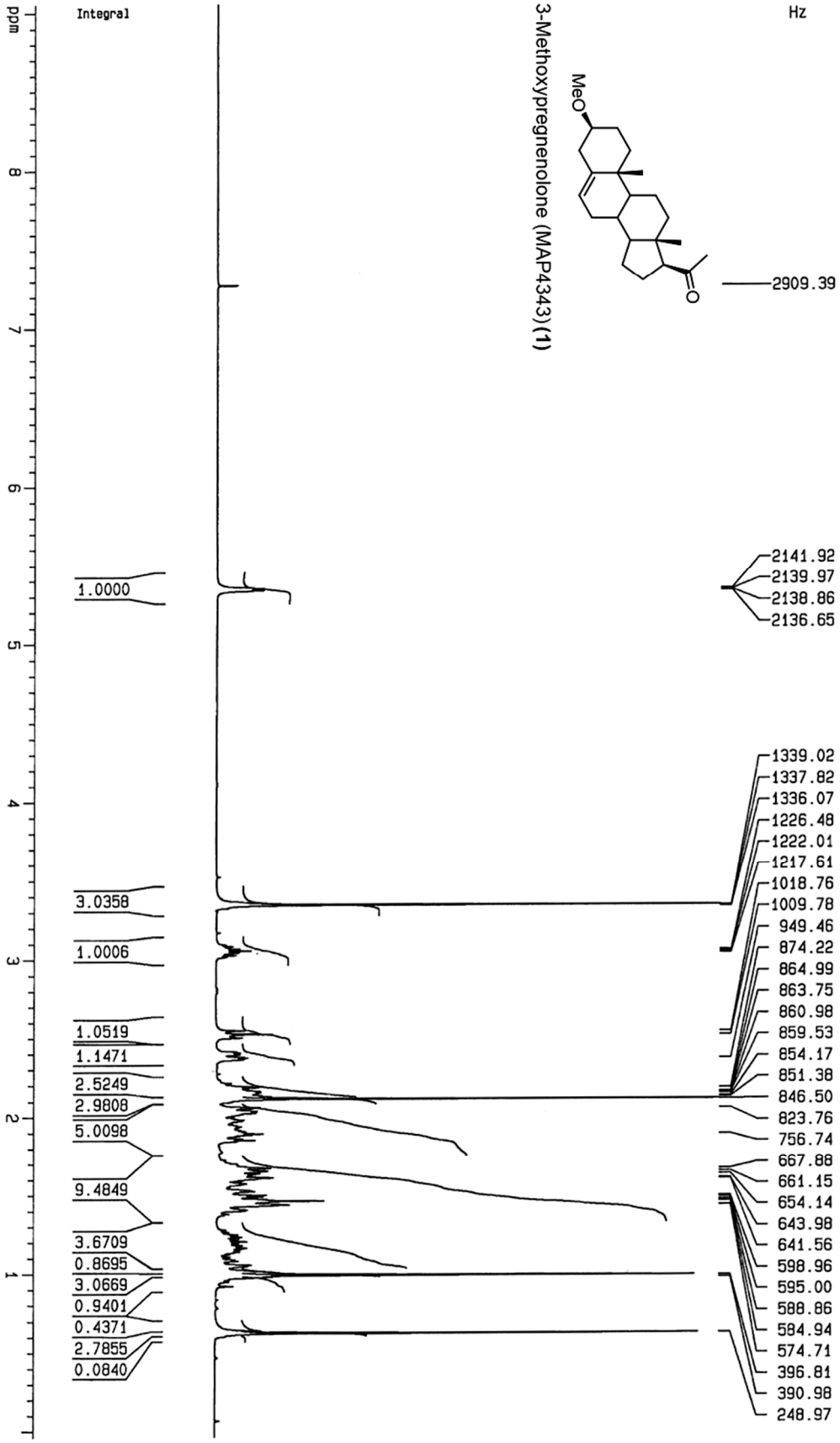
Scheme S2. Integrated multistep flow synthesis of pregnenolone (**2**) and MAP4343 (**1**) from diosgenin (**3**). ***BPR*$_{1\text{-}5}$*:*** back pressure regulators; ***CC*$_{1\text{-}2}$*:*** automated flash chromatography; ***MS*$_{1\text{-}3}$*:*** liquid-liquid membrane separators; ***P*$_{1\text{-}13}$*:*** pumps; ***R*$_{1\text{-}5}$*:*** 10 mL reactor coils; ***RC*$_1$*:*** reactor column; ***RTR*$_{1\text{-}2}$*:*** residence time reactors; ***SC*$_{1\text{-}2}$**:** scavenging columns; ***UV-d:*** UV detectors. The preparation of 16-DPA (**8**) from diosgenin (**3**) in flow reactors was reported in ref. 3.



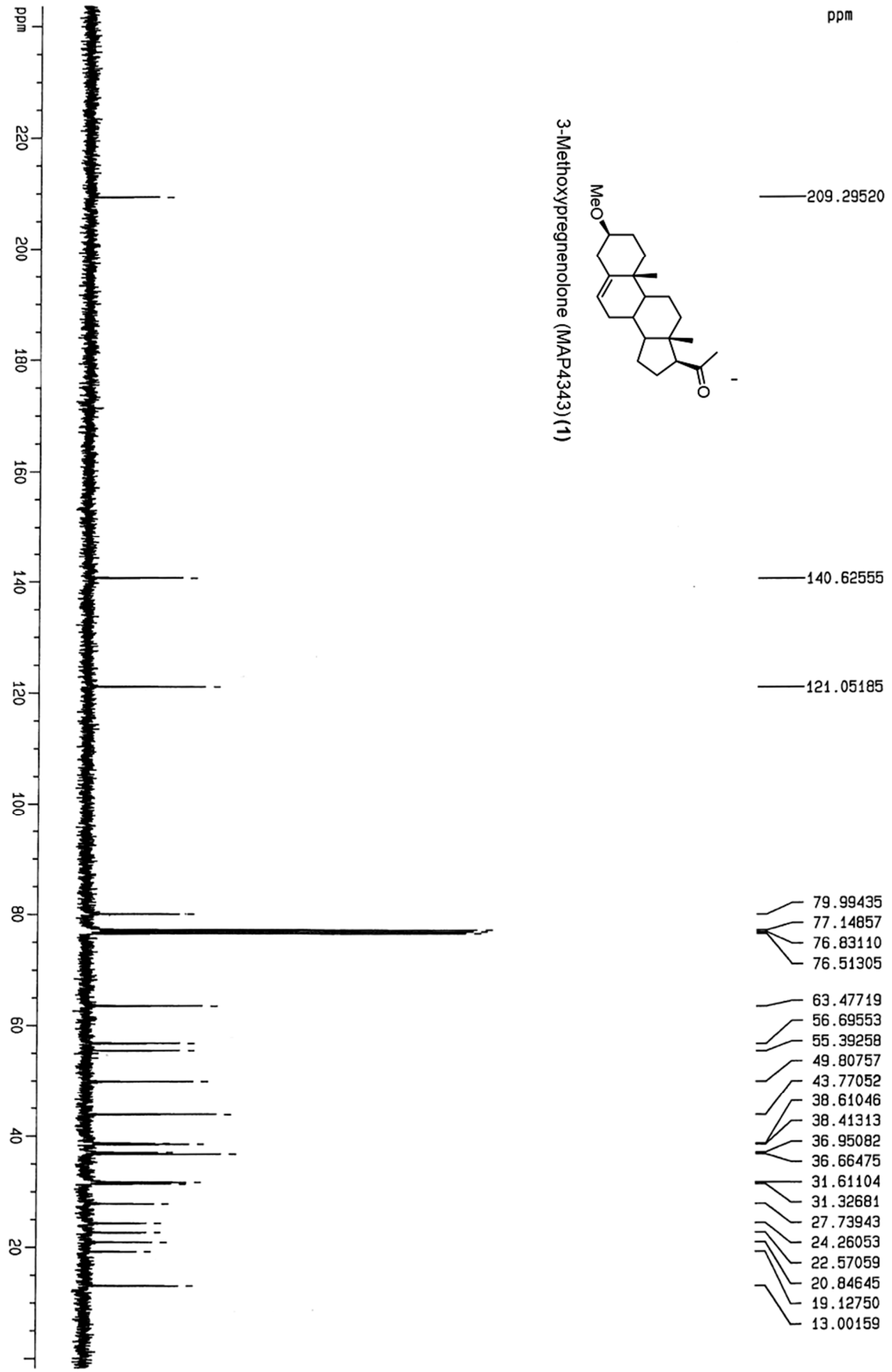
Scheme S3. Set-up for the continuous flow synthesis of MAP4343 designed by Asia Manager PC Software™.

NMR Spectra

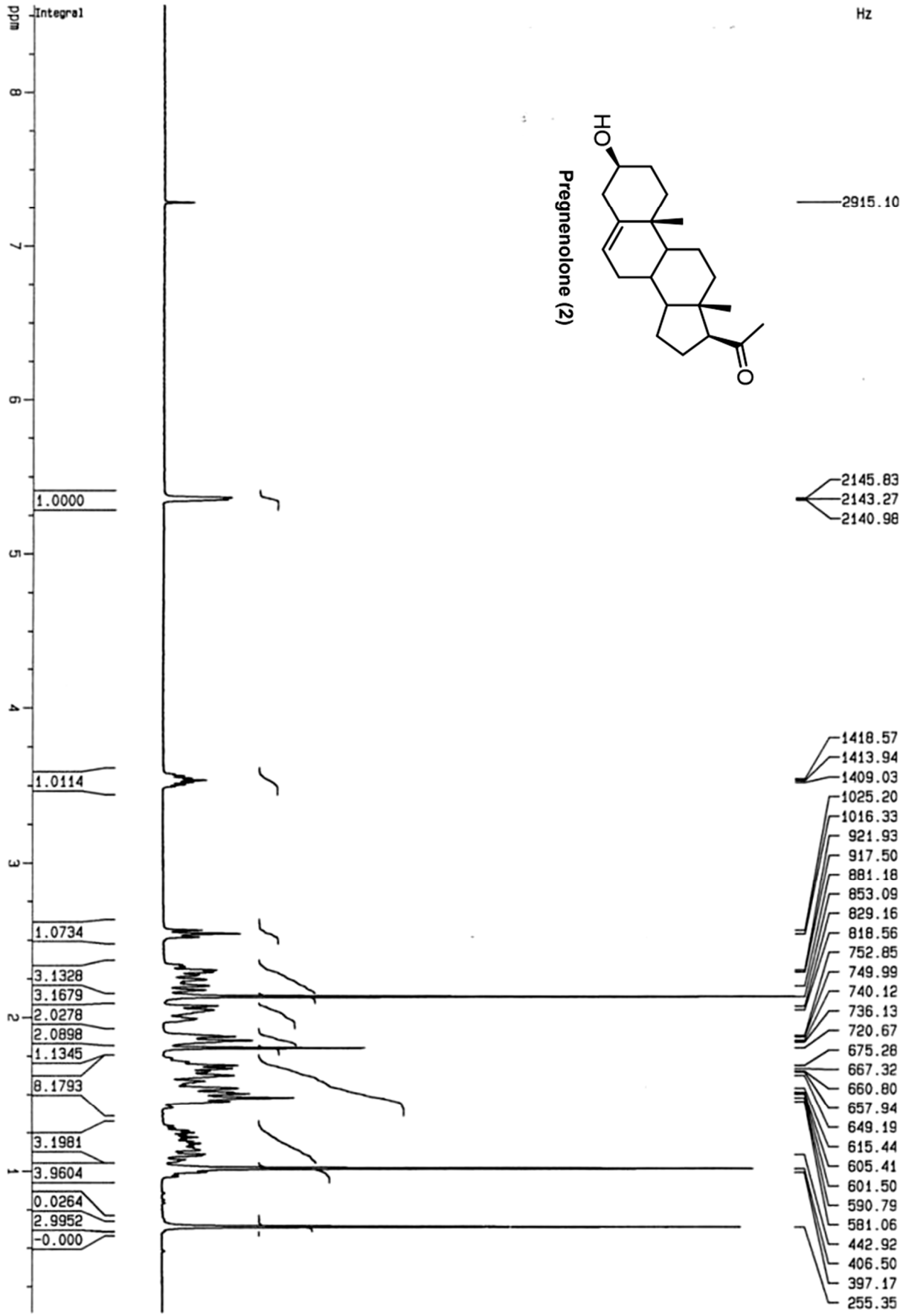
^{1}H-NMR ($CDCl_3$, 400 MHz) of 3-methoxypregnenolone (MAP4343, 1)



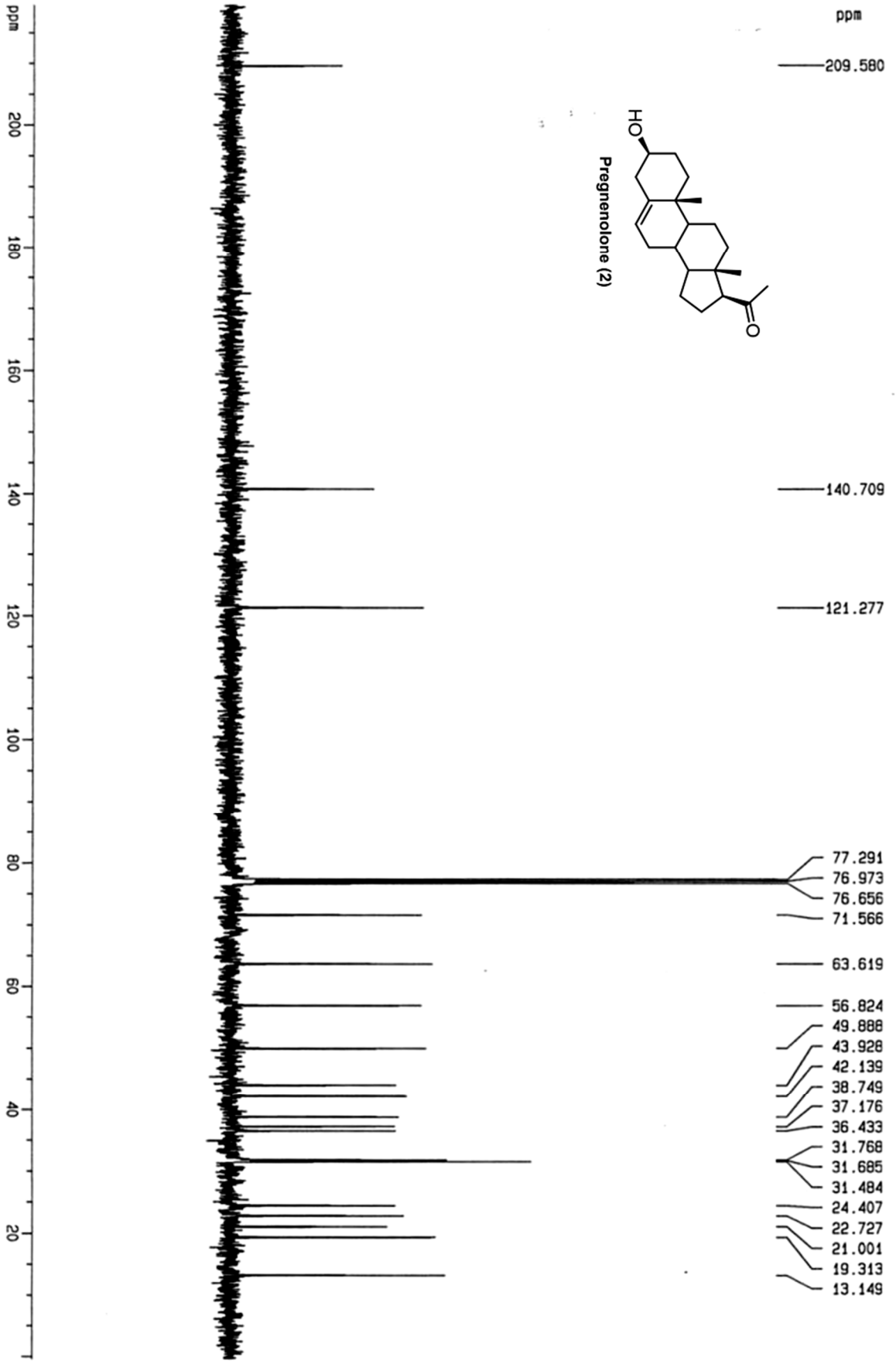
^{13}C-NMR ($CDCl_3$, 100.6 MHz) of 3-methoxypregnenolone (MAP4343, 1)



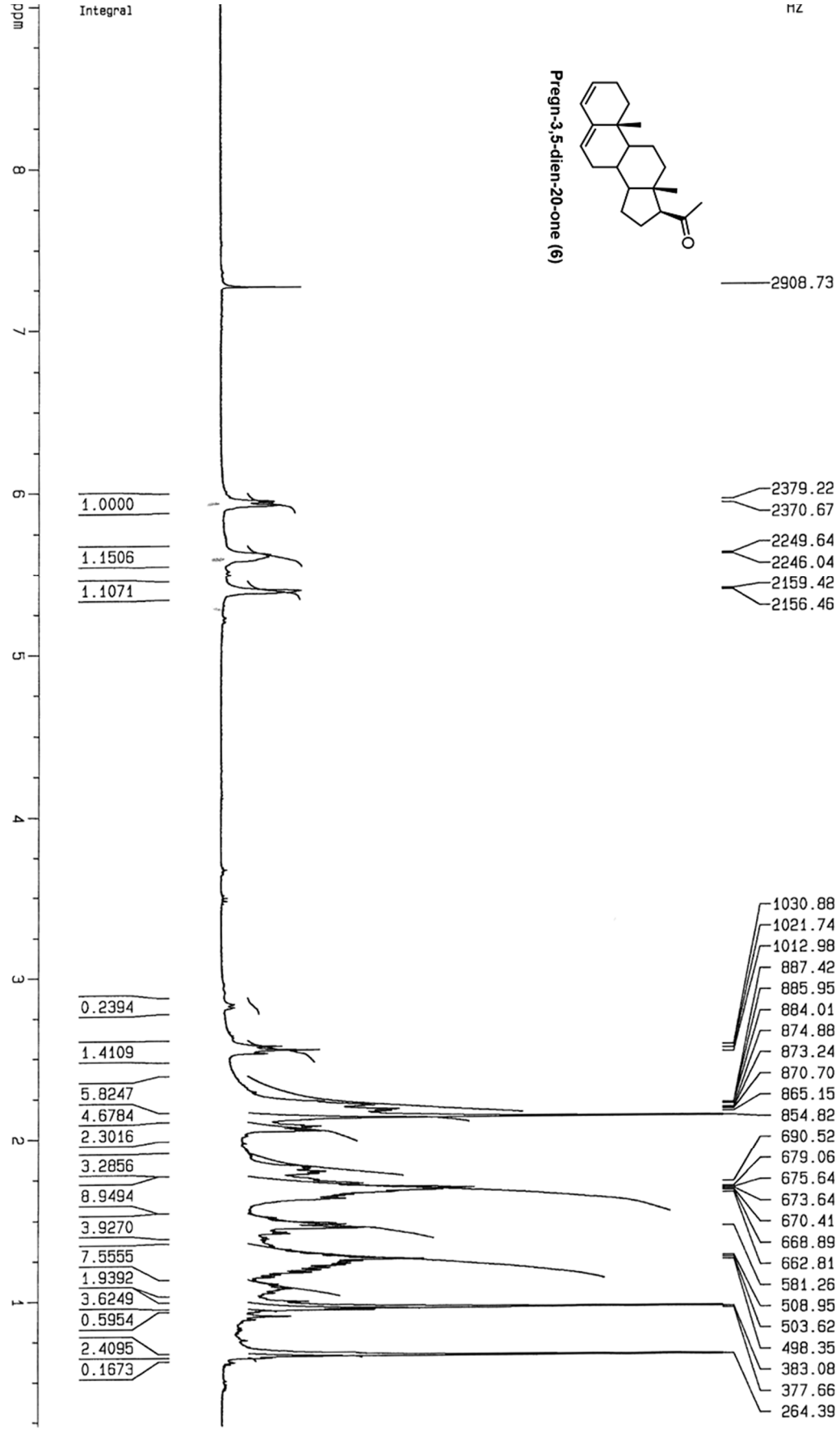
^{1}H-NMR ($CDCl_3$, 400 MHz) of pregnenolone (2)



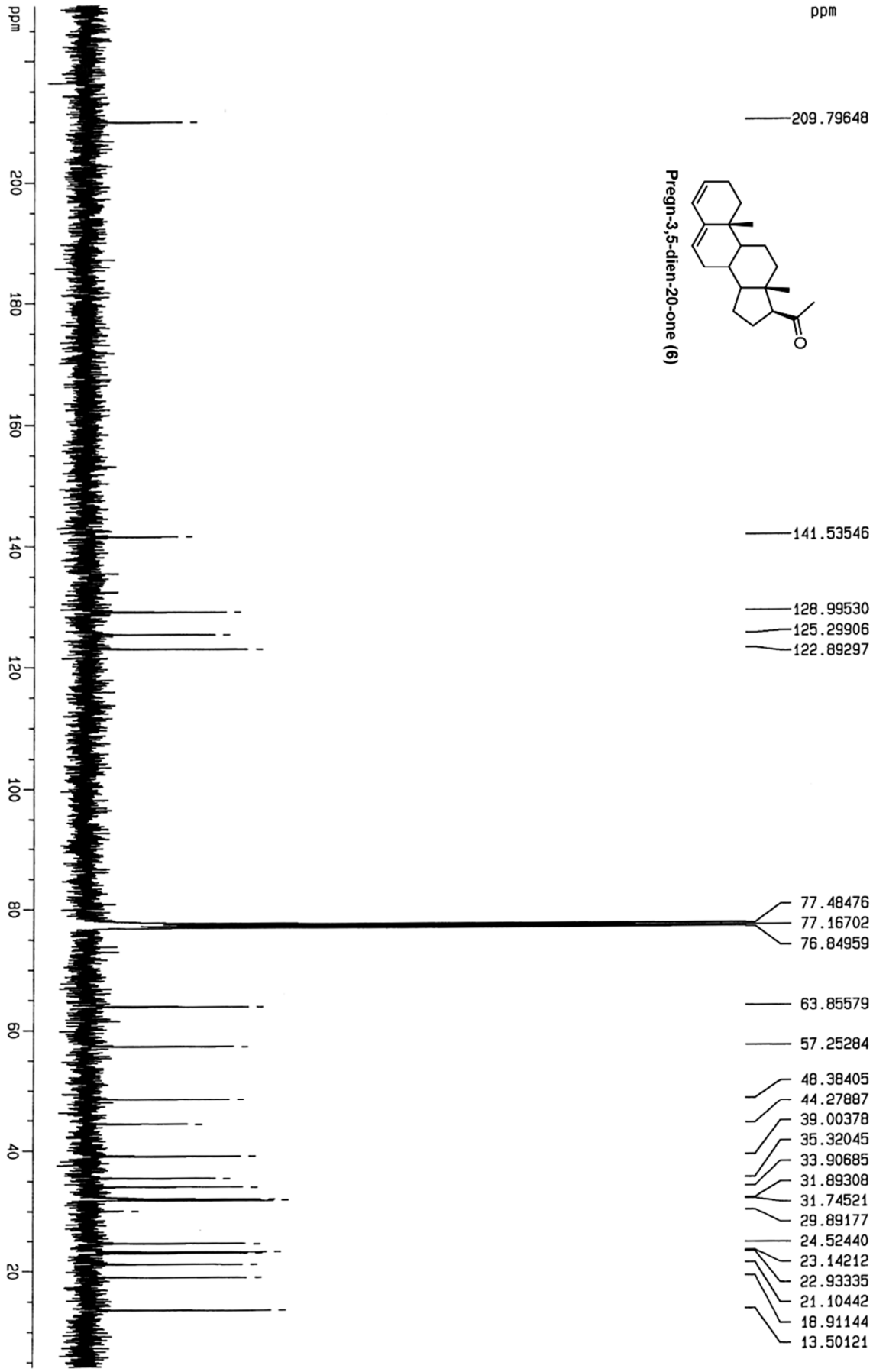
^{13}C-NMR ($CDCl_3$, 100.6 MHz) of pregnenolone (2)



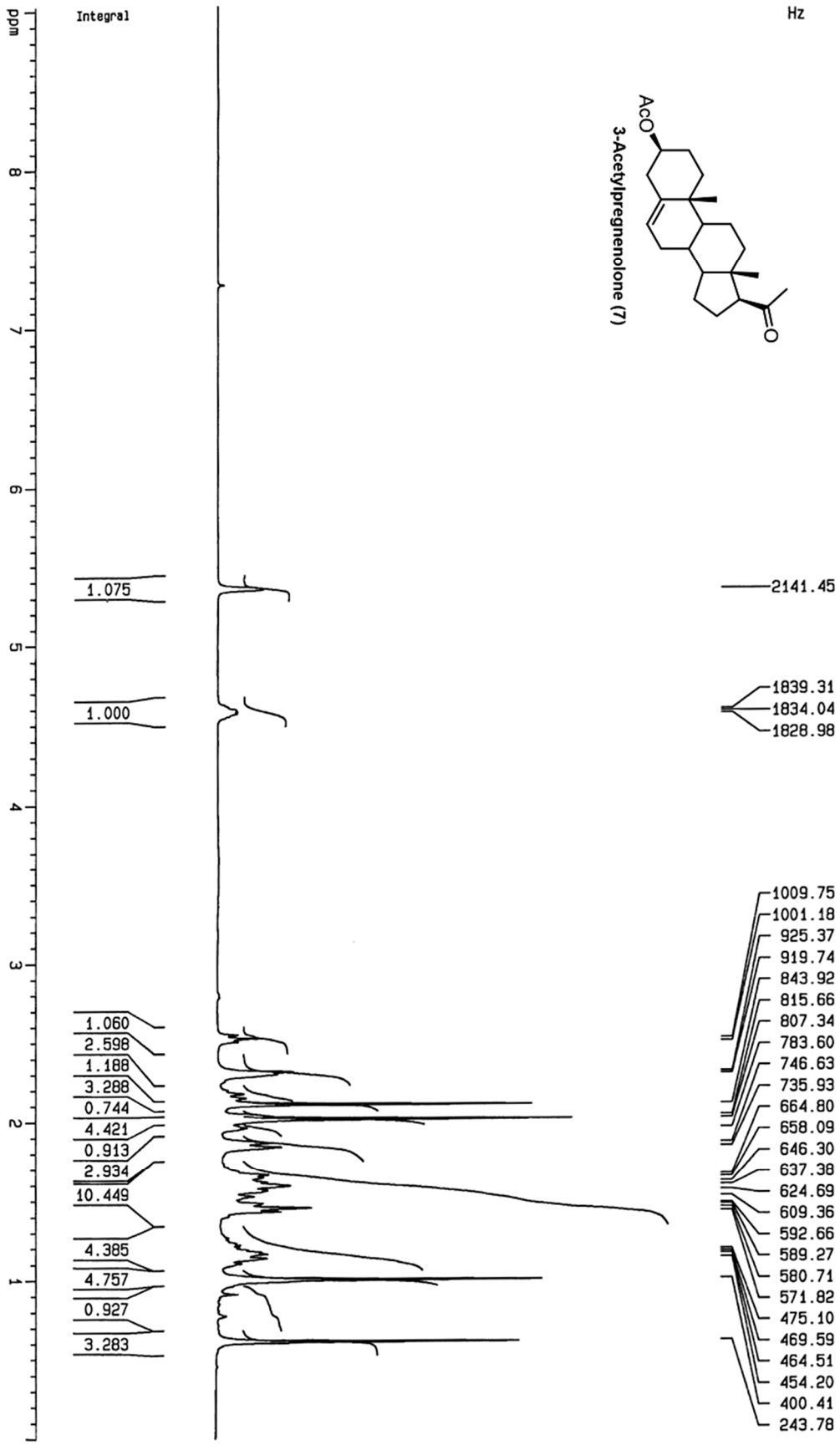
^{1}H-NMR ($CDCl_3$, 400 MHz) of pregn-3,5-dien-20-one (6)



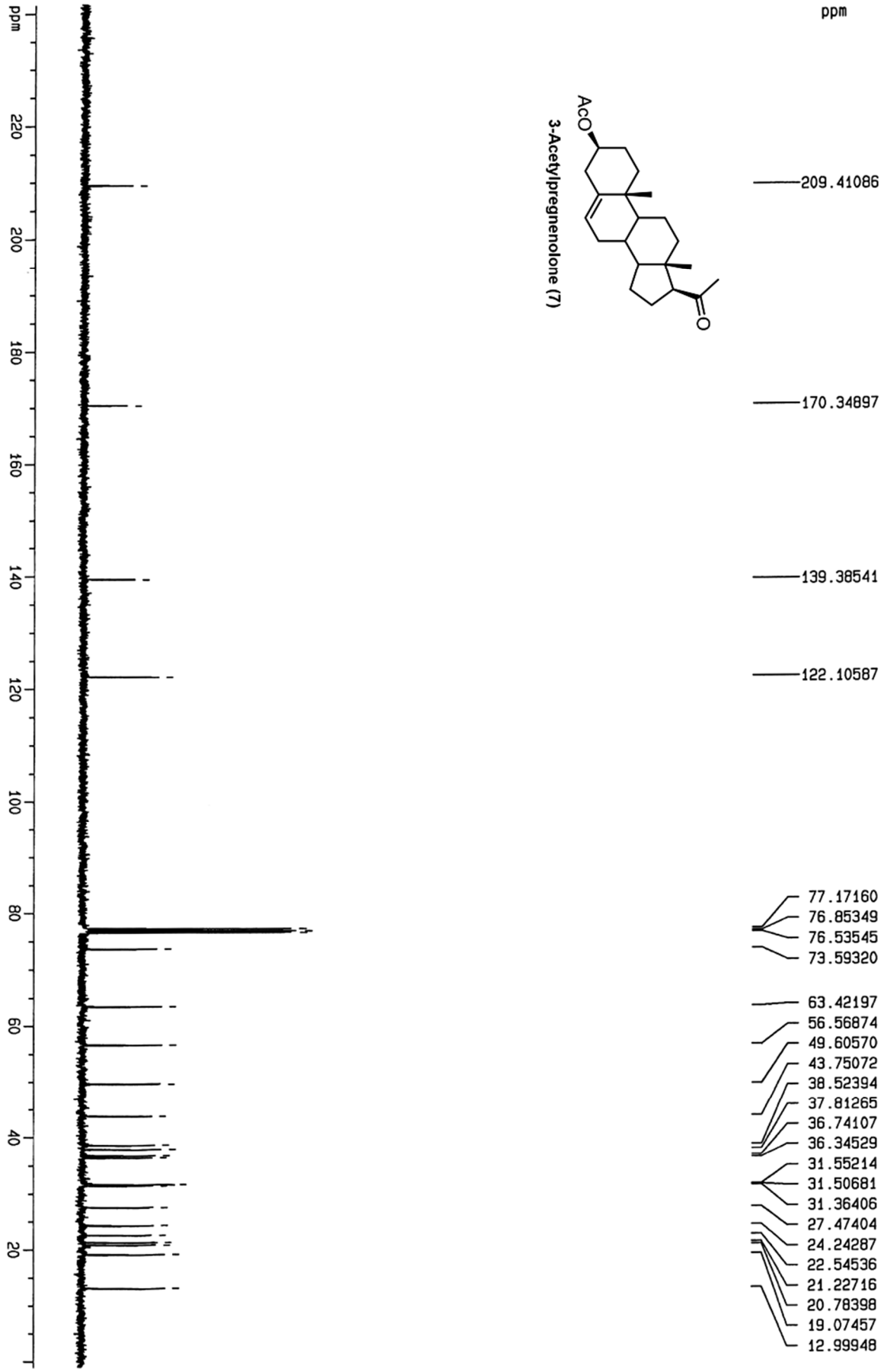
^{13}C-NMR ($CDCl_3$, 100.6 MHz) of pregn-3,5-dien-20-one (6)



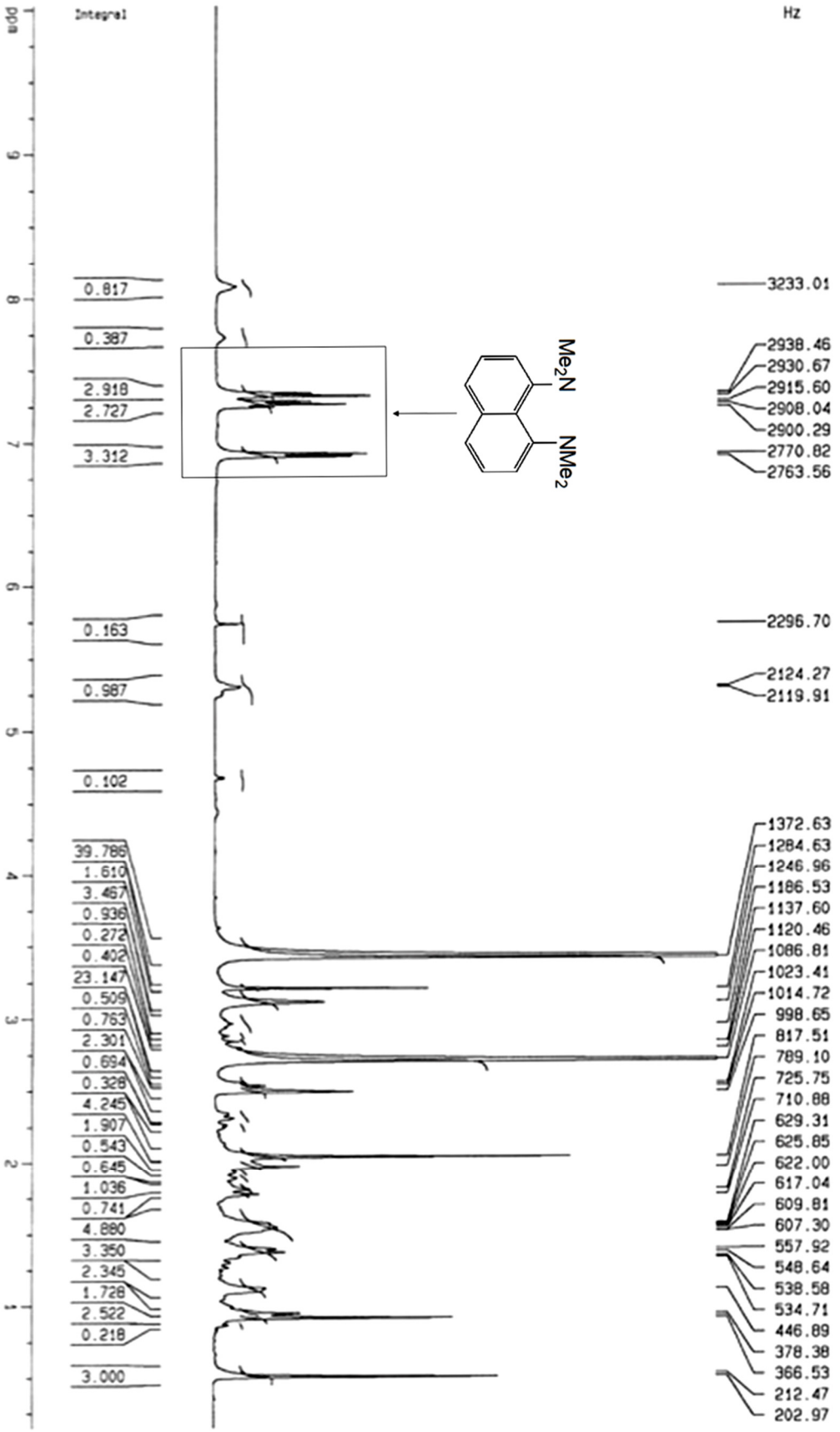
^{1}H-NMR ($CDCl_3$, 400 MHz) of 3-acetylpregnenolone (7)



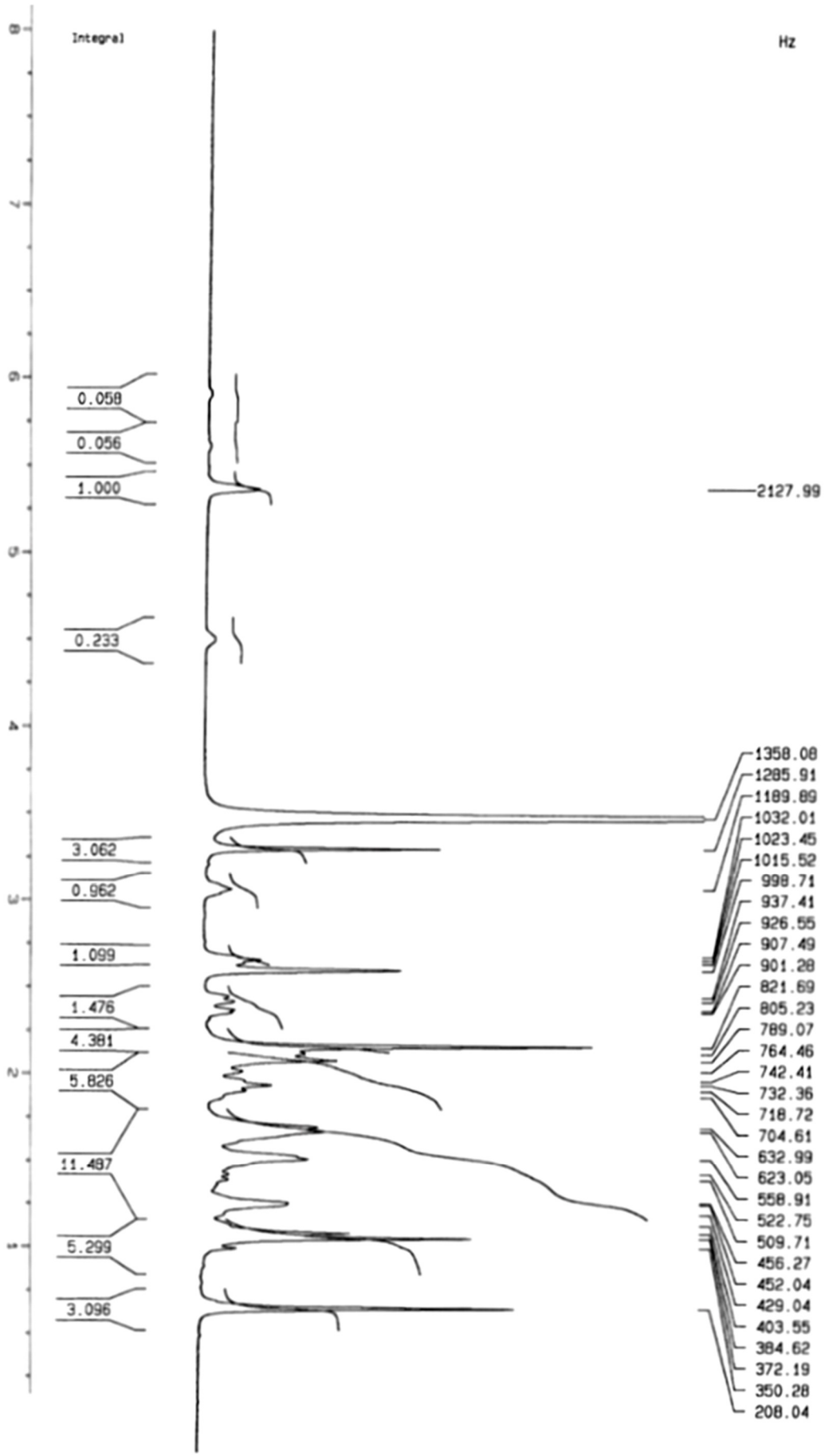
^{13}C-NMR ($CDCl_3$, 100.6 MHz) of 3-acetylpregnenolone (7)



^{1}H-NMR (d^{6}-DMSO, 400 MHz) of crude 3-methoxypregnenolone (MAP4343, 1) before scavenging

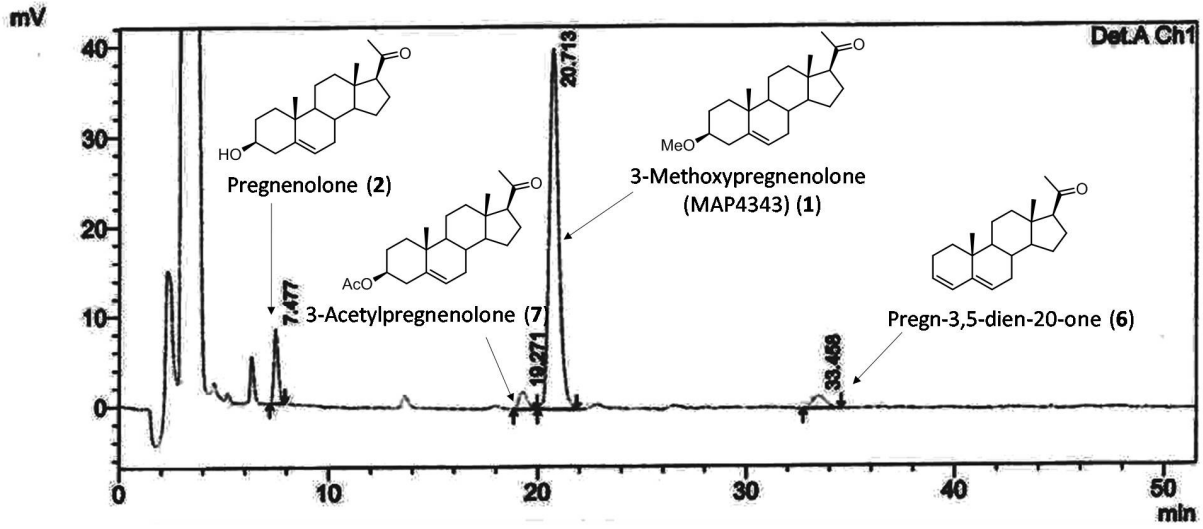


^{1}H-NMR (d^{6}-DMSO, 400 MHz) of crude 3-methoxypregnenolone (MAP4343, 1) after scavenging



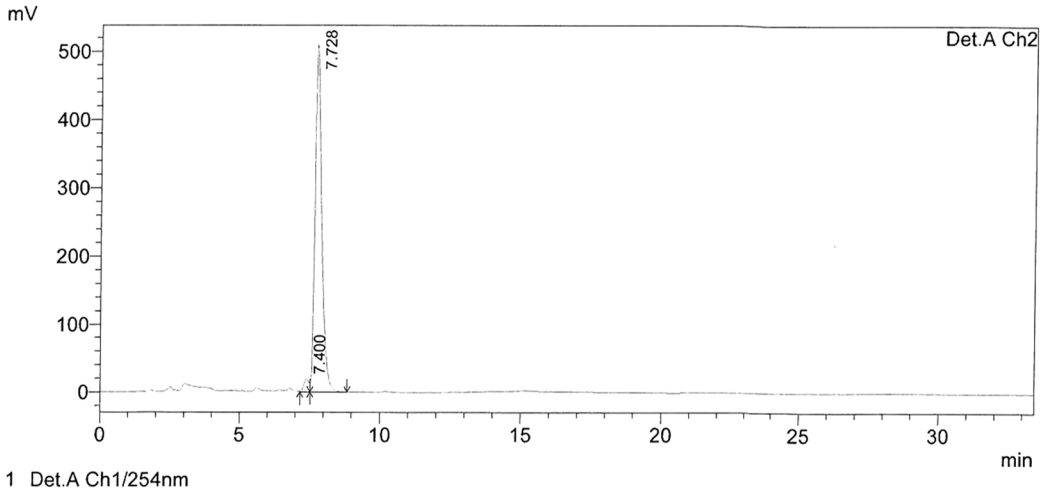
HPLC Analysis

HPLC chromatogram with peaks area, normalized area (mM) and yield (%) of crude MAP4343 (1) obtained by continuous flow synthesis after downstream operations



Chemical Entitites	Peaks Area	Normalized Area (mM)	Yield %
Pregnenolone (**2**)	120723	0.04274222	7.8 %
MAP4343 (1)	1246021	0.467780449	86.2 %
Pregn-3,5-dien-20-one (**6**)	64136	0.023728521	4.4 %
3-Acetylpregnenolone (**7**)	56595	0.008300292	1.6 %

HPLC chromatogram of crude pregnenolone (2) obtained by catalytic transfer flow hydrogenation and alkaline hydrolysis of 16-dehydropregnenolone acetate (16-DPA, 8)

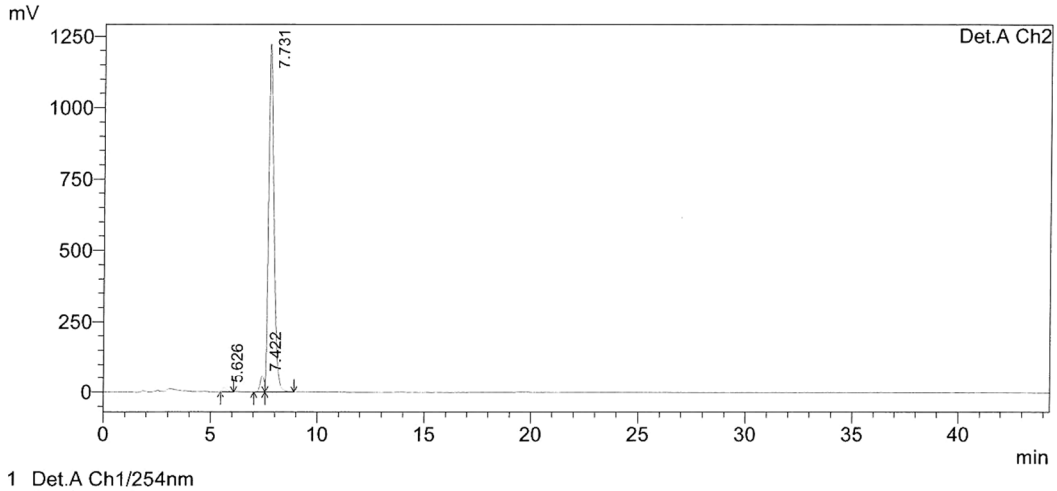


PeakTable

Detector A Ch2 210nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	7.400	247121	19717	2.816	3.722
2	7.728	8527807	510082	97.184	96.278
Total		8774928	529799	100.000	100.000

HPLC chromatogram of crude pregnenolone (2) obtained by Pd-catalyzed flow hydrogenation and alkaline hydrolysis of 16-dehydropregnenolone acetate (16-DPA, 8)



PeakTable

Detector A Ch2 210nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.626	196266	17756	0.882	1.364
2	7.422	757060	60015	3.402	4.610
3	7.731	21299849	1224149	95.716	94.026
Total		22253175	1301919	100.000	100.000

References

1. Z. Szendi, P. Forgò and F. Sweet, *Steroids*, 1995, 60, 442–446.
2. M.-E. Rafestin-Oblin, M. Alami, H. Loosfelt, A. Hamze, A. J. Khan, A. Tikad, M. Lombes and J.-D. Brion, *WO Patent*, 2011138460, 2011.
3. V. Mancino, B. Cerra, A. Piccinno and A. Gioiello, *Org. Process Res. Dev.*, 2018, 22, 600–607.
4. J. N. Moorthy, N. Singhal and K. Senapati, *Tet. Lett.*, 2008, 49, 80–84.