Title: Identification of overlapping, but differential binding sites for the high-affinity CXCR3 antagonists NBI-74330 and VUF11211

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Supplemental Table 1: Sequences of primers used to construct indicated mutants

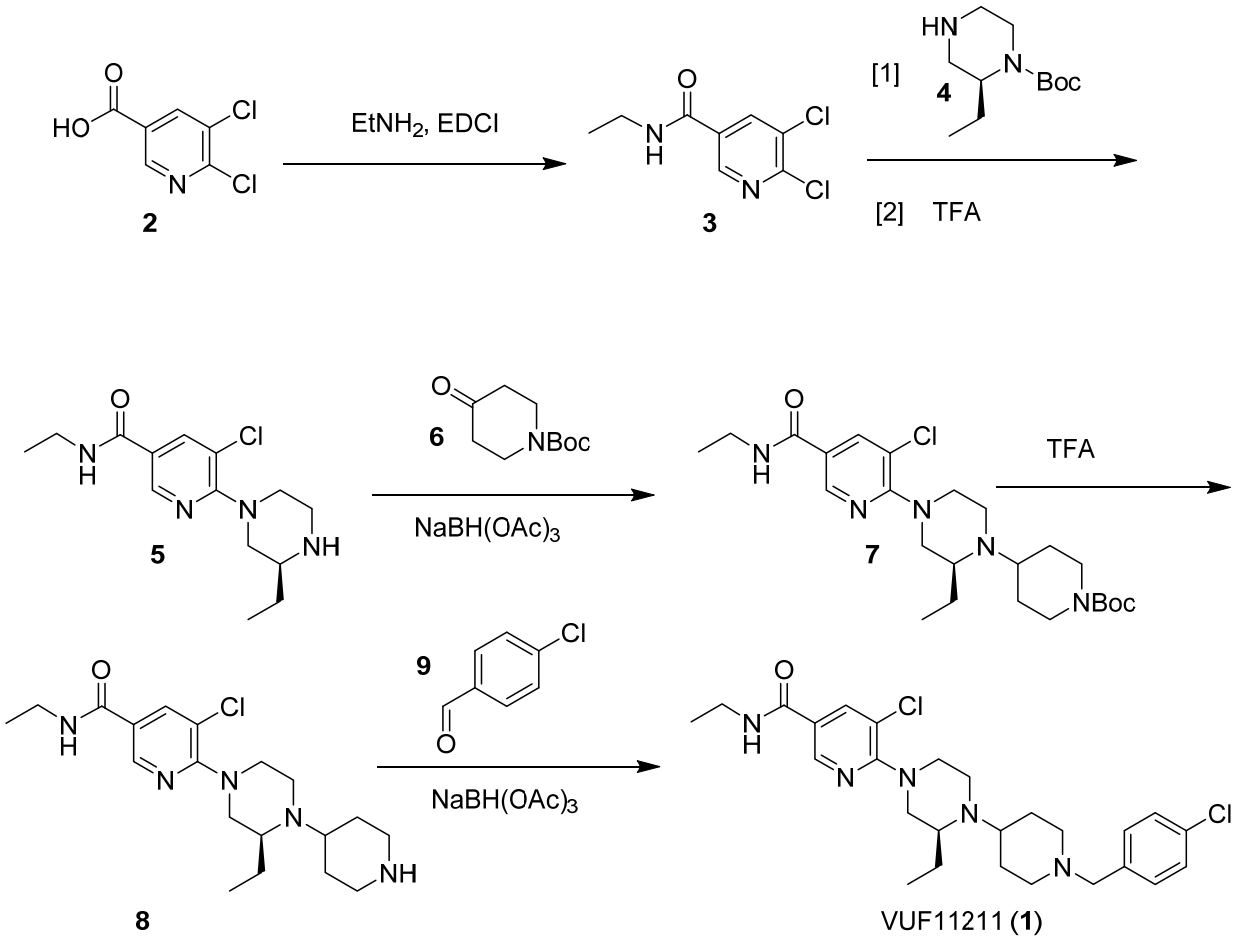
Mutant	**Forward primer (5'-3')**
D46N	CCTCCCCGCCCTGCCCACAGAATTTCAGCCTGAACTTCGACCG
D52N	CTTCAGCCTGAACTTCAACCGGGCCTTCCTGC
D89N	GCCCTGAGCAGCACCAACACCTTCCTGCTCC
D112N	ACACTGCCGCTCTGGGCAGTGAATGCTGCCGTCCAGTGGGTCTTT
D186N	GCTTTTCGCCCTCCCAAACTTCATCTTCCTGTCG
D195N	CTGTCGGCCCACCACAACGAGCGCCTCAACG
E196N	GGCCCACCACGACAACCGCCTCAACGCCAC
D278N	CCTGGTGGTGCTGGTGAACATCCTCATGGACC
D282N	GCTGGTGGACATCCTCATGAACCTGGGCGCTTTGGCCCGC
E293N	GCAACTGTGGCCGAAACAGCAGGGTAGACGTG
D297N	CCGAGAAAGCAGGGTAAACGTGGCCAAGTCGG
Y60A	CTTCCTGCCAGCCCTCGCCAGCCTCCTCTTTCTG
Y60F	CTTCCTGCCAGCCCTCTTCAGCCTCCTCTTTCTG
W109Q	CTGACACTGCCGCTCCAGGCAGTGGACGCTG
G128H	CCTCTGCAAAGTGGCACACGCCCTCTTCAACATC
F131A	GTGGCAGGTGCCCTCGCCAACATCAACTTCTAC
F131H	GTGGCAGGTGCCCTCCACAACATCAACTTCTAC
F135A	CTCTTCAACATCAACGCCTACGCAGGAGCCCTC
W268Q	GCCTTTGCCCTCTGCCAGACCCCCTATCACCTG
Y271A	CTCTGCTGGACCCCCGCCCACCTGGTGGTGCTG
K300I	GCAGGGTAGACGTGGCCATCTCGGTCACCTCAGGCCTGG
S301A	GTAGACGTGGCCAAGGCCGTCACCTCAGGCCTG
S304A	GCCAAGTCGGTCACCGCCGGCCTGGGCTACATG
S304E	GCCAAGTCGGTCACCGAGGGCCTGGGCTACATG
S304L	CGTGGCCAAGTCGGTCACCCTGGGCCTGGGCTACATGCAC
Y308A	CACCTCAGGCCTGGGCGCCATGCACTGCTGCCTC
Y308F	CCTCAGGCCTGGGCTTCATGCACTGCTGCCTC

Note: the reverse primer is the "reverse complemented" version of the primers indicated above.

Supplemental Methods: Synthesis and chemical analysis of VUF11211

Approach

The synthesis of VUF11211 was carried out according to the general procedures patented by Merck (see patent citation in main text). The scheme below depicts the approach.



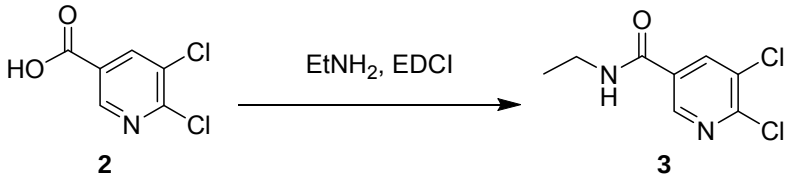
Overview of synthetic approach

General synthetic remarks

THF, toluene and CH_2Cl_2 were distilled freshly from CaH_2. All other solvents were used as received. Unless indicated otherwise, all reactions were carried out under an inert nitrogen atmosphere. TLC analyses were performed with Merck F254 Alumina Silica Plates using UV visualization or staining. Microwave reactions were carried out on a Biotage® Initiator. Column purifications were carried out using the Biotage® equipment. All HRMS spectra were recorded on Bruker micrOTOF MS using ESI in positive ion mode. The ^{1}H-, ^{13}C- and 2D-NMR spectra were recorded on a Bruker 250, 400 or 500 MHz spectrometer. Systematic names for molecules were generated using ChemBioDraw Ultra 12. Chemical purities were measured with the aid of analytical LC-MS using a Shimadzu LC-20AD liquid chromatograph pump system with a Shimadzu SPD-M20A diode array detector with the MS detection performed with a Shimadzu LCMS-2010EV mass spectrometer. The column used was an Xbridge (C18) 5 µm column (50 mm × 4.6 mm). Elution program: solvent B (MeCN/0.1% formic acid) and solvent A (water/0.1% formic acid), flow rate of 1.0 mL/min, start 5% B, linear gradient to 90% B in 4.5 min, then 1.5 min at 90% B, then linear gradient to 5% B in 0.5 min, then 1.5 min at 5% B, total run time of 8 min. Compound purities were calculated as the percentage peak area of the analyzed compound by UV detection at (unless mentioned otherwise) 230 nm. Unless reported otherwise, all compounds have a purity ≥ 95 % as measured by these LC-MS analyses. Optical purities were measured with the aid of chiral chromatography. The column used was a Chiralcel OD-H 250x4.6 mm. The eluent used was *n*-hexane (+0.05% diethylamine)/2-propanol = 90/10 with a flow rate of 0.7 mL/min at 40 ºC. The run time was 30 min and UV detection was conducted at 211 nm. Specific optical rotations were measured with a sodium lamp and are reported as follows: $[\alpha]_D^{23}$ (c = g/100 mL, solvent). *Tert*-butyl (S)-2-ethylpiperazine-1-carboxylate (**4**) was from Porse Fine Chemical (> 98 % e.e). Its optical rotation $[\alpha]_D^{23}$ (c = 4.79 g/100 mL, MeOH) was +41.7º. Racemic *tert*-butyl (S)-2-ethylpiperazine-1-carboxylate hydrochloride (***rac*-4.HCl**) was from Acesys Pharmatech. As expected, its optical rotation measured under similiar conditions as with **4** amounted to zero. All other chemicals were from Sigma-Aldrich.

Synthetic procedures

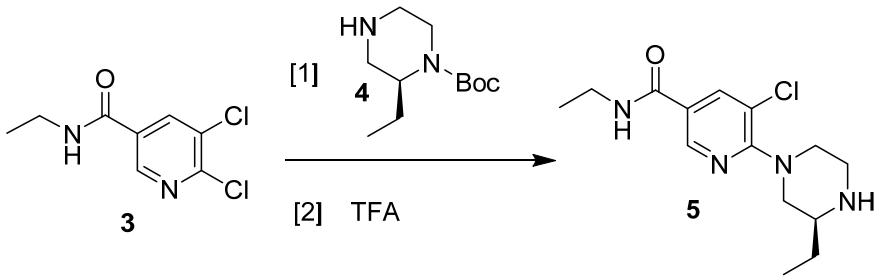
5,6-Dichloro-N-ethylnicotinamide (3).



2 **3**

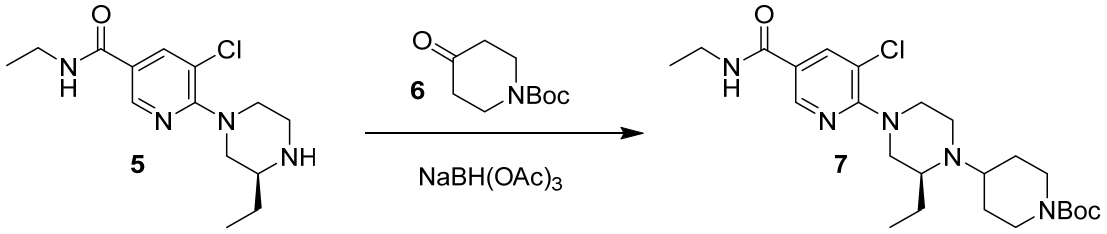
Acid **2** (10.0 g, 52.1 mmol) and $EtNH_2$.HCl (8.5 g, 104 mmol) were dissolved in DMF (200 mL, dried over molsieves). EDCI (10.0 g, 52.2 mmol) and Et_3N (15.0 mL, 108 mmol) were added and the mixture was stirred overnight at room temperature. DMF was removed under reduced pressure. The residue was dissolved in DCM (200 mL) and washed with 1.0 M aq. Na_2CO_3 (200 mL) and water (200 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum. Column chromatography was performed (eluent: EtOAc/heptane = 1/1) to give the product as a white solid (5.35 gr, 47 %).
LC-MS purity: 98+ %. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.61 (d, 1H, J=2.1 Hz), 8.19 (d, 1H, J=2.1 Hz), 6.23-6.12 (br, 1H), 3.51 (dq, 2H, J_1=7.3 Hz), 1.27 (t, 3H, J=7.3 Hz).

(S)-5-Chloro-N-ethyl-6-(3-ethylpiperazin-1-yl)nicotinamide (5).



Chloride **3** (5.35 g, 24.4 mmol) was dissolved in DMF (50 mL, dried over molsieves). Piperazine **4** (5.0 g, 23.3 mmol) was added, followed by the addition of K_2CO_3 (8.0 gr, 58 mmol). The mixture was stirred overnight at 80°C. The DMF was evaporated under reduced pressure. The residue was dissolved in DCM (100 mL) and washed with water (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum to give crude Boc-protected product. This was dissolved in DCM (50 mL) and CF_3COOH (5 mL) was added dropwise. The mixture was stirred for 1 hour at room temperature and subsequently concentrated under vacuum. The residue was dissolved in DCM (25 mL) and washed with 1.0 M aq. NaOH (25 mL) and water (25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum. Column chromatography (gradient: EtOAc/Et_3N 99/1 to EtOAc/MeOH/Et_3N 80/20/1) afforded the product as a white solid (3.35 g, 46 %).
LC-MS purity: 98+ %. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.48 (d, 1H, J=2.1 Hz), 7.97 (d, 1H, J=2.1 Hz), 6.09 (br t, 1H), 4.00-3.89 (m, 2H), 3.48 (dq, 2H, J_1=7.3 Hz), 3.11-2.89 (m, 3H), 2.82-2.72 (m, 1H), 2.58 (dd, 1H, J_1=12.4 Hz, J_2=10.0 Hz), 1.52-1.35 (m, 2H), 1.23 (t, 3H, J=7.3 Hz), 0.97 (t, 3H, J=7.5 Hz).

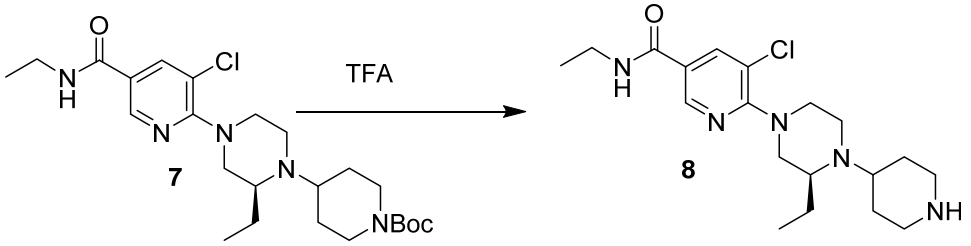
***Tert*-butyl (S)-4-(4-(3-chloro-5-(ethylcarbamoyl)pyridin-2-yl)-2-ethylpiperazin-1-yl)piperidine-1-carboxylate (7).**



Amine **5** (3.30 g, 11.1 mmol) was dissolved in dry DCM (75 mL). Piperidone **6** (4.4 g, 22.2 mmol) was added stepwise, followed by the addition of $NaBH(OAc)_3$ (4.7 g, 22.2 mmol). The mixture was stirred for 16 hours at room temperature. LC-MS inspection of an aliquot revealed incomplete conversion. Therefore, more piperidone **6** (2.0 g, 10.0 mmol) and

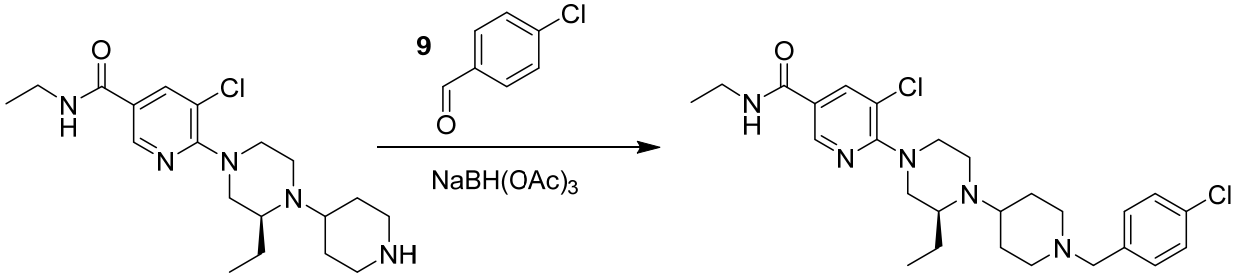
$NaBH(OAc)_3$ (2.0 g, 9.4 mmol) were added. Stirring was continued for another 24 hours at room temperature. Aq. 1.0 M NaOH (75 mL) was added to quench the reaction. The organic layer was collected and the water layer was extracted once with DCM (75 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. Column chromatography (eluent: EtOAc) afforded the product as a white solid (4.35 g, 81 %).
LC-MS purity: 98+ %. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.47 (d, 1H, J=2.1 Hz), 7.97 (d, 1H, J=2.1 Hz), 5.94 (br t, 1H), 4.22-4.08 (br, 2H), 3.75-3.64 (m, 2H), 3.52-3.44 (m, 2H), 3.31-3.22 (m, 1H), 3.22-3.12 (m, 1H), 2.91-2.59 (m, 6H), 1.75-1.65 (m, 2H), 1.65-1.53 (m, 3H), 1.48-1.33 (m, 1H), 1.46 (s, 9H), 1.24 (t, 3H), 0.94 (t, 3H, J=7.4 Hz).

(S)-5-Chloro-N-ethyl-6-(3-ethyl-4-(piperidin-4-yl)piperazin-1-yl)nicotinamide (8).



Boc-protected amine **7** (4.35 g, 9.1 mmol) was dissolved in DCM (50 mL). CF_3COOH (5 mL) was added drop wise. The mixture was stirred for 1 hour at room temperature and subsequently concentrated under vacuum. The residue was dissolved in DCM (25 mL) and washed with 1.0 M aq. NaOH (25 mL) and water (25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum. Column chromatography (gradient: EtOAc/Et_3N 99/1 to EtOAc/MeOH/Et_3N 80/20/1) gave the product as a yellow foam (2.3 g, 67 %).
LC-MS purity: 98+ %. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.47 (d, 1H, J=2.1 Hz), 7.97 (d, 1H, J=2.1 Hz), 5.97 (br t, 1H, J=5.0 Hz), 3.77-3.68 (m, 2H), 3.48 (dq, 2H, J_1=7.3 Hz), 3.29-3.23 (m, 1H), 3.22-3.11 (m, 3H), 2.91-2.80 (m, 2H), 2.78-2.71 (m, 1H), 2.71-2.55 (m, 3H), 1.79-1.70 (m, 2H), 1.68-1.50 (m, 3H), 1.48-1.36 (m, 1H), 1.24 (t, 3H, J=7.1 Hz), 0.93 (t, 3H, J=7.5 Hz).

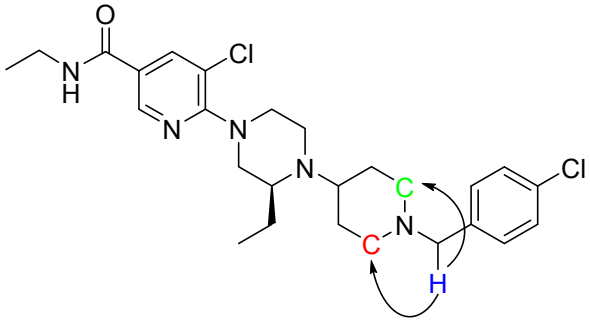
(S)-5-Chloro-6-(4-(1-(4-chlorobenzyl)piperidin-4-yl)-3-ethylpiperazin-1-yl)-N-ethylnicotinamide (1, VUF11211).



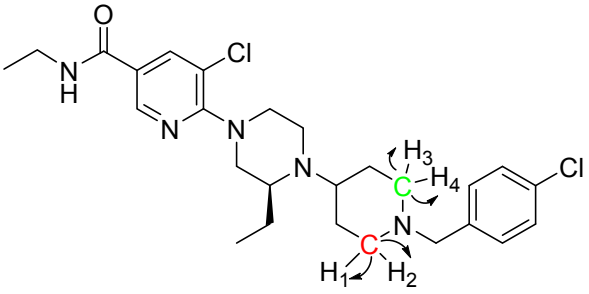
Amine **8** (800 mg, 2.1 mmol) was dissolved in DCM (20 mL). Aldehyde **9** (300 mg, 2.1 mmol) was added, followed by the addition of $NaBH(OAc)_3$ (1.0 g, 4.7 mmol). The reaction mixture was stirred overnight at room temperature. The mixture was quenched with 1.0 M aq. NaOH (20 mL). The organic layer was collected, washed with water (20 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. Column chromatography (eluent: EtOAc / MeOH / TEA = 100/0/1 to 80/20/1) afforded an oil. The obtained oil was crystallized from hot EtOAc to give the product as a white solid (550 mg, 52 %). The compound tends to retain traces of EtOAc (1-5 mole %) even after extensive drying.
LC-MS purity: 99+ %. Chiral LC purity: one peak at 15.22 min (co-injection with ***rac*-1** confirms identity of peak). HR-MS: $[M+H]^+$ $C_{26}H_{36}Cl_2N_5O^+$ calc: 504.2291, found: 504.2269. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 8.60 (d, 1H, J=2.1 Hz), 8.47 (t, 1H, J=5.5 Hz), 8.12 (d, 1H, J=2.1 Hz), 7.36 (d, 2H, J=8.5 Hz), 7.30 (d, 2H, J=8.5 Hz), 3.56-3.49 (m, 2H), 3.42 (app s, 2H),

3.24 (dq, 2H, J1=7.1 Hz), 3.20-3.13 (m, 1H), 3.13-3.06 (m, 1H), 2.85-2.77 (m, 3H), 2.70-2.64 (m, 1H), 2.64-2.60 (m, 1H), 2.60-2.53 (m, 1H), 2.01-1.88 (m, 2H), 1.66-1.55 (m, 3H), 1.55-1.46 (m, 2H), 1.37 (dq, 1H, J1=11.8 Hz), 1.10 (t, 3H, J=7.1 Hz), 0.84 (t, 3H, J=7.4 Hz).
^{13}C-APT-NMR (DMSO-d_6 (↑), 125 MHz) δ 163.6 (↑), 159.0 (↑), 145.8 (↓), 138.2 (↑), 138.2 (↓), 131.8 (↑), 130.9 (↓), 128.6 (↓), 124.2 (↑), 119.7 (↑), 61.6 (↑), 56.7 (↓), 55.5 (↓), 53.4 (↑), 52.9 (↑), 52.1 (↑), 49.2 (↑), 44.2 (↑), 34.5 (↑), 30.7 (↑), 25.4 (↑), 20.4 (↑), 15.2 (↓), 10.4 (↓).
Hardcopies of selected spectra are available at the end of this Supporting Information.

2D-NMR spectroscopy: From the APT spectrum it became clear that the piperidine CH_2 groups in compound **1** are non-equivalent (making the total amount of expected aliphatic carbons to be 14), perhaps due to severely restricted rotation around N-CH bond (low level force field calculations using MOE software and MMF94x protocol suggest a C-N rotation barrier of about 16 kcal/mole). Heating the NMR sample to 373 K had little effect. Non-equivalency of the piperidine CH_2 groups was proven by 2D NMR spectroscopy. Key evidence involves [1] two HMBC couplings of equal intensity between the benzyl CH_2 protons and two different aliphatic CH_2 carbons (colored green and red) [2] HSQC coupling of these two CH_2 aliphatics carbons with four different protons (termed H_1 to H_4).

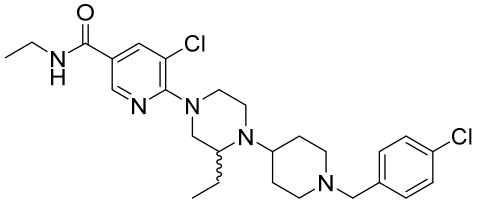


Key HMBC couplings



Key HSQC couplings

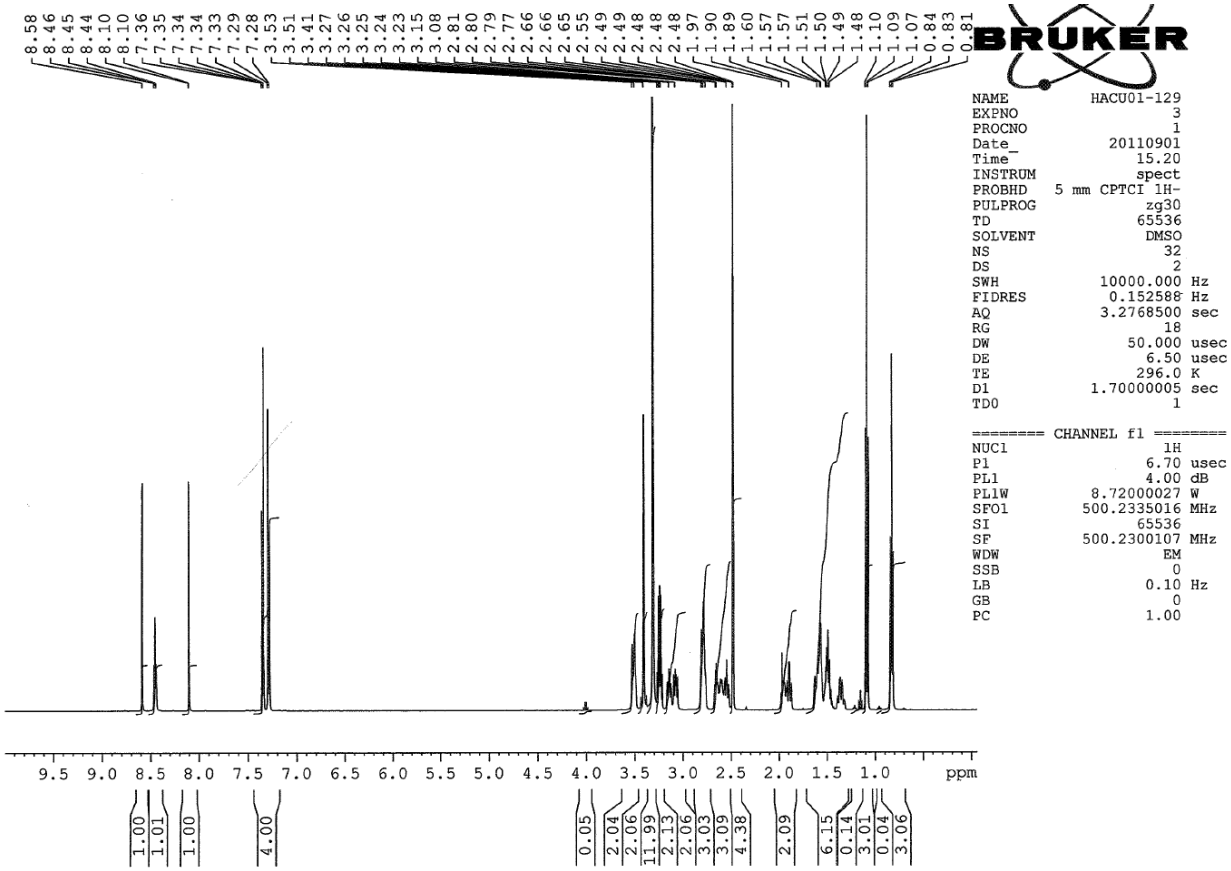
rac-5-Chloro-6-(4-(1-(4-chlorobenzyl)piperidin-4-yl)-3-ethylpiperazin-1-yl)-N-ethylnicotinamide (_rac_-1, _rac_-VUF11211).



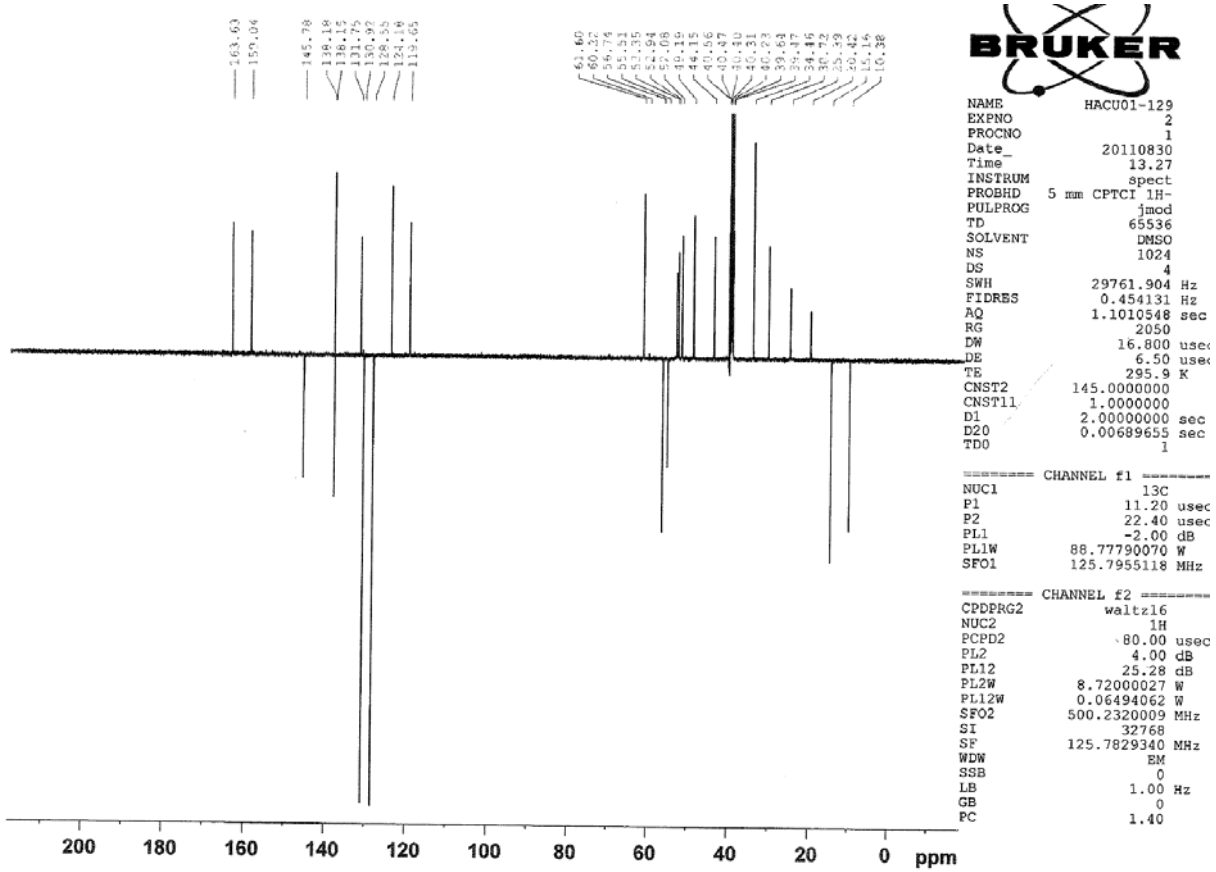
rac-VUF11211 (***rac*-1**)

This compound was prepared using the same route as described for VUF11211 (**1**), but using ***rac*-4.HCl** instead of enantiopure **4**. As expected, features of ***rac*-1** from NMR and achiral LC-MS analyses are identical to those of enantiopure **1**.
LC-MS purity: 98+ %. LR-MS: $[M+H]^+$ $C_{26}H_{36}Cl_2N_5O^+$ calc: 504.2, found: 504.1. Chiral LC purity: two peaks of equal area at 13.84 min and 15.32 min.

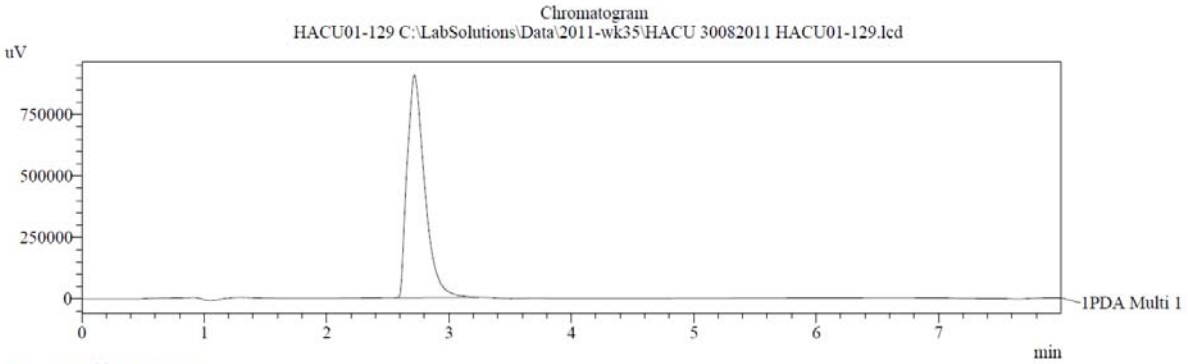
Exemplary spectra and chromatograms



*^{1}H-NMR spectrum of **1** in DMSO-d_6. A trace of EtOAc (2.5 mole %) is visible.*



*^{13}C-APT-NMR spectrum of **1** in DMSO-d_6.*



1 PDA Multi 1 / 230nm 4nm

PeakTable

PDA Ch1 230nm 4nm

Peak#	Name	Ret. Time	Area	Area %
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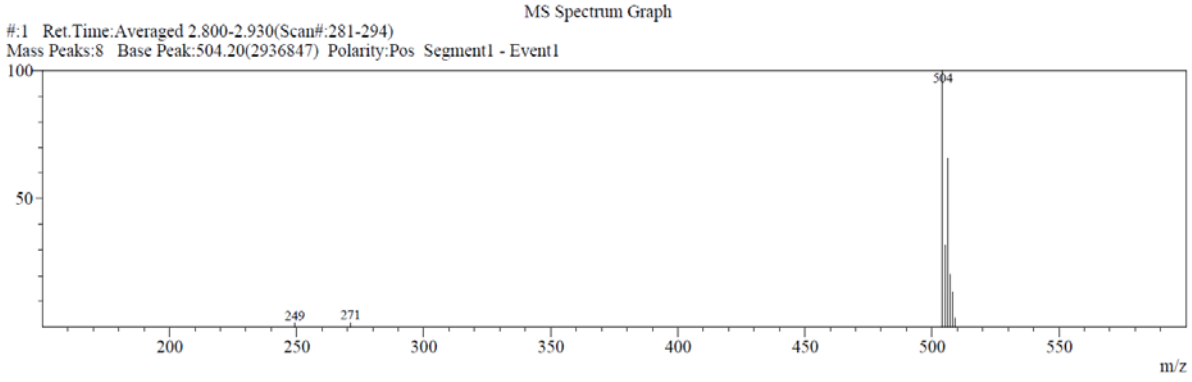

#:1 Ret.Time:Averaged 2.800-2.930(Scan#:281-294)
Mass Peaks:8 Base Peak:504.20(2936847) Polarity:Pos Segment1 - Event1

MS Spectrum Table

#:1 Ret.Time:
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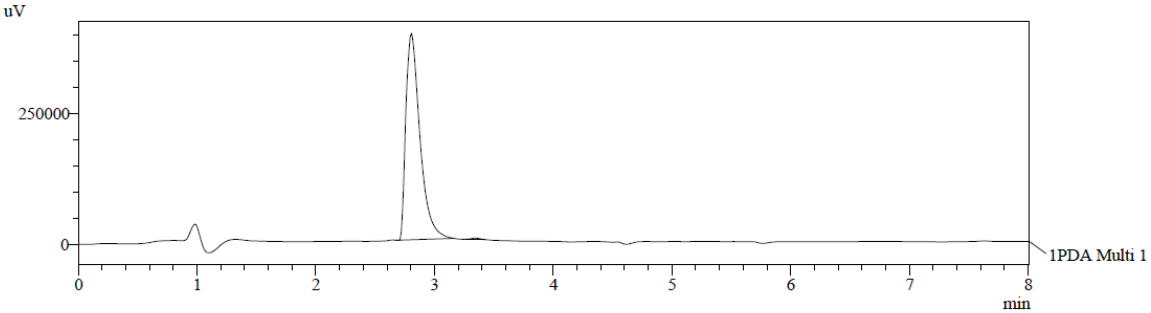
Mass Peaks:8 Base Peak:504.20(2936847) Polarity:Pos Segment1 - Event

#	m/z	Abs.Inten.	Rel.Inten.	Charge	Polarity	Monoisotopic	#	m/z	Abs.Inten.	Rel.Inten.	Charge	Polarity	Monoisotopic
1	249.15	46502	1.58				5	506.20	1942501	66.14			
2	271.10	51416	1.75				6	507.20	597522	20.35			
3	504.20	2936847	100.00				7	508.20	401205	13.66			
4	505.20	930253	31.68				8	509.25	103031	3.51			

*LC-MS chromatogram of **1**.*



1 PDA Multi 1 / 230nm 4nm

PeakTable

PDA Ch1 230nm 4nm

Peak#	Name	Ret. Time	Area	Area %
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2		3.340	13215	0.405

MS Spectrum Graph

#:1 Ret.Time:Averaged 2.880-2.980(Scan#:289-299)
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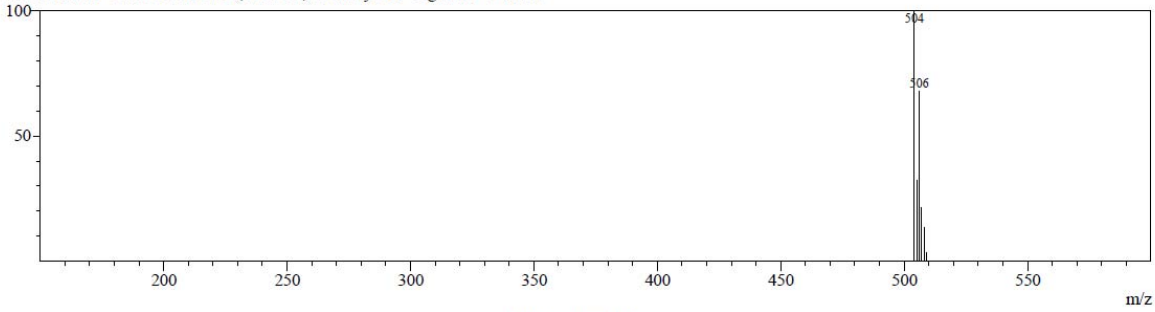
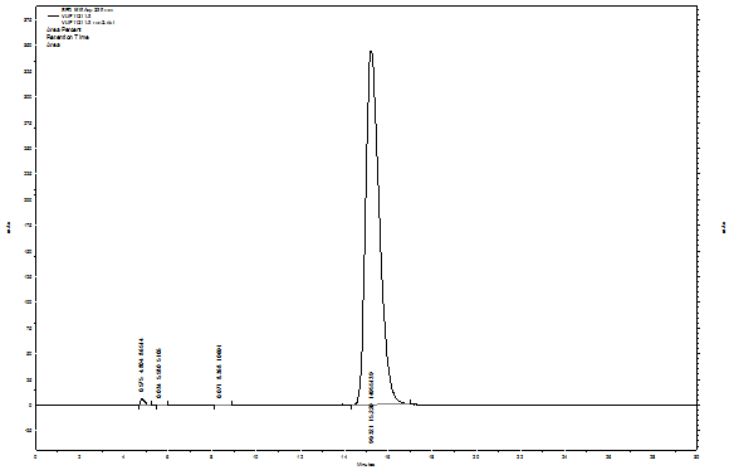


MS Spectrum Table

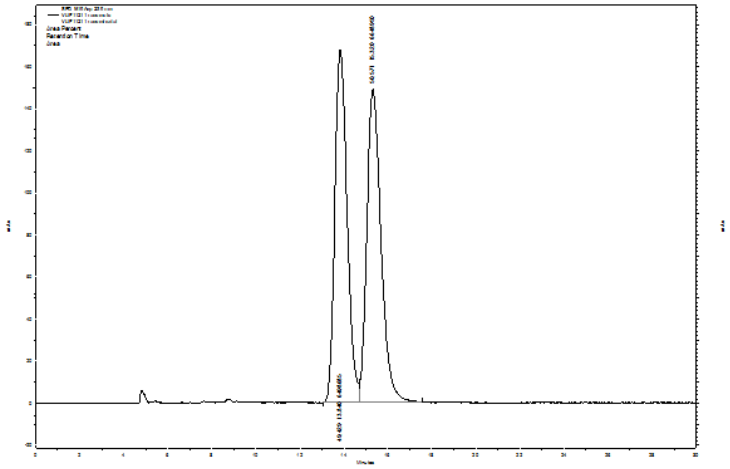
#:1 Ret.Time:
BG Mode:None
Mass Peaks:6 Base Peak:504.05(1155803) Polarity:Pos Segment1 - Event

#	m/z	Abs.Inten.	Rel.Inten.	Charge	Polarity	Monoisotopic	#	m/z	Abs.Inten.	Rel.Inten.	Charge	Polarity	Monoisotopic
1	504.05	1155803	100.00				4	507.05	245028	21.20			
2	505.05	374091	32.37				5	508.10	154922	13.40			
3	506.05	785607	67.97				6	509.05	36308	3.14			

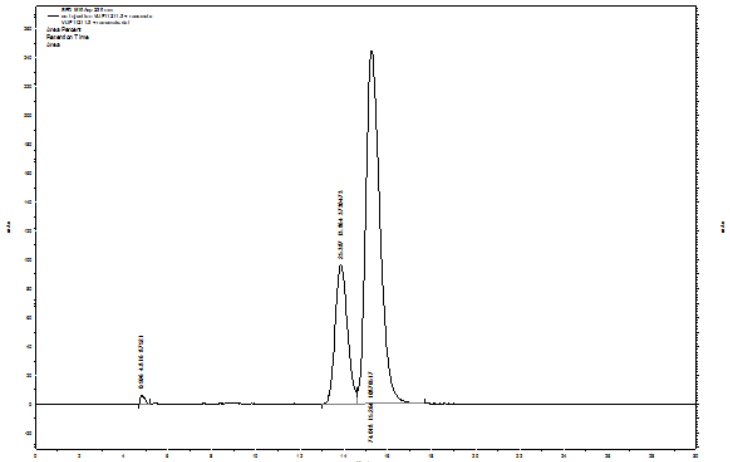
*LC-MS chromatogram of co-injected equimolar amounts of enantiopure **1** and **rac-1.***



*Chiral LC chromatogram of enantiopure **1**.*



*Chiral LC chromatogram of **rac-1**.*



*Chiral LC chromatogram of co-injected equimolar amounts of enantiopure **1** and **rac-1.***