

Supporting Information

An Insight into Tetrahydro- β -carboline-Tetrazole Hybrids: Synthesis and Bioevaluation as Potent Antileishmanial Agents

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Table of content

Experimental section for Antileishmanial activity	S2 – S4
Experimental section for Pharmacokinetics study	S5 – S6
References	S6
Spectra of compounds	S7 – S27

Experimental section for antileishmanial activity: Each compound was evaluated for its *in vitro* activity against WHO reference strain (MHOM/IN/80/Dd8) of extracellular promastigotes and intramacrophagic amastigotes (expressing luciferase firefly reporter gene) of *L. donovani*. The *in vitro* cytotoxicity assay was performed using murine macrophage J-774A.1 cell line.

***In vitro* antileishmanial assay:** *Leishmania donovani* promastigotes (WHO designation MHOM/IN/80/Dd8), originally obtained as a gift from (late) Prof. P. C. C. Garnham and routinely maintained at the institute in golden hamsters, were used in the present study.

Promastigotes were grown in medium 199 medium (Sigma) supplemented with 10 % heat-inactivated fetal calf serum (GBL) and 1% penicillin (50 U/ml) and streptomycin (50 mg/ml) solution (Sigma) at 24 °C.¹ Luciferase tagged with promastigotes maintained at 25 ± 1 °C in medium 199 (Sigma Chemical) supplemented with 10 % Fetal Calf Serum (Gibco, Gaithersburg, MD, USA) and G418 (20 µg/ml) were used for *in vitro* evaluation of antileishmanial activity.²

Antipromastigote activity: The *in vitro* effect of test compounds on the growth of promastigotes was assessed by monitoring the luciferase activity of viable cells after treatment.³ The transgenic promastigotes of late log phase were seeded at 5 X10⁵ cells/well in 96- well flat-bottomed microtitre (MT) plates (CELLSTAR Greiner Bio-one GmbH, Monroe, NC, USA) and incubated for 72 h in medium, in absence (control) or the presence of test compounds (stock prepared in 100 % DMSO, initial concentration, followed by serial dilution in media). The test compounds were added at 2-fold dilutions in up to 7 points in fresh complete medium starting from a 100 µM concentration. After incubation, an aliquot (50 µL) of promastigote suspension was aspirated from each well of a 96-well plate and mixed with an equal volume of Steady Glo (R) reagent (Promega, Madison, WI, USA) and luminescence was measured in luminometer.

Antiamastigote activity: For assessing the activity of compounds against amastigote stage of the parasite, mouse macrophage cell line (J-774A.1), infected with promastigotes expressing firefly luciferase reporter gene was used. Macrophage cells were seeded in a 96-well plate (5×10^4 cells / 200 μ l / well) in RPMI-1640 containing 10 % fetal calf serum. The plates were incubated at 37 °C in a CO₂ incubator. After 24 h, the medium was replaced with fresh medium containing stationary phase transgenic promastigotes (2.5×10^5 / 200 μ L / well). Promastigotes invade the macrophage and are transformed into amastigotes. The test compounds were added at 2-fold dilutions in up to 7 points in fresh complete medium starting from a 100 μ M concentration, and the plates were incubated at 37 °C in a CO₂ incubator for 72 h. After incubation, the drug containing medium was decanted and 50 μ l phosphate-buffered saline (PBS) was added in each well and mixed with an equal volume of Steady-Glo luciferase assay substrate dissolved in Steady-Glo luciferase assay buffer. After gentle shaking for 1 to 2 min, the readings were recorded in a luminometer. The values were expressed as relative luminescence

The IC₅₀ value of each compound was calculated by non-linear regression analysis of the concentration–response curve using the four-parameter Hill equations.

Cytotoxicity assay: The macrophage cell viability was determined using the MTT assay.⁴ Exponentially growing cells (J774) ($1-2 \times 10^5$ cells/100 μ l/well) were incubated with test compounds. The test compounds are added at three fold dilutions up to 7 points in complete medium starting from 500 μ M concentrations, and were incubated at 37 °C in a humidified mixture of CO₂ and 95 % air in an incubator. Control wells containing dimethyl sulfoxide (DMSO) without compounds were also included in the experiment. After incubation, 25 μ L of MTT reagent (5 mg/mL) in PBS medium and incubated at 37 °C for 2 h. At the end of the incubation period, the supernatant were removed by inverting the plate completely without

disturbing cell layer and 150 μ L of pure DMSO was added to each well. After 15 min. of shaking the readings were recorded as absorbance at 544 nm on a micro plate reader. The cytotoxic effect were expressed as 50 % lethal dose (i.e., as the concentration of a compound which provoked a 50 % reduction in cell viability compared to cell in culture medium alone). CC_{50} values were estimated as described by Huber *et al.*⁵ The selectivity index (SI) for each compound was calculated as the ratio between, cytotoxicity (CC_{50}) and activity (IC_{50}) against *Leishmania* amastigotes. Compounds with SI index > 5 were considered as safe in this cytotoxic assay.

***In vivo* antileishmanial activity in hamster model:** The modified method was used for *in vivo* screening. Golden male hamsters (Inbred strain) weighing 40-45 gm were infected intra-cardially with 1×10^7 amastigotes per animal. Pre-treatment spleen biopsy of all the animals was carried out to assess the degree of infection on day 20th of infection. The animals with +1 infection (5-15 amastigotes/100 spleen cell nuclei) were included in the experiment. The infected animals were randomized into several groups of six animals each. Drug treatment by intraperitoneal route was initiated after 2 days of biopsy and continued for 5 consecutive days. Negative control group hamsters were administered 0.2 ml of saline solution. Post-treatment biopsies were done on day 7th and 28th day of the last drug administration and amastigote counts were assessed after Giemsa staining. Intensity of infection in both, treated and untreated animals, and also the initial count in treated animals was compared and the efficacy was expressed in terms of percent inhibition (PI) using the following formula:

$$PI = 100 - [ANAT \times 100 / (INAT \times TIUC)]$$

Where PI is percent inhibition of amastigotes multiplication, ANAT is actual number of amastigotes in treated animals, INAT is initial number of amastigotes in treated animals and TIUC is times increase of parasites in untreated control animals.

Experimental Section for Pharmacokinetics:

Material and methods: The pharmacokinetics of **14t** was studied in golden Syrian hamsters weighing 100 ± 10 gm. The hamsters were obtained from Laboratory Animal Division of the Institute and were housed in plastic cages under standard laboratory conditions with a regular **12 h** day-night cycle. Suspension formulation containing 12.5 mg/ml of compound was prepared by triturating **14t**, gum acacia (1%, w/v) and water (drop wise addition) in a mortar and pestle. A single 50 mg/kg oral dose was given to conscious hamsters using feeding needle and blood samples were withdrawn at various predefined times up to **24 h** post dose. Serum samples were harvested from blood. All samples were stored at -80 °C until analysis. The serum (50 μ L; blank, spiked or test) was extracted twice with 1 ml of extraction solvent (*n*-hexane: ethyl acetate, 60:40, % v/v) followed by vortex-mixing and centrifugation. The supernatant was evaporated to dryness in Turbovap LX (Caliper, Massachusetts, USA). The residue was reconstituted in 100 μ L mobile phase and centrifuged. Clear supernatant (80 μ L) was transferred into HPLC vials and 50 μ L was injected on to the LC-MS/MS system.

A Shimadzu UFLC pump (LC-20AD) with online degasser (DGU-20A3), an auto-sampler (SIL-HTc) with a temperature-controlled peltier-tray and a API 4000 Q trap mass spectrometer (Applied Biosystems, Toronto, Canada) was used for analysis on a Discovery HS C-18 column (5 μ m, 50 x 4.6 mm id) preceded with a guard column (5 μ m, 20 x 4.0 mm, id) packed with the same material under isocratic condition at a flow rate of 0.7 mL/min. The mobile phase [85 % acetonitrile in aqueous ammonium acetate buffer (0.01 M)] was degassed by ultrasonication for

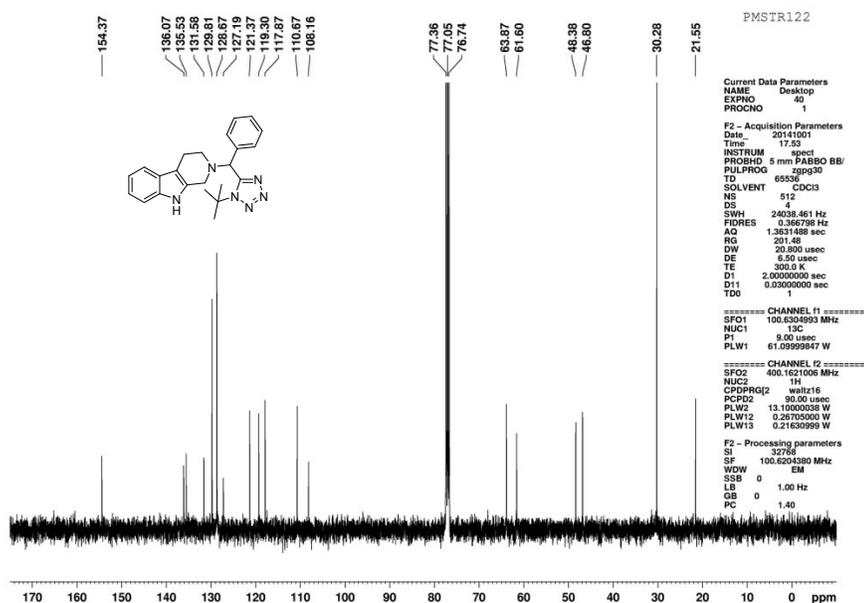
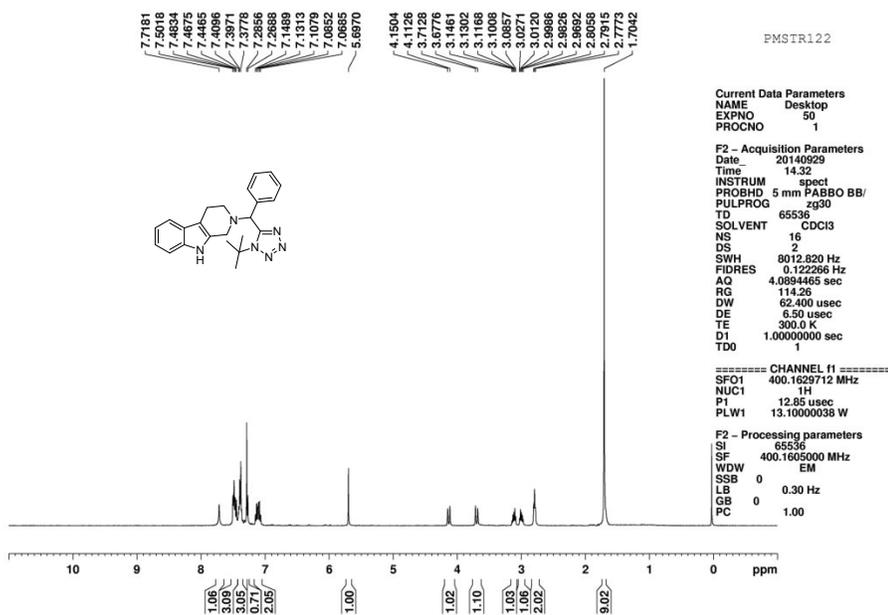
15 min before use. LC-MS/MS system was equilibrated for approximately 20 min before commencement of analysis. The column oven temperature was 40 °C. Total analysis time was 4 min per sample. Multiple reactions monitoring (MRM) was used to monitor the transitions m/z 503.2 → 331.1 for **14t** and m/z 180.1 → 138.1 for phenacetin (Internal Standard) at 5500 V spray voltage. Data acquisition and quantitation were performed using analyst software (version 1.4.2; AB Sciex, Toronto, Canada). The method was linear over the concentration range of 1-200 ng/mL with recovery of >70 % and acceptable accuracy and precision.

References:

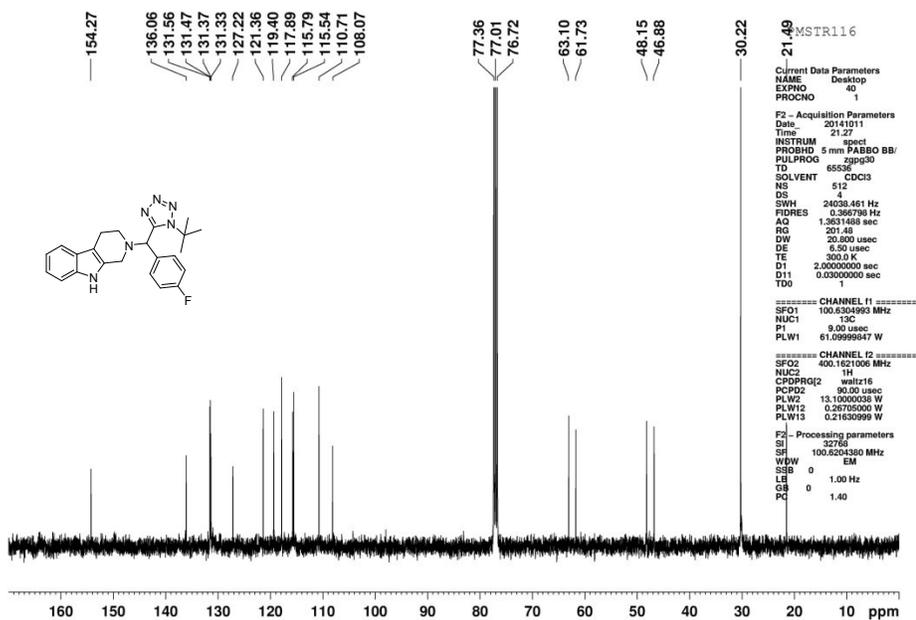
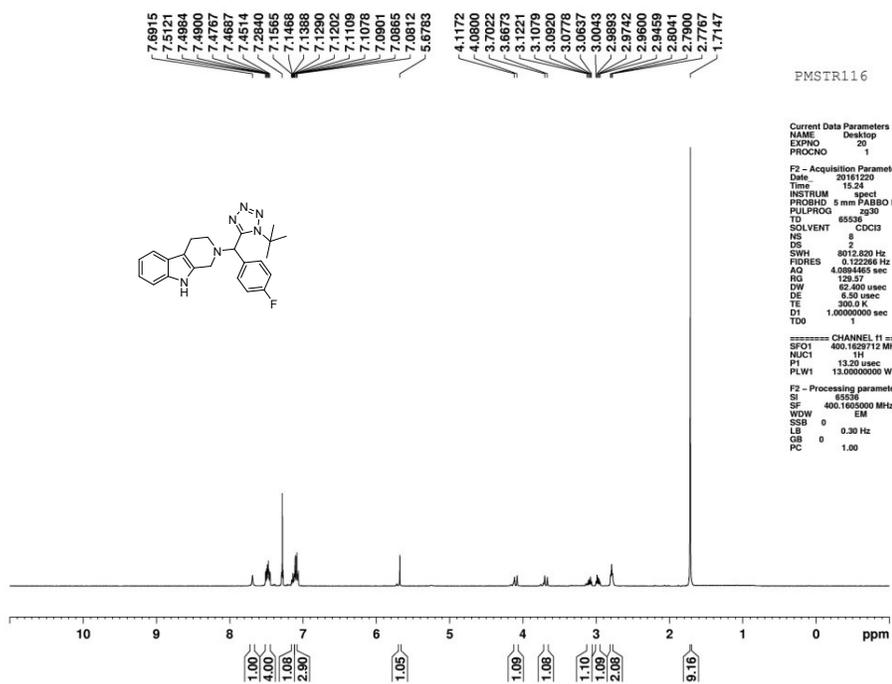
1. A. Debrabant, M. Gottlieb, D. M. Dwyer, *Mol Biochem Parasitol.*, 1995, **71**, 51-63.
2. Ashutosh, S. Gupta, Ramesh, S. Sundar, N. Goyal, *Antimicrob. Agents Chemother.*, 2005, **49**, 3776-3783.
3. T. Mosmann, *J. Immunol. Methods.*, 1983, **65**, 55-63.
4. W. Huber, J. C. Koella, *Acta Trop.*, 1993, **55**, 257-261.
5. S. Bhatnagar, P. Y. Guru, J. C. Katiyar, R. Srivastava, A. Mukherjee, M. S. Akhtar, M. Seth, A. P. Bhaduri, *Indian J Med Res.*, 1989, **89**, 439-44.

Supplementary Spectra of compounds:

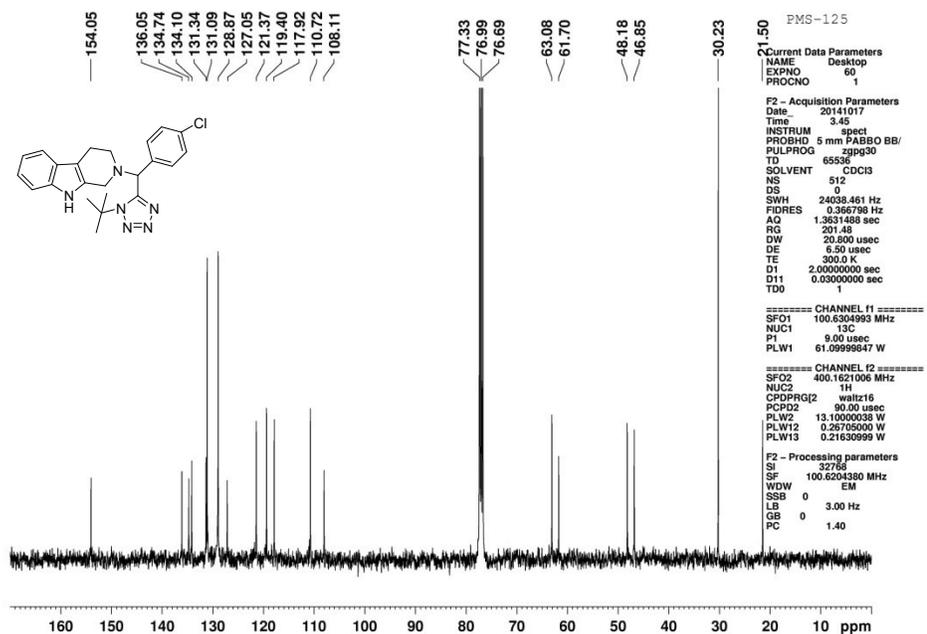
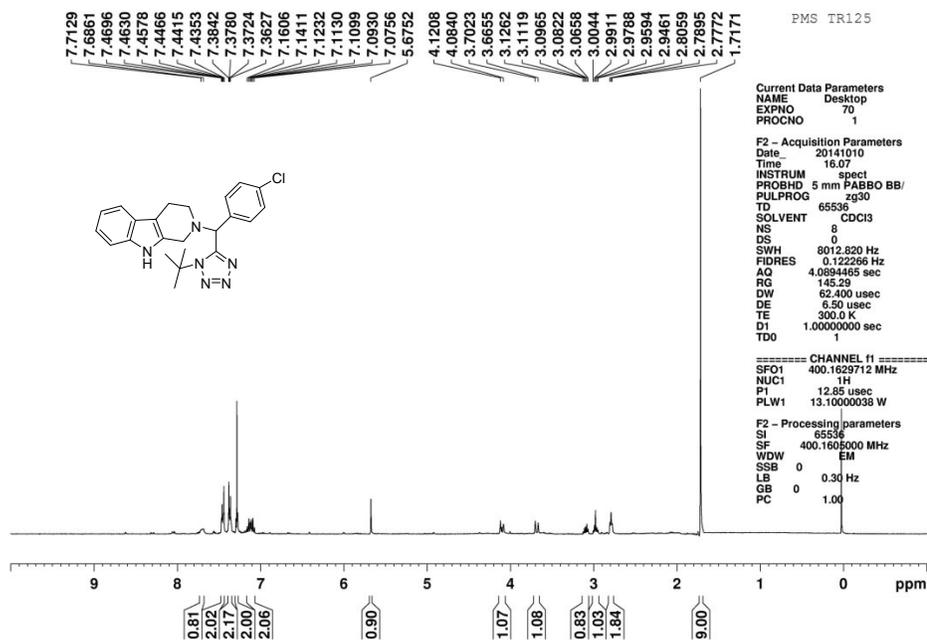
¹H and ¹³C NMR spectra of 14a



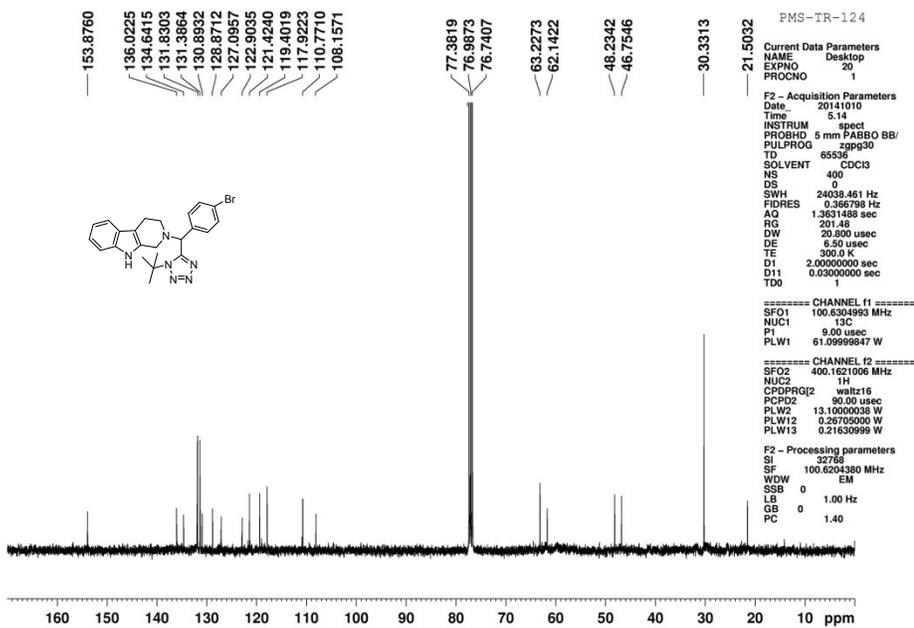
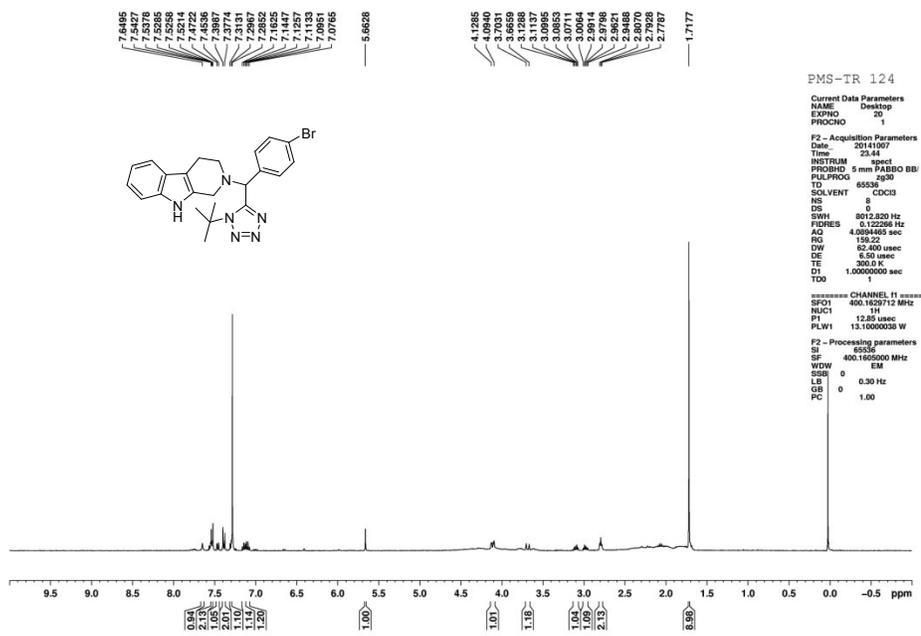
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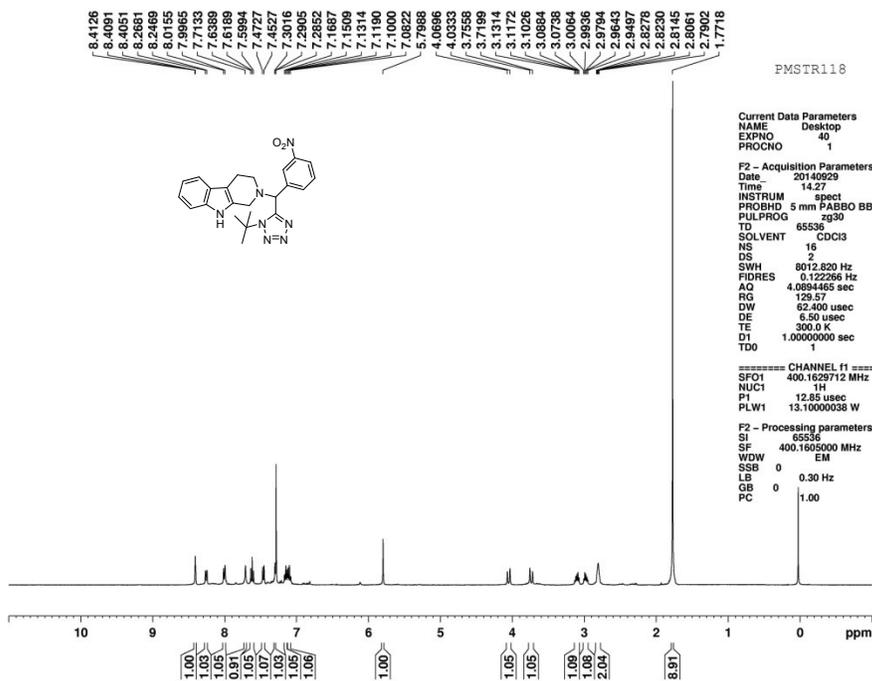
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¹H and ¹³C NMR spectra of 14d



¹H and ¹³C NMR spectra of 14e



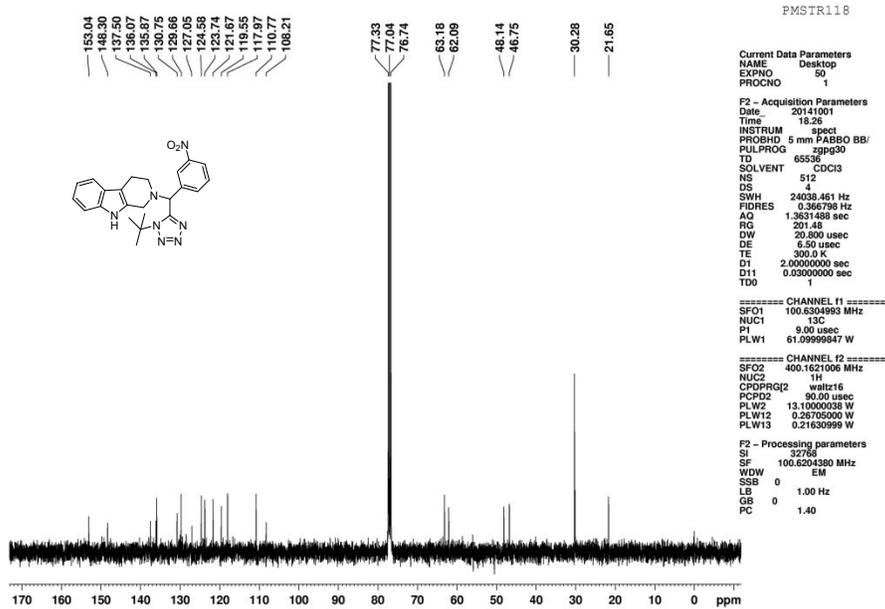
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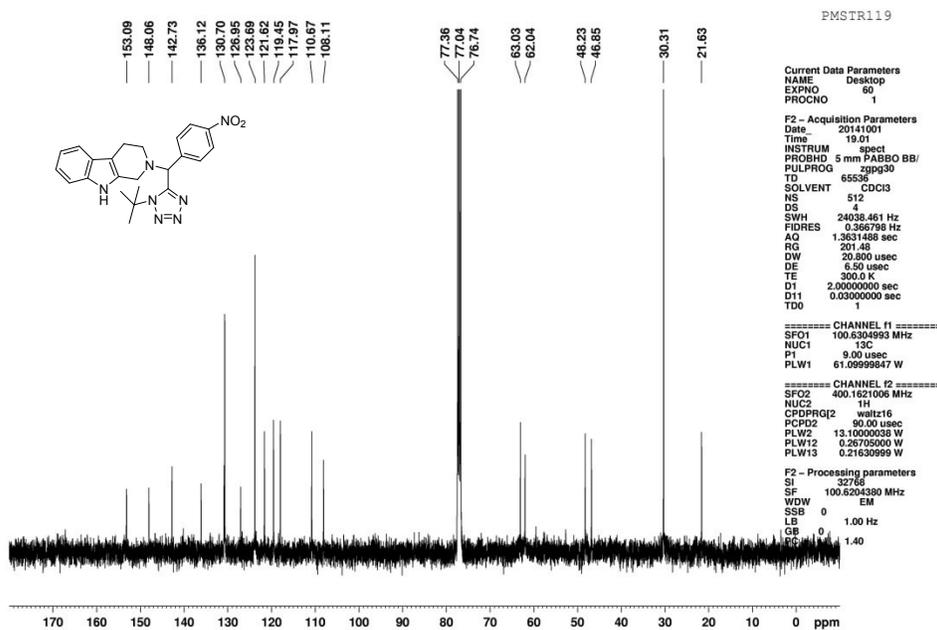
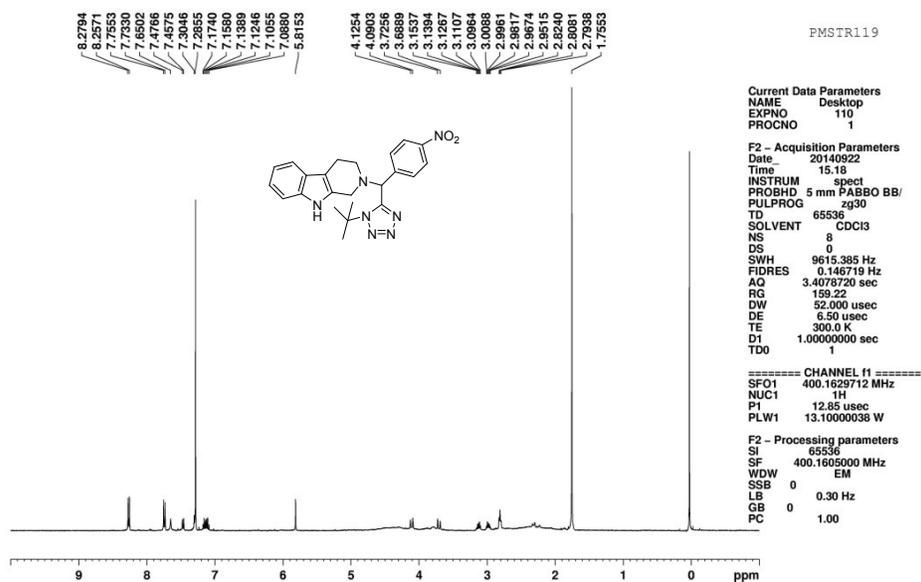
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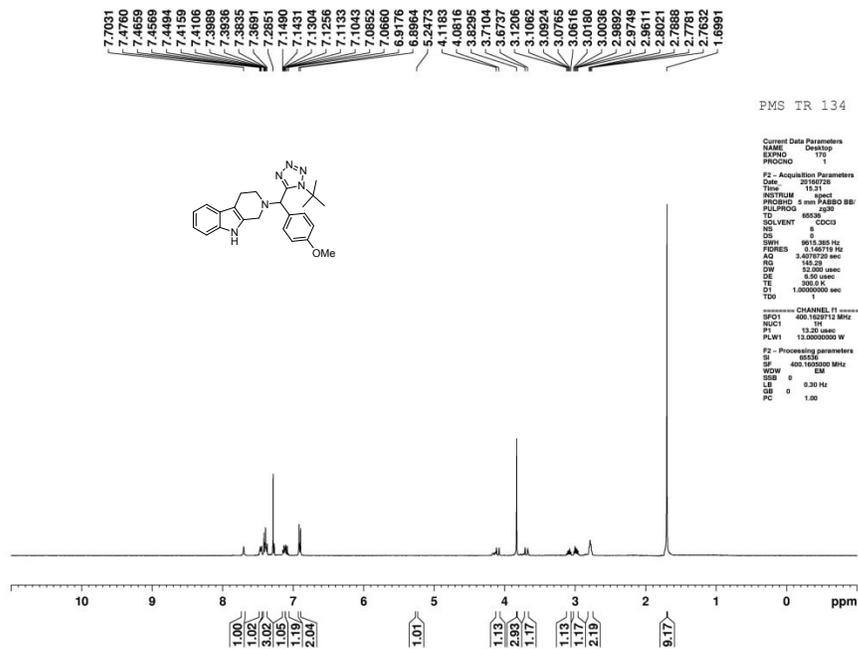
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¹H and ¹³C NMR spectra of 14f



¹H and ¹³C NMR spectra of 14h



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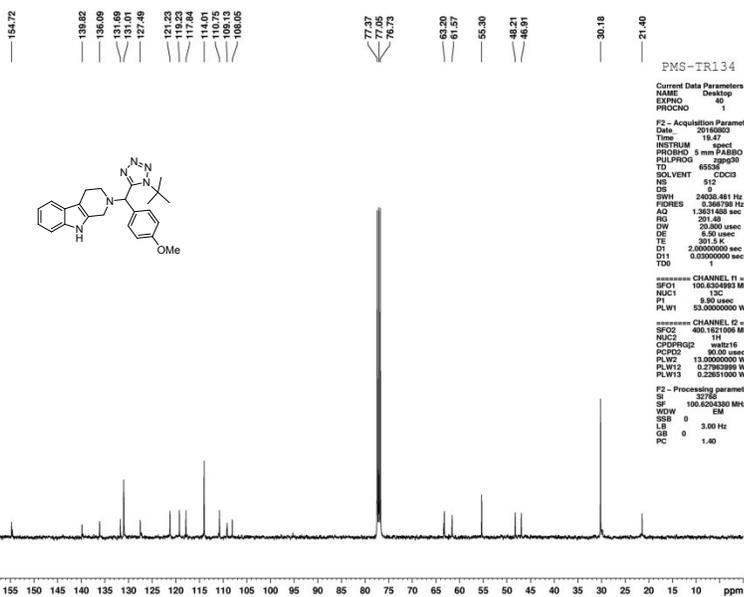
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PMS-TR134

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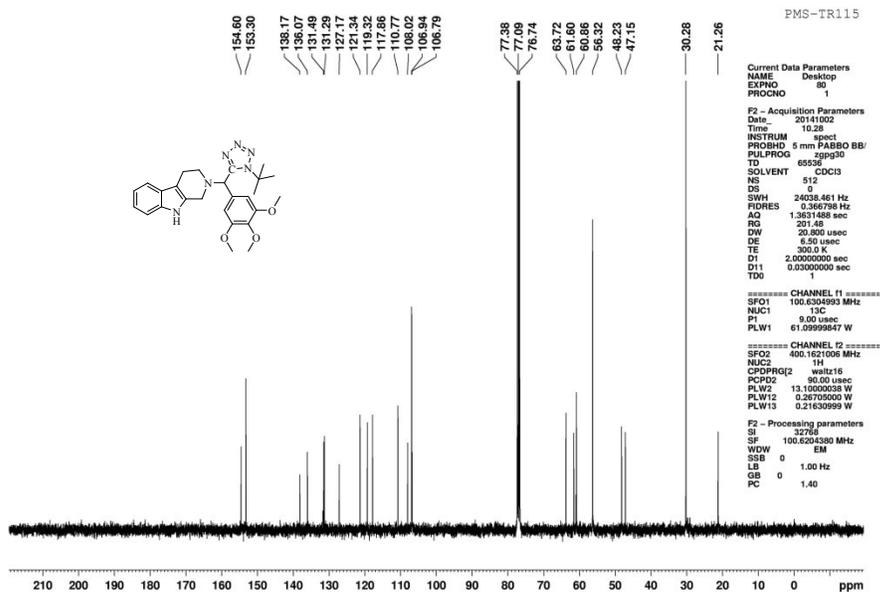
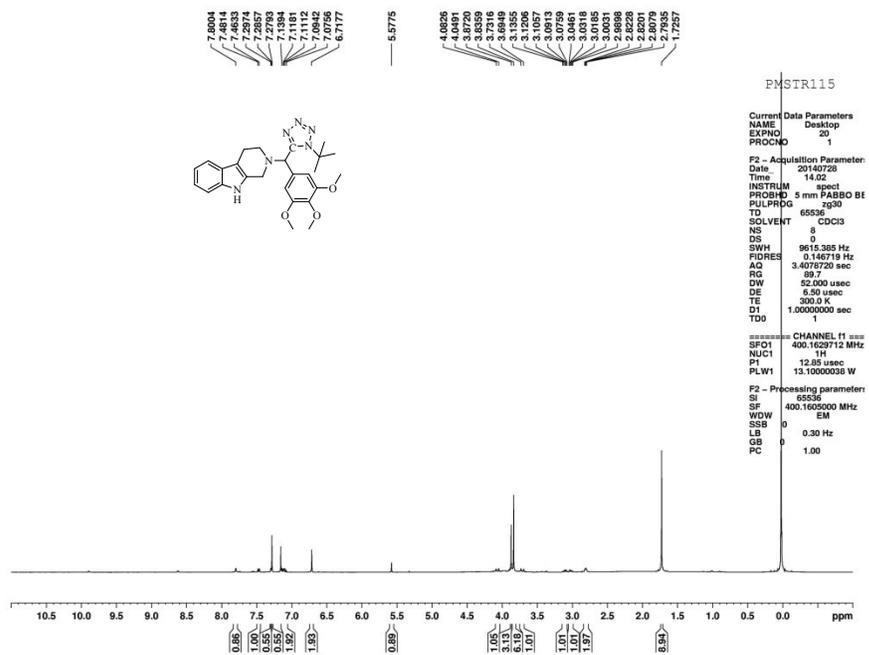
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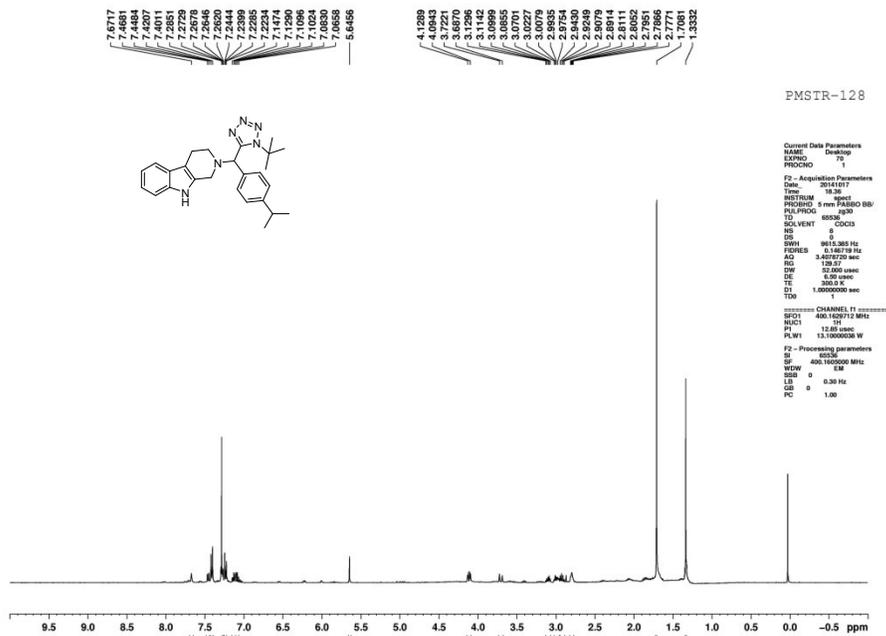
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¹H and ¹³C NMR spectra of 14j

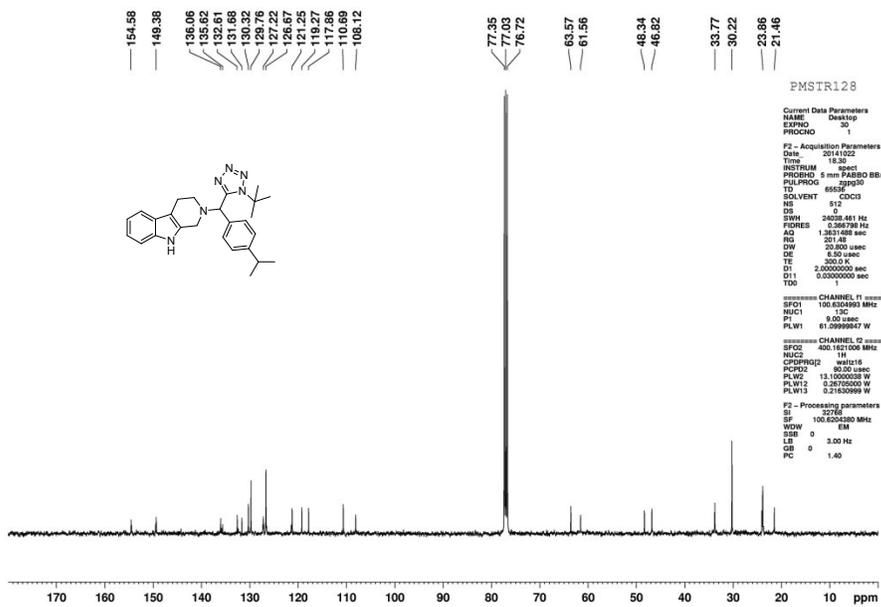


¹H and ¹³C NMR spectra of 14k



PMSTR-128

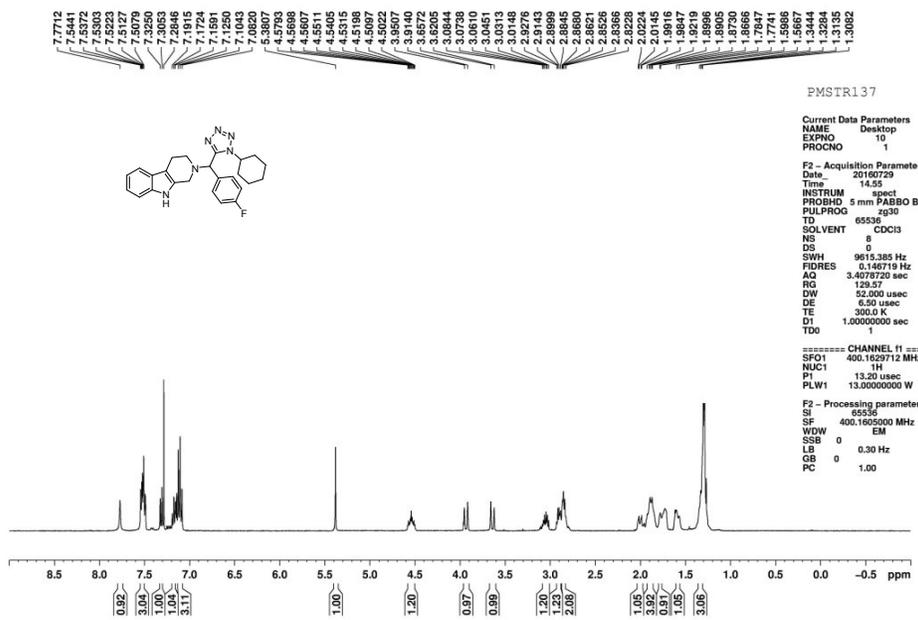
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¹H and ¹³C NMR spectra of 14l



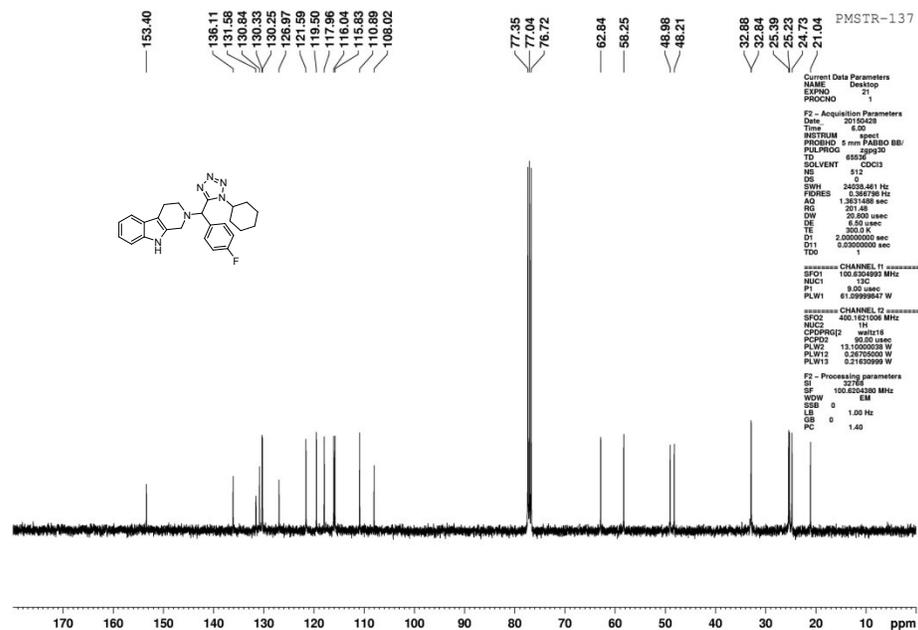
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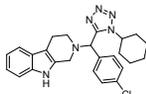
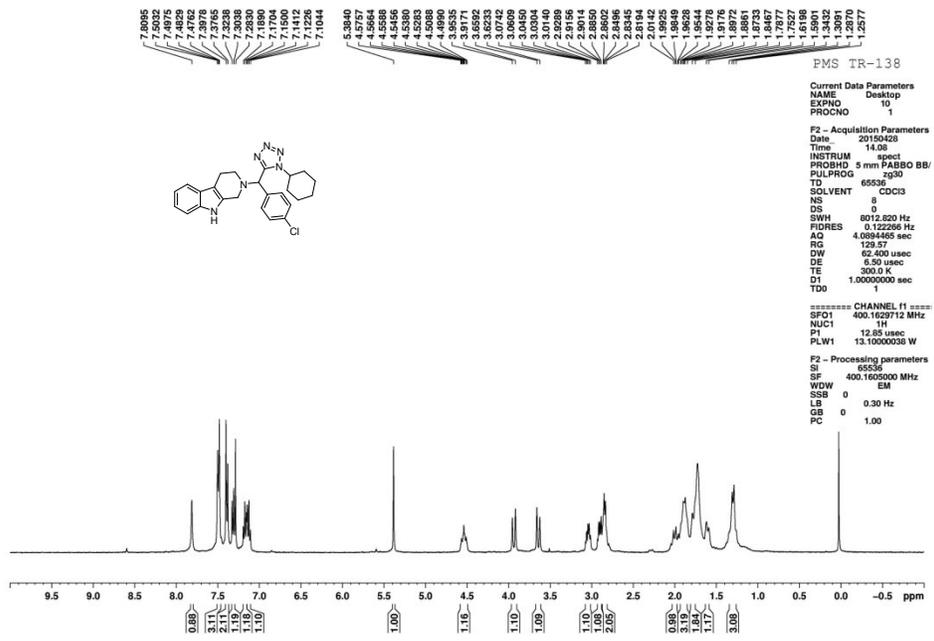
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¹H and ¹³C NMR spectra of 14m



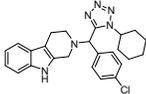
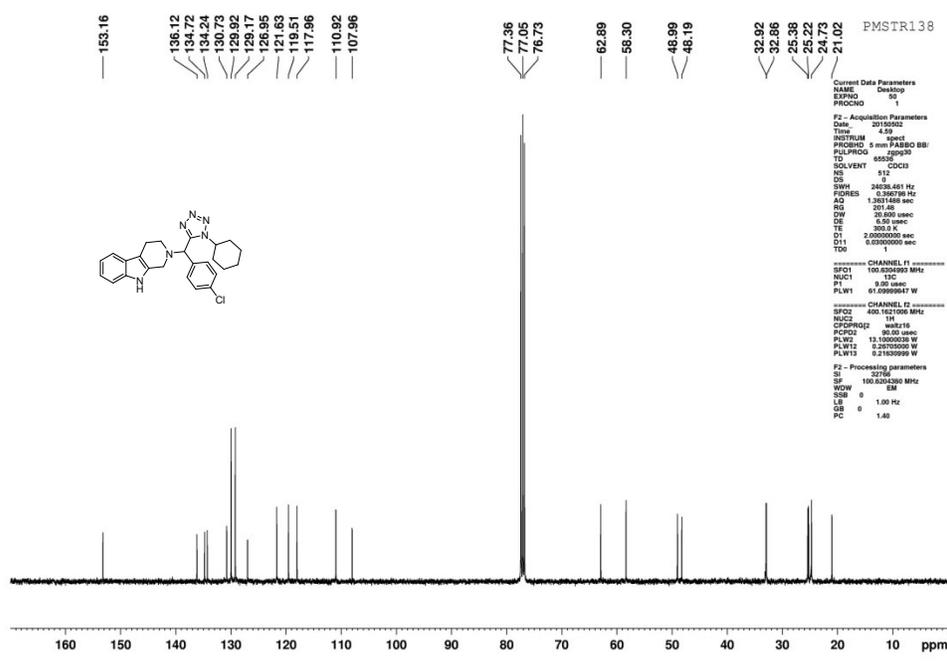
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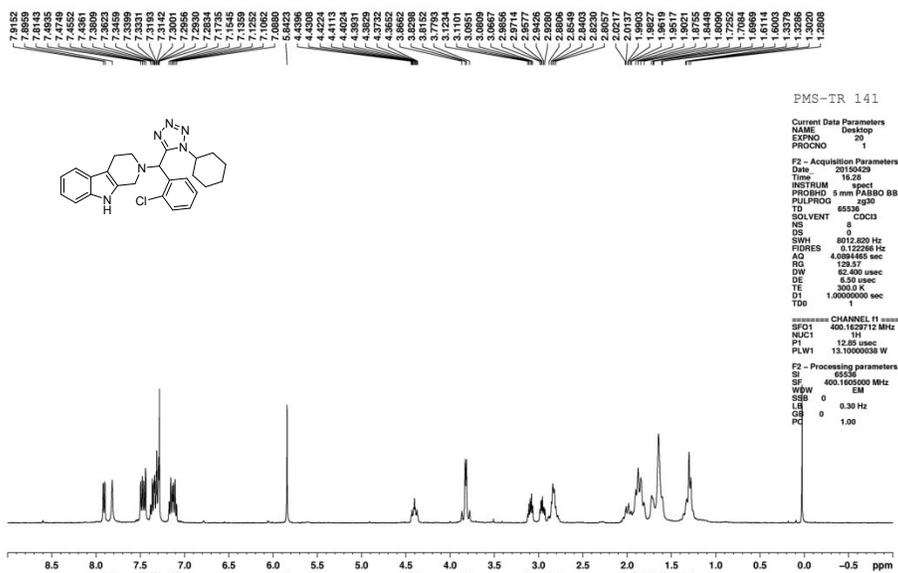
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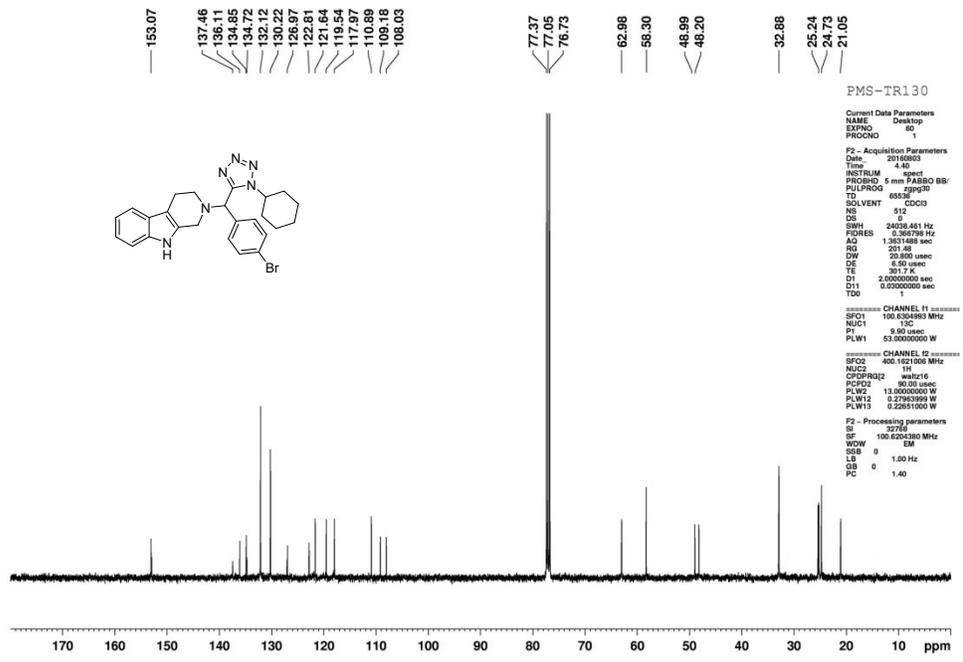
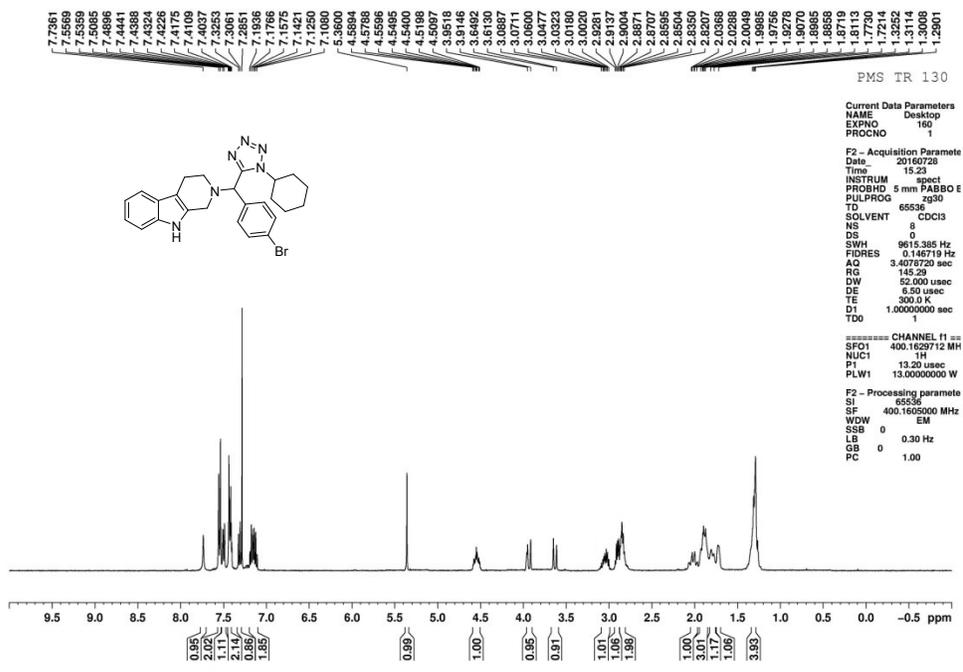
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 GB 0
 PC 1.40

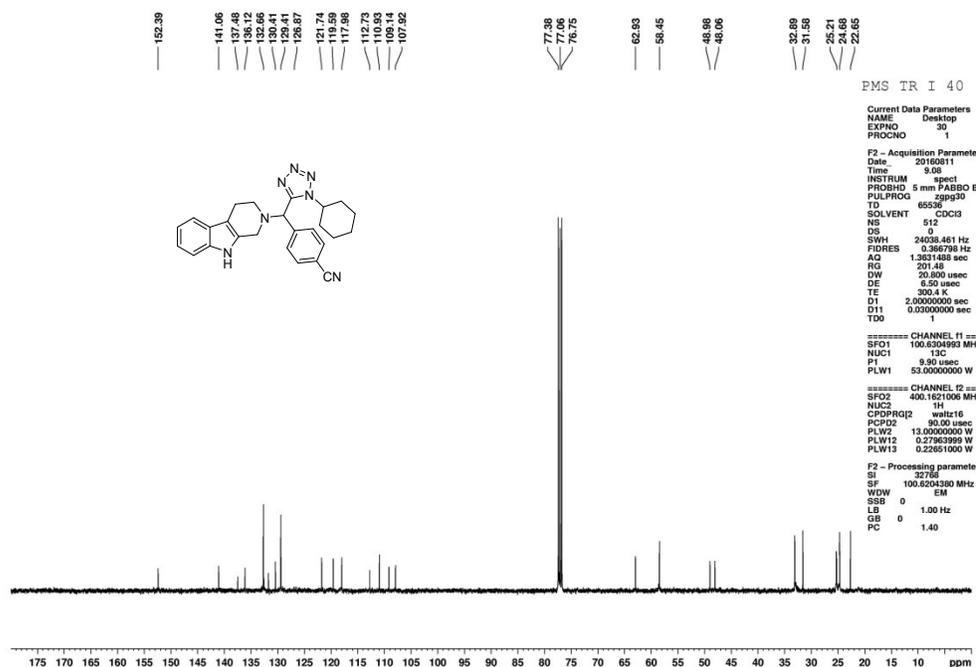
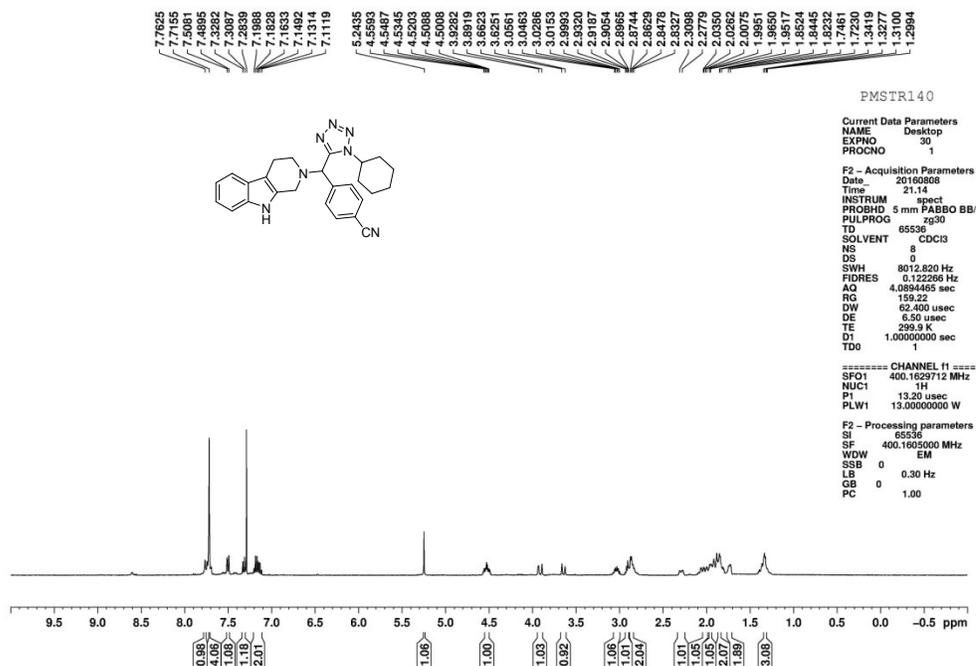
¹H and ¹³C NMR spectra of 14n



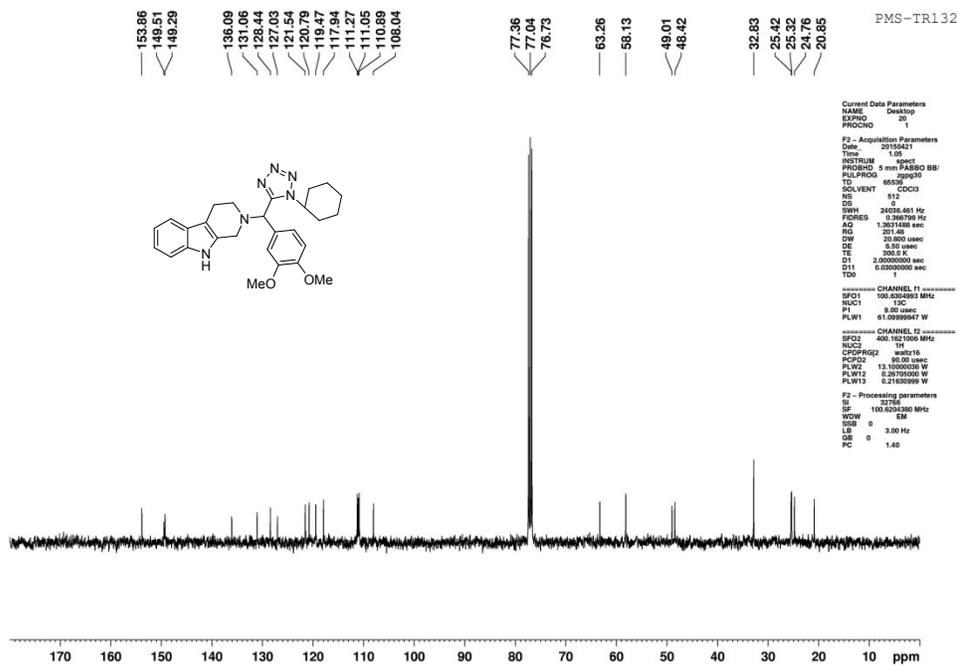
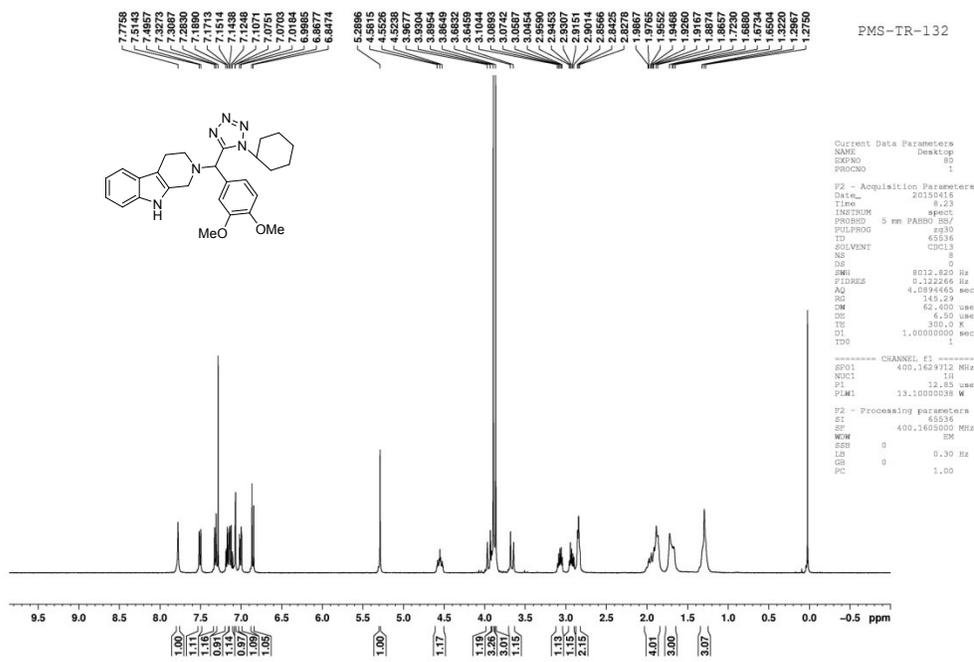
¹H and ¹³C NMR spectra of 14o



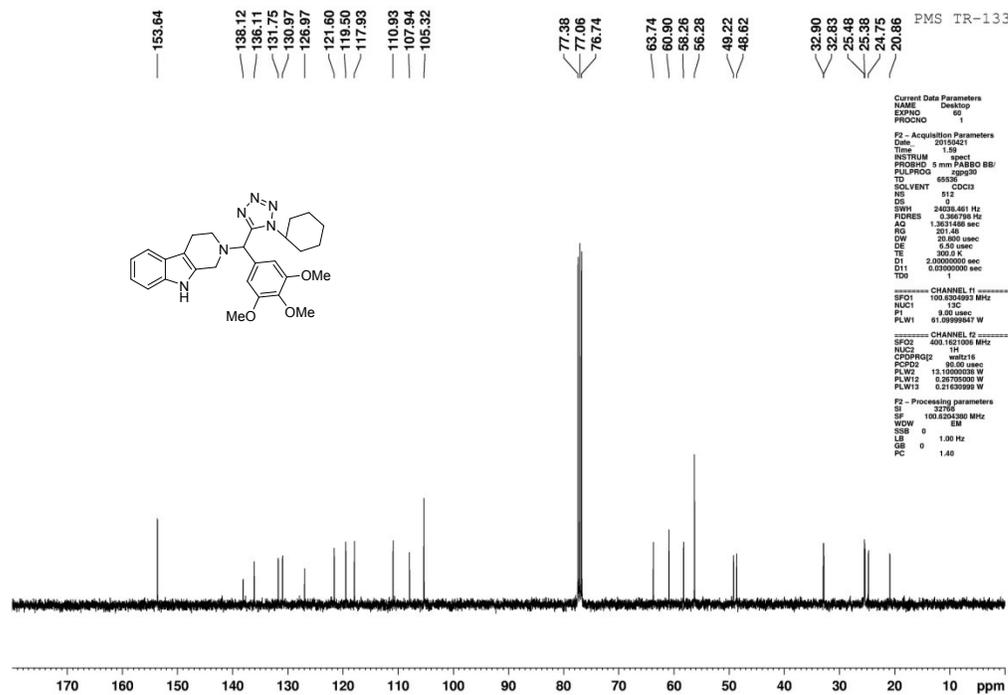
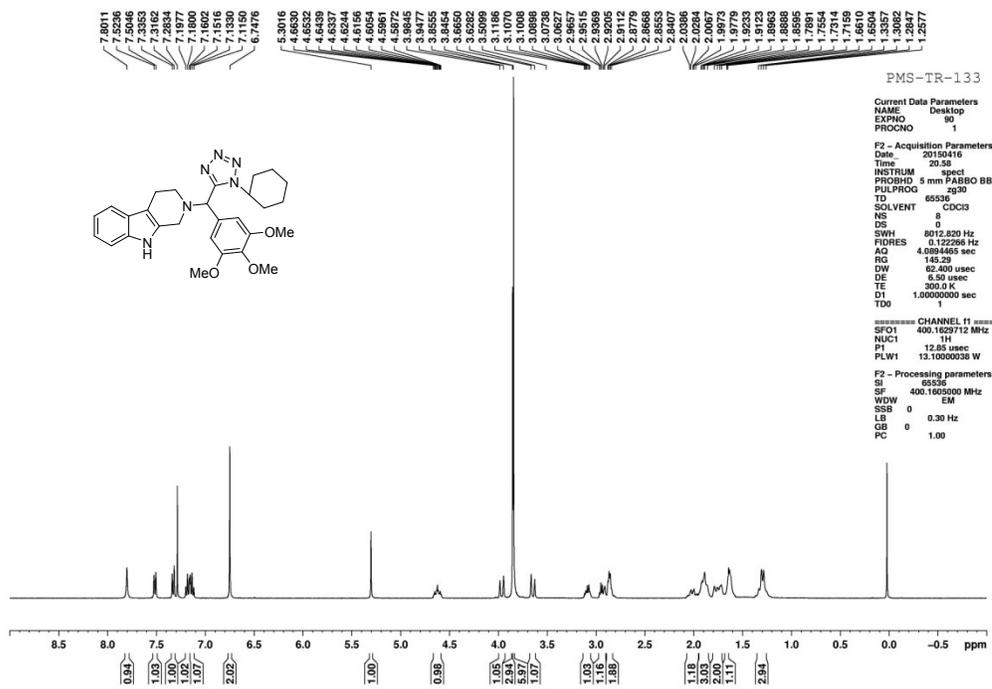
¹H and ¹³C NMR spectra of 14q



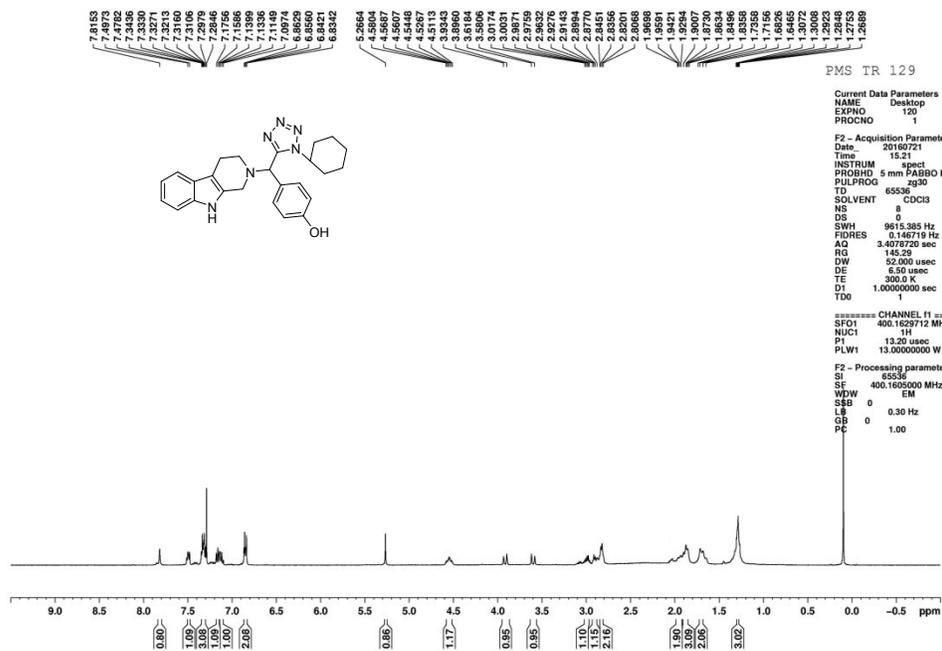
¹H and ¹³C NMR spectra of 14s



¹H and ¹³C NMR spectra of 14t



¹H and ¹³C NMR spectra of 14u



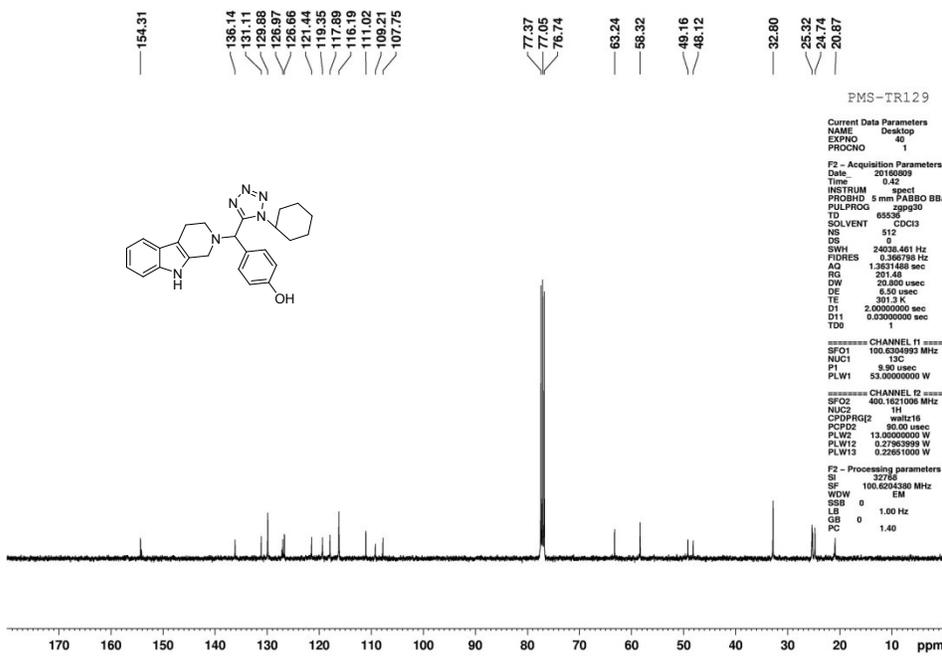
PMS TR 129

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F2 - Processing parameter:
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PMS-TR129

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F2 - Processing parameters
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