Supplementary Materials

Dynamic Covalent Bonding (DCB): The Bond Lability of Alkoxyamines as Drugs Against *Schistosoma mansoni* and *Plasmodium falciparum*

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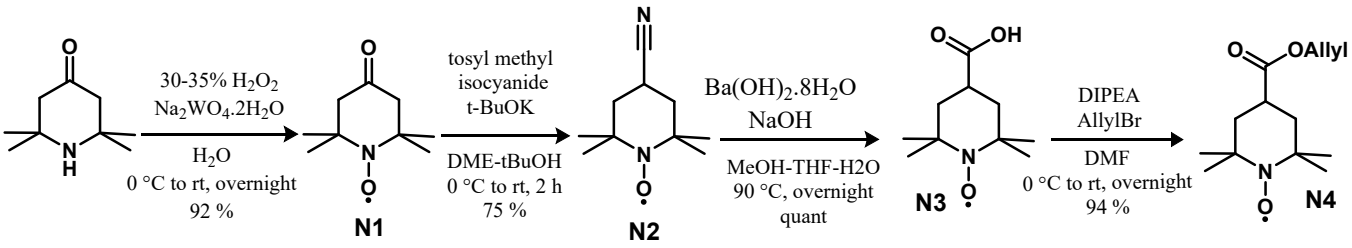
Synthesis and protection of 4-carboxy-TEMPO



The synthesis of 4-carboxy-TEMPO **N3** is described by Shingo Sato et al, Synthesis and fluorescence properties of six fluorescein-nitroxide radical hybrid-compounds, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **(2016)**, 169, 66–71.

4-Oxo-2,2,6,6-tetramethyl-1-piperidin-N-oxy radical

(N1)

A solution of 2,2,6,6-tetramethylpiperidine-4-one (30.000 g, 193.249 mmol, 1.0 eq) and $Na_2WO_4.2H_2O$ (3.187 g, 9.662 mmol, 0.050 eq) in 250 mL of water is cooled to 0°C and 30–35% H_2O_2 (40.7 mL, 1352.743 mmol, 7.0 eq) is added dropwise (one drop every seven seconds) with vigorous stirring. The temperature did not exceed 25°C. The suspension is stirred vigorously for 24 h at ambient temperature. Then, it is saturated with potassium carbonate and extracted with ether. The ether phase is dried over magnesium sulfate, filtered, and evaporated. The purification by gel column chromatography PE/EtOAc (9:1) afforded **N1** as a red solid (30.25 g, 92 %).

4-Carbonitril-2,2,6,6-tetramethyl-4-piperidin-N-oxyl radical

(N2)

N1 (13.030 g, 76.584 mmol, 1.0 eq) is dissolved in DME (200 mL) and tosyl methyl isocyanide (15.700 g, 80.413 mmol, 1.05 eq) is added. After cooling to 0 °C the reaction mixture is treated with a solution of *t*-BuOK (17.240 g, 153.168 mmol, 2.0 eq) in DME (50 mL) and *t*-BuOH (50 mL). The reaction is stirred at the same temperature for 45 min and then 1 h at rt. Afterwards H_2O (100 mL) is added and the mixture is extracted with Et_2O (3 x 80 mL). The combined organic layers are dried over $MgSO_4$, and solvents are then removed in vacuo. The gel column chromatography EtOAc/PE afforded **N2** as red needles (10.40 g, 75 %).

4-Carboxy-2,2,6,6-tetramethylpiperidin-N-oxy radical

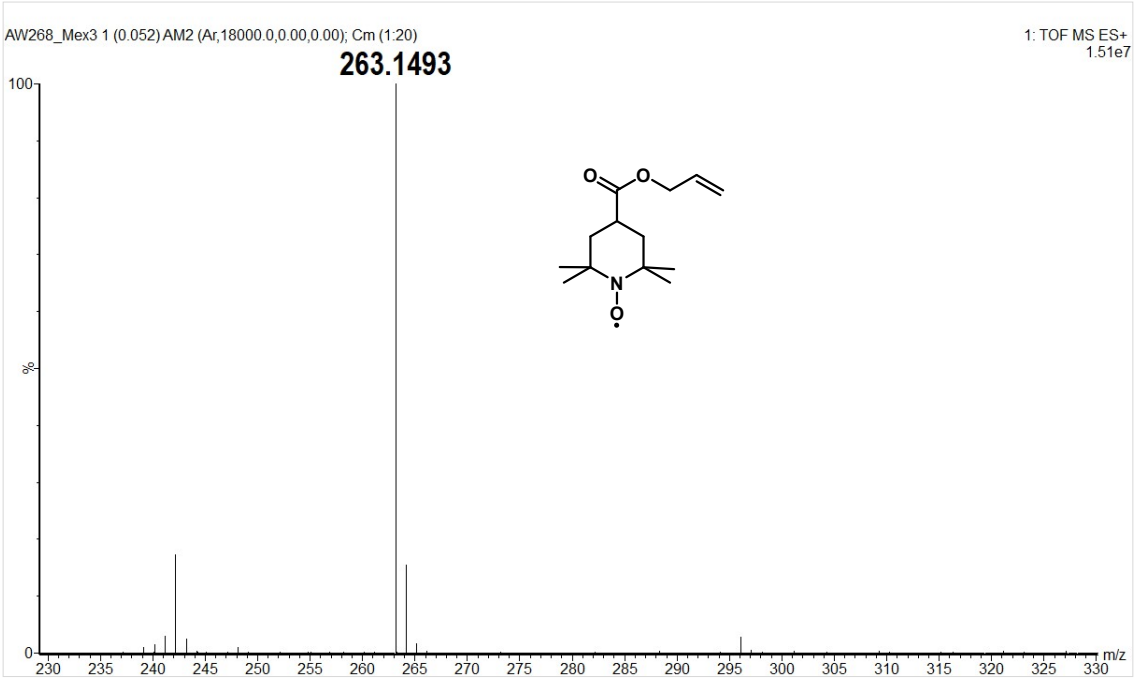
(N3)

N2 (4.600 g, 25.396 mmol, 1.0 eq) is dissolved in MeOH/THF (50:15 mL, 10:3) and a solution of $Ba(OH)_2.8H_2O$ (19.226 g, 60.950 mmol, 2.4 eq) and NaOH (1.218 g, 30.475 mmol, 1.2 eq) in H_2O (140 mL) is added. The mixture is heated to reflux (90 °C) for 24 h, cooled to rt, and extracted with $CHCl_3$. The aqueous phase is acidified with HCl (aq. 10%) and extracted with $CHCl_3$ (3 x (70 mL). The combined organic layers are dried over $MgSO_4$ and evaporated to dryness, giving **N3** (10.20 g, quant) as red solid which is used without further purification.

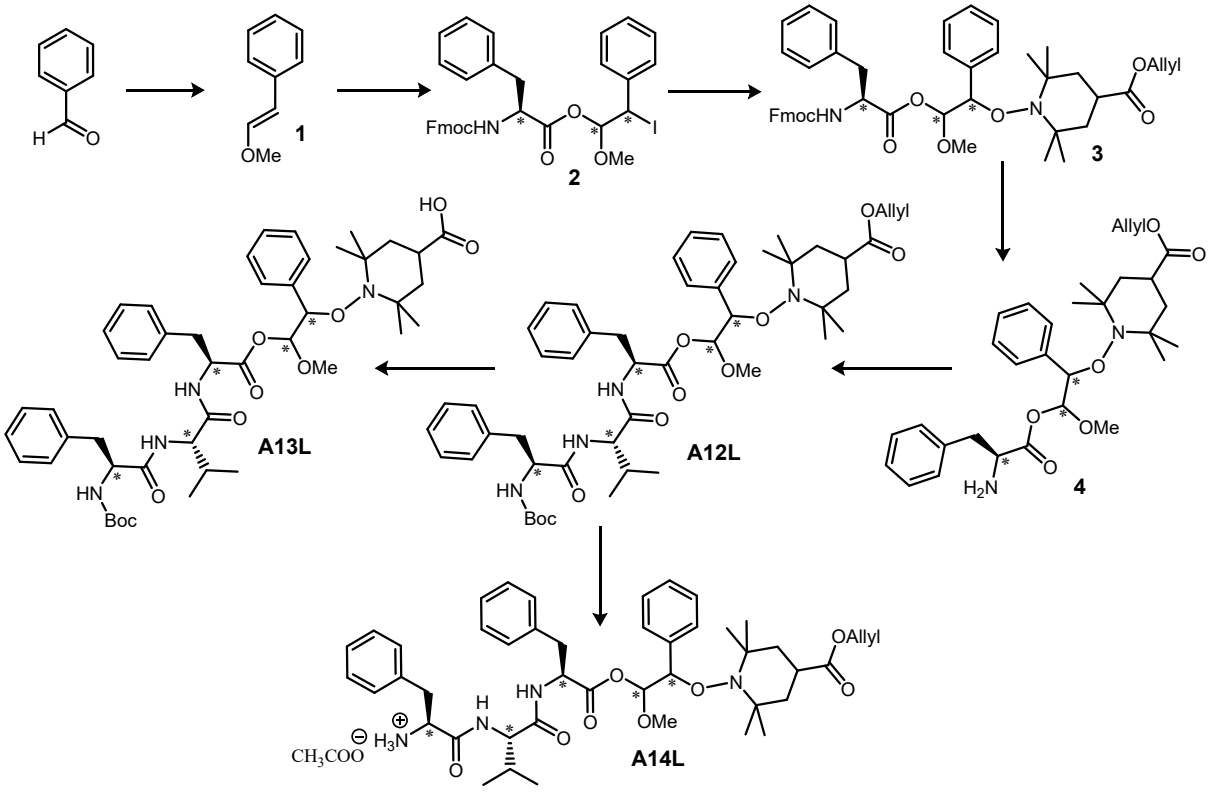
4-Allyl carboxylate-2,2,6,6-tetramethyl-4-piperidin-N-oxyl radical

(N4)

HRMS (ESI)



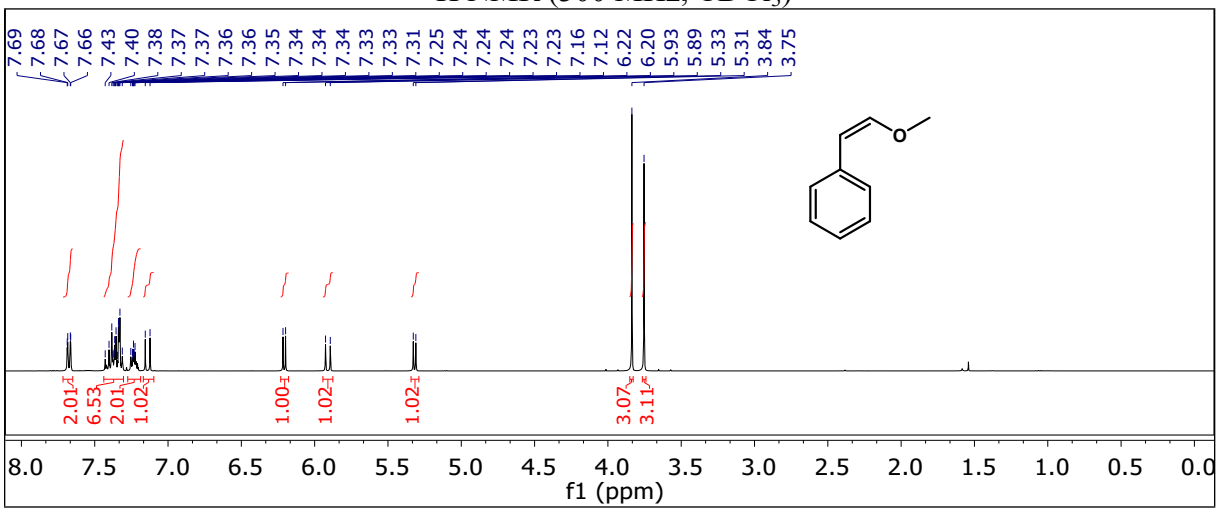
Peptide-Alkoxyamine acetal synthesis



(2-methoxyvinyl)benzene

(1)

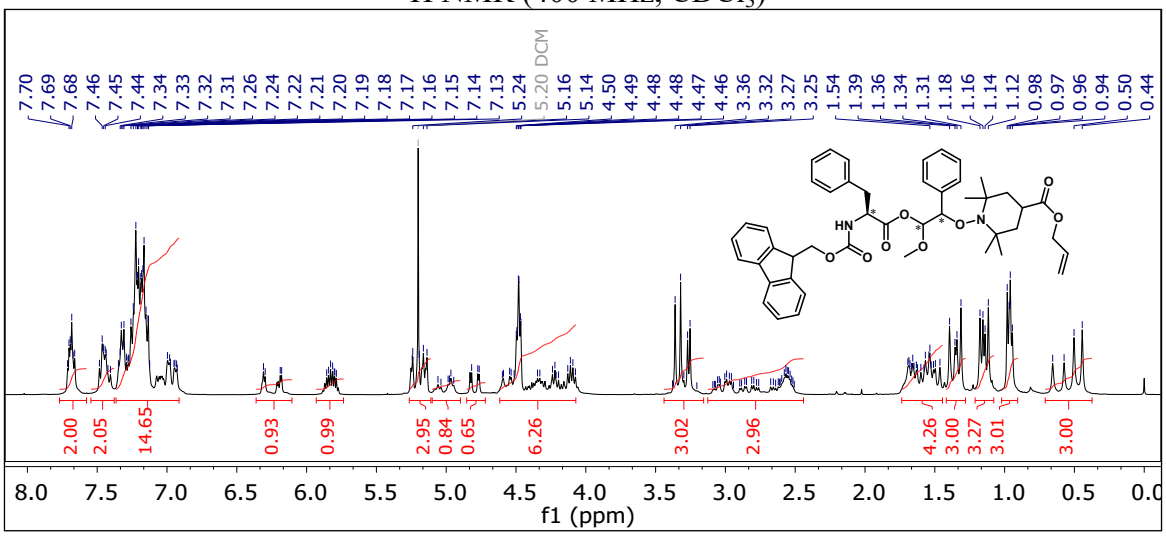
^{1}H NMR (300 MHz, $CDCl_3$)



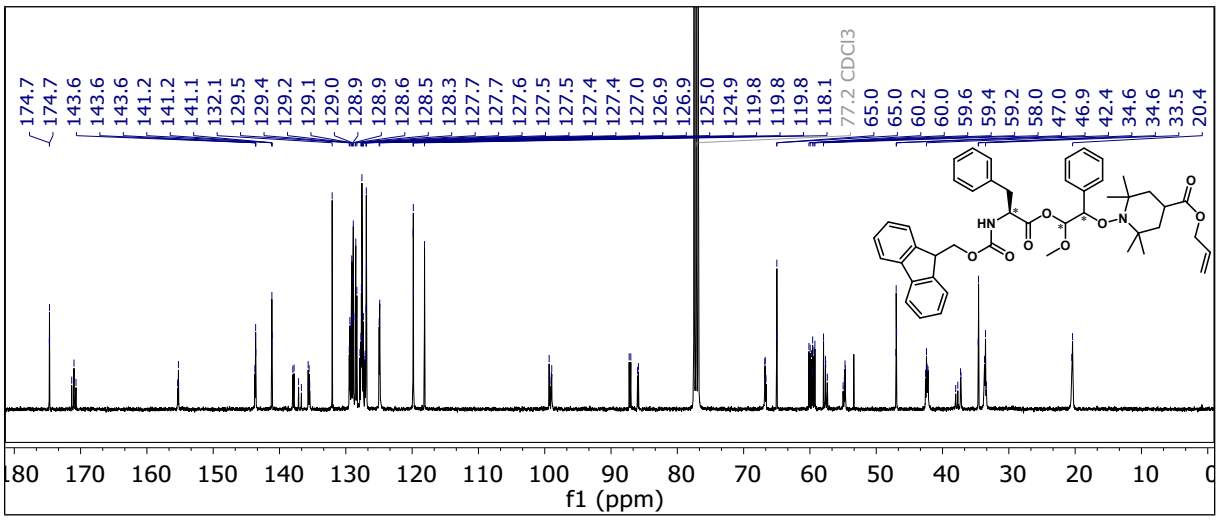
allyl 1-(2-(((((9H-fluoren-9-yl)methoxy)carbonyl)-L-phenylalanyl)oxy)-2-methoxy-1-phenylethoxy)-2,2,6,6-tetramethylpiperidine-4-carboxylate

(3)

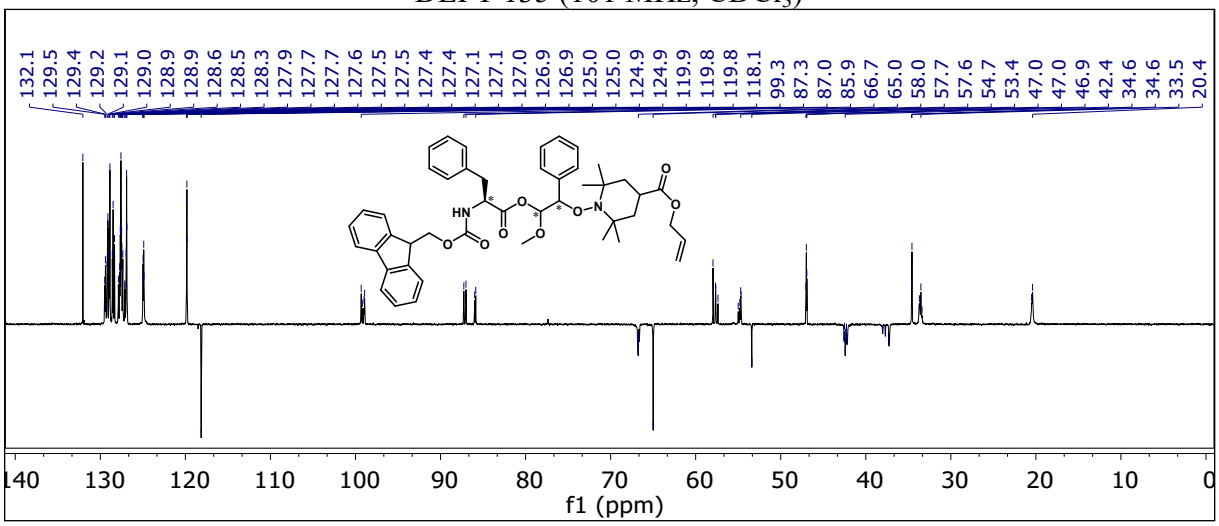
^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (101 MHz, $CDCl_3$)

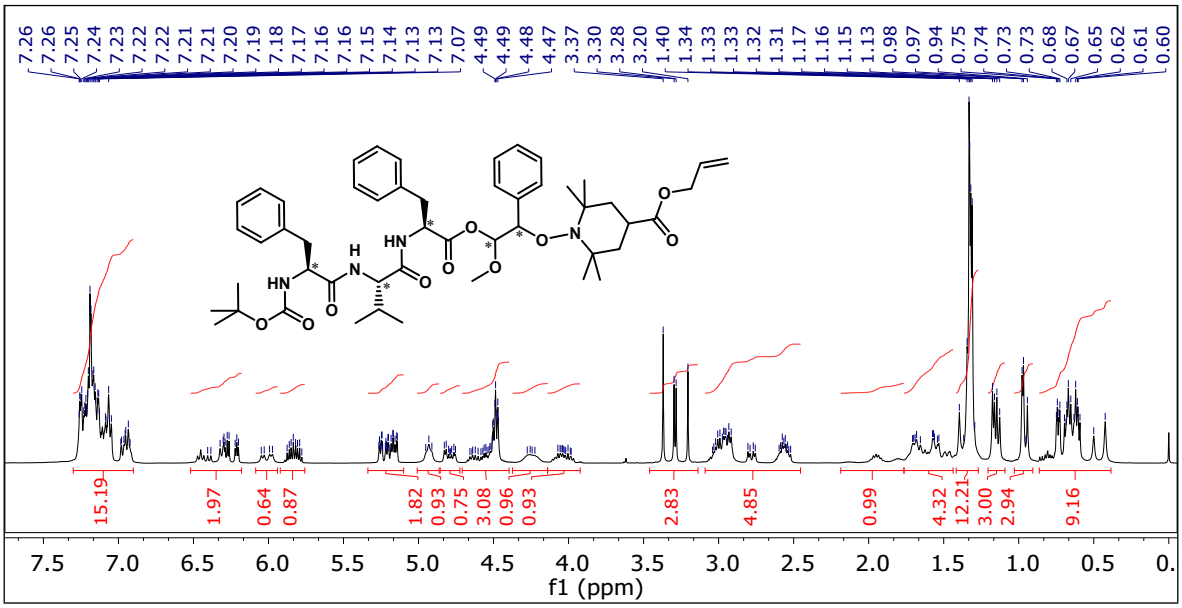


DEPT 135 (101 MHz, $CDCl_3$)

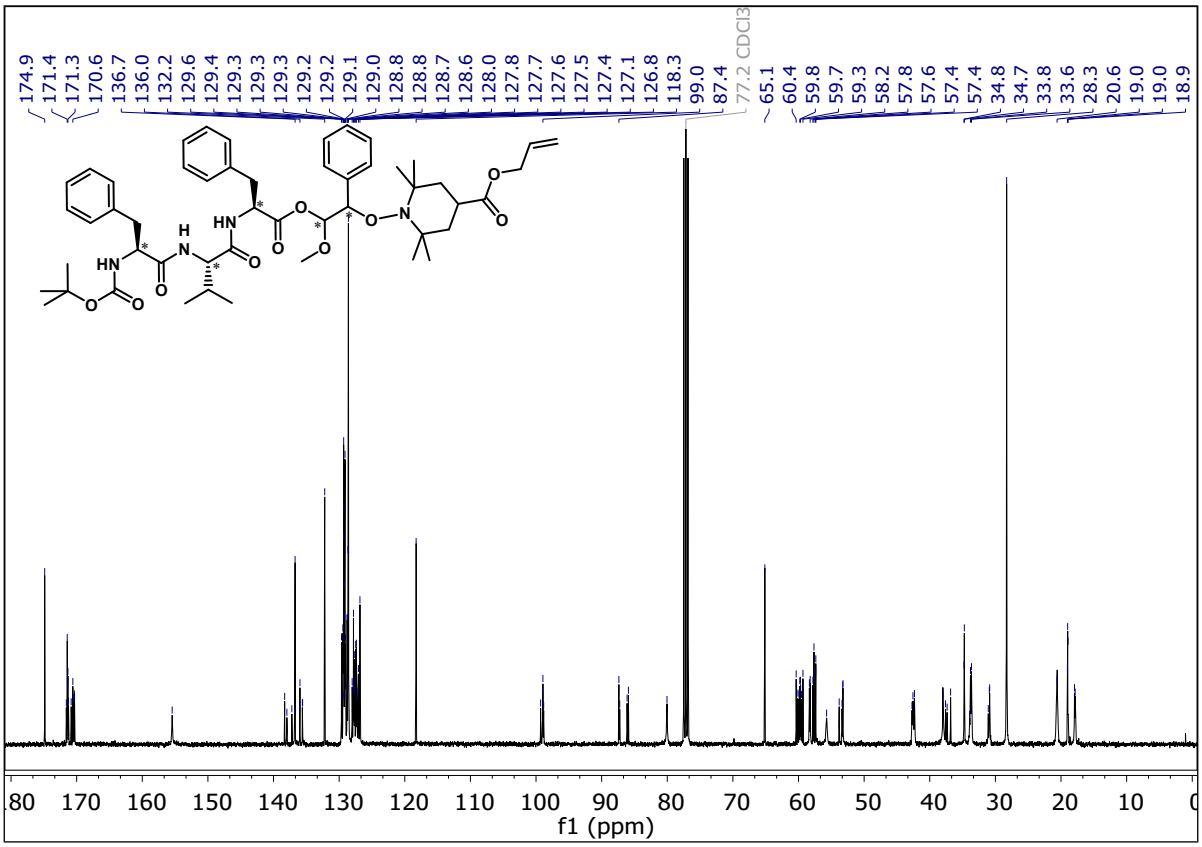


allyl 1-(((6S,9S,12S)-6,12-dibenzyl-9-isopropyl-15-methoxy-2,2-dimethyl-4,7,10,13-tetraoxo-16-phenyl-3,14-dioxa-5,8,11-triazahexadecan-16-yl)oxy)-2,2,6,6-tetramethylpiperidine-4-carboxylate
(A12L)

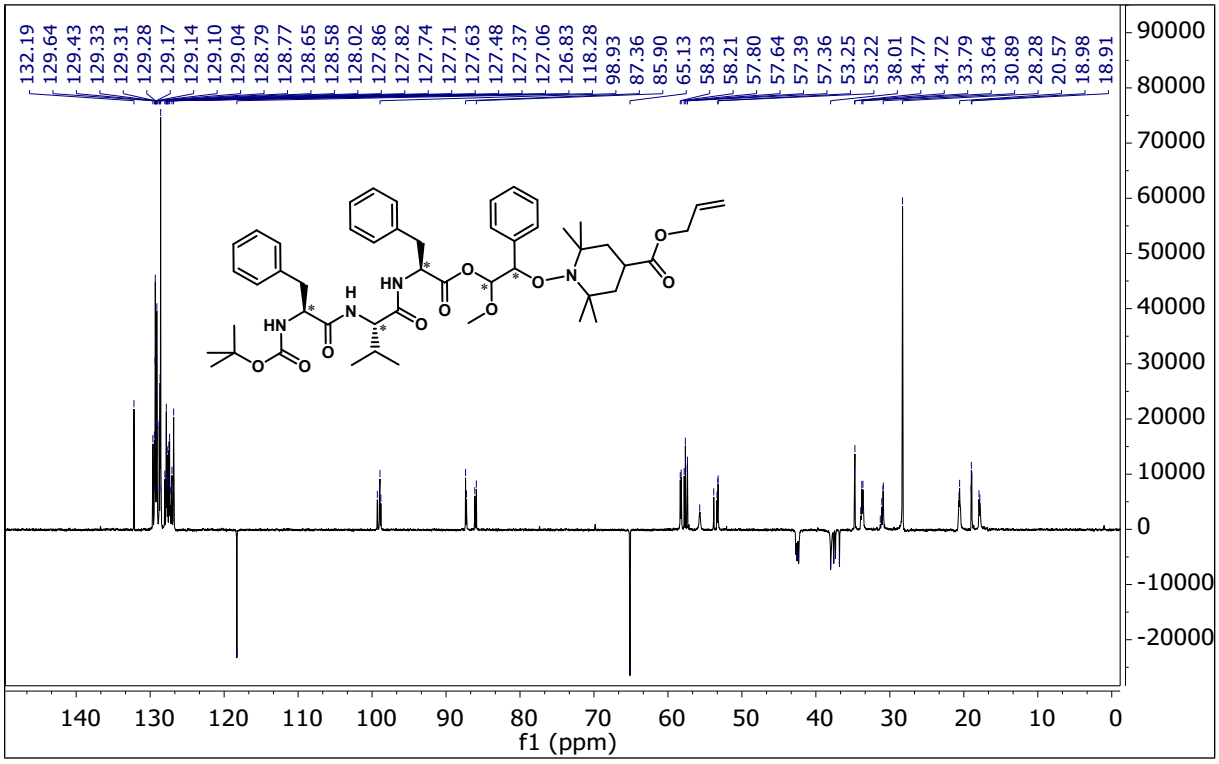
^{1}H NMR (400 MHz, $CDCl_3$)



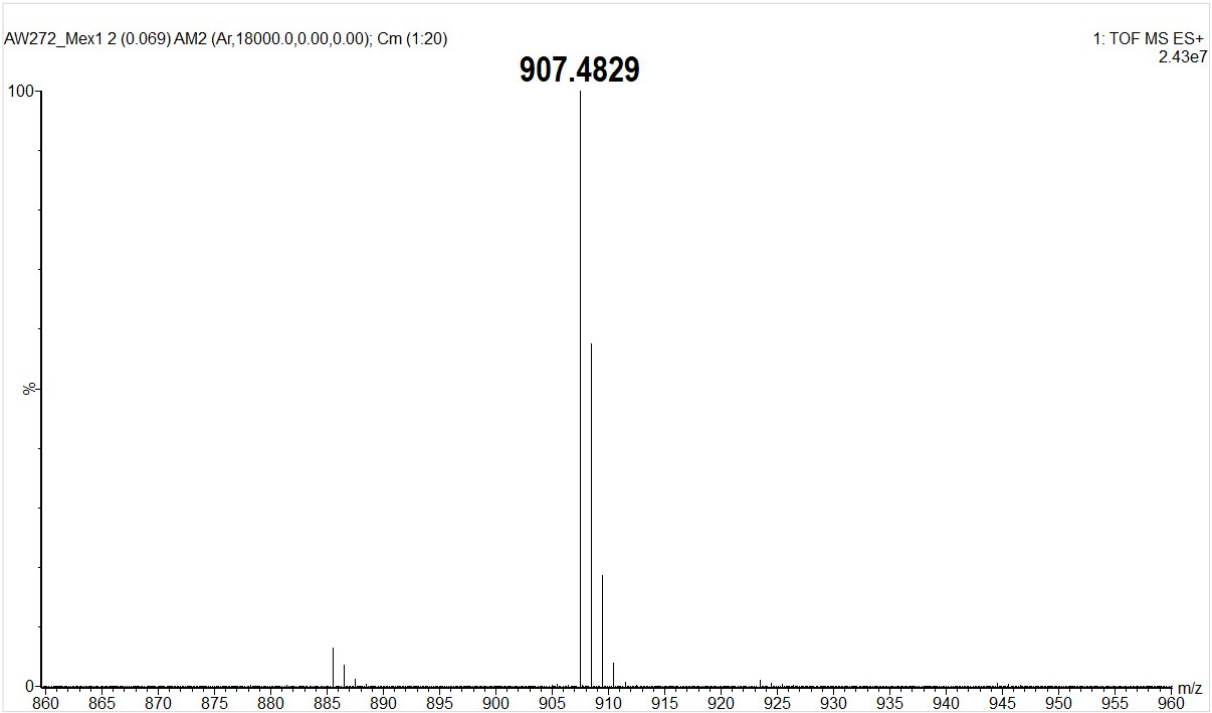
^{13}C NMR (101 MHz, $CDCl_3$)



DEPT 135 (101 MHz, $CDCl_3$)

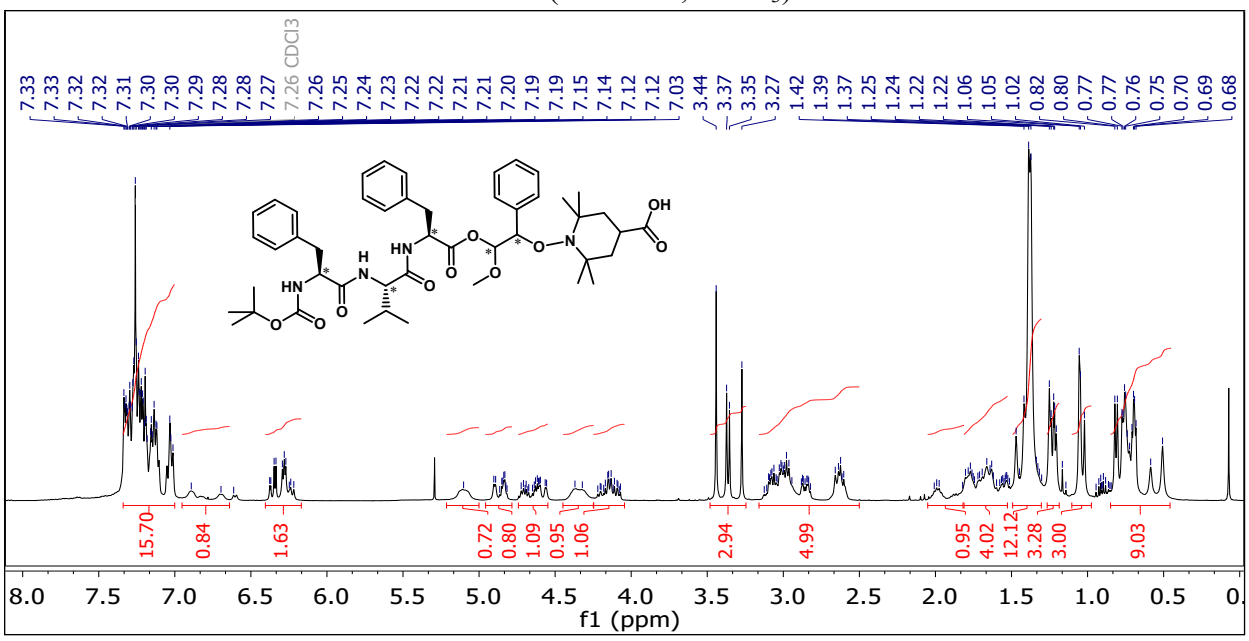


HRMS (ESI)

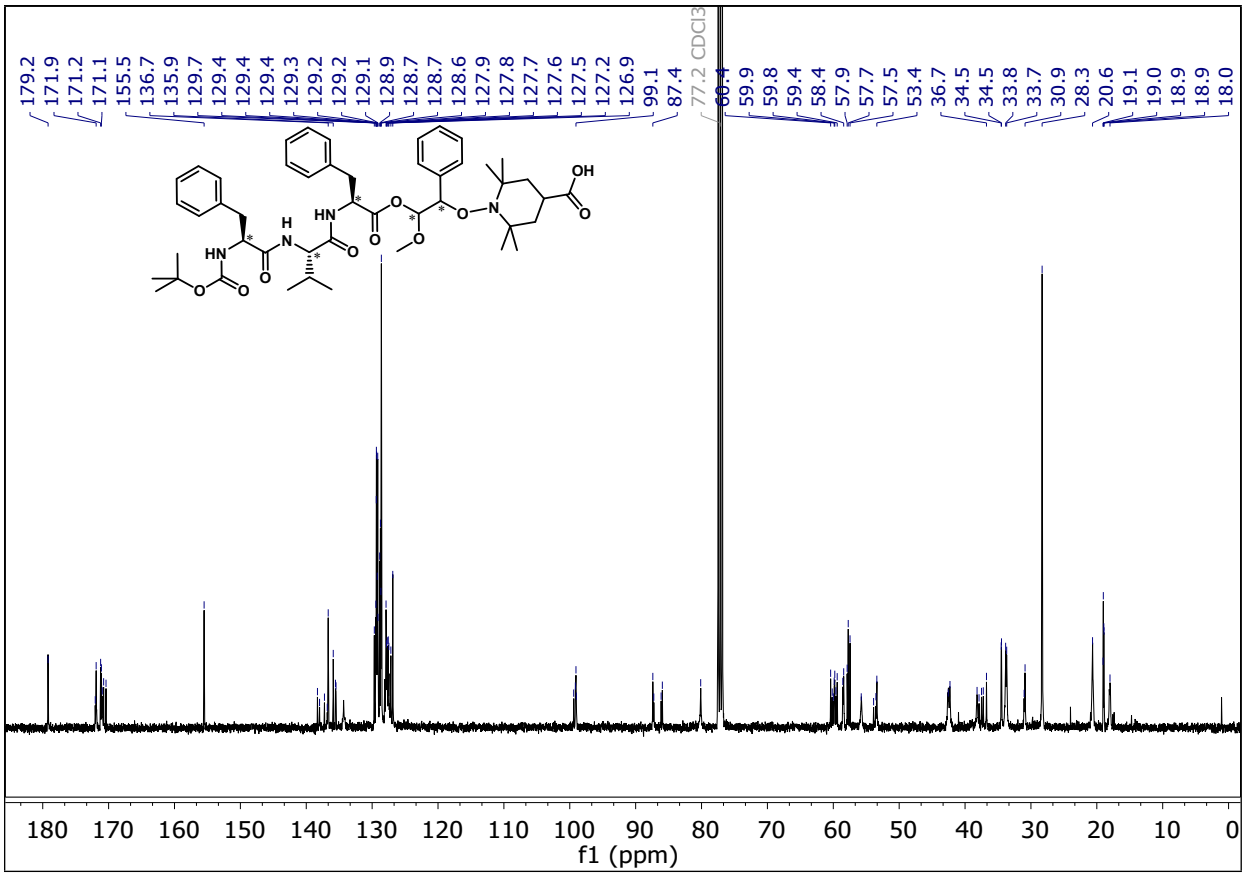


1-(((6S,9S,12S)-6,12-dibenzyl-9-isopropyl-15-methoxy-2,2-dimethyl-4,7,10,13-tetraoxo-16-phenyl-3,14-dioxa-5,8,11-triazahexadecan-16-yl)oxy)-2,2,6,6-tetramethylpiperidine-4-carboxylic acid
(A13L)

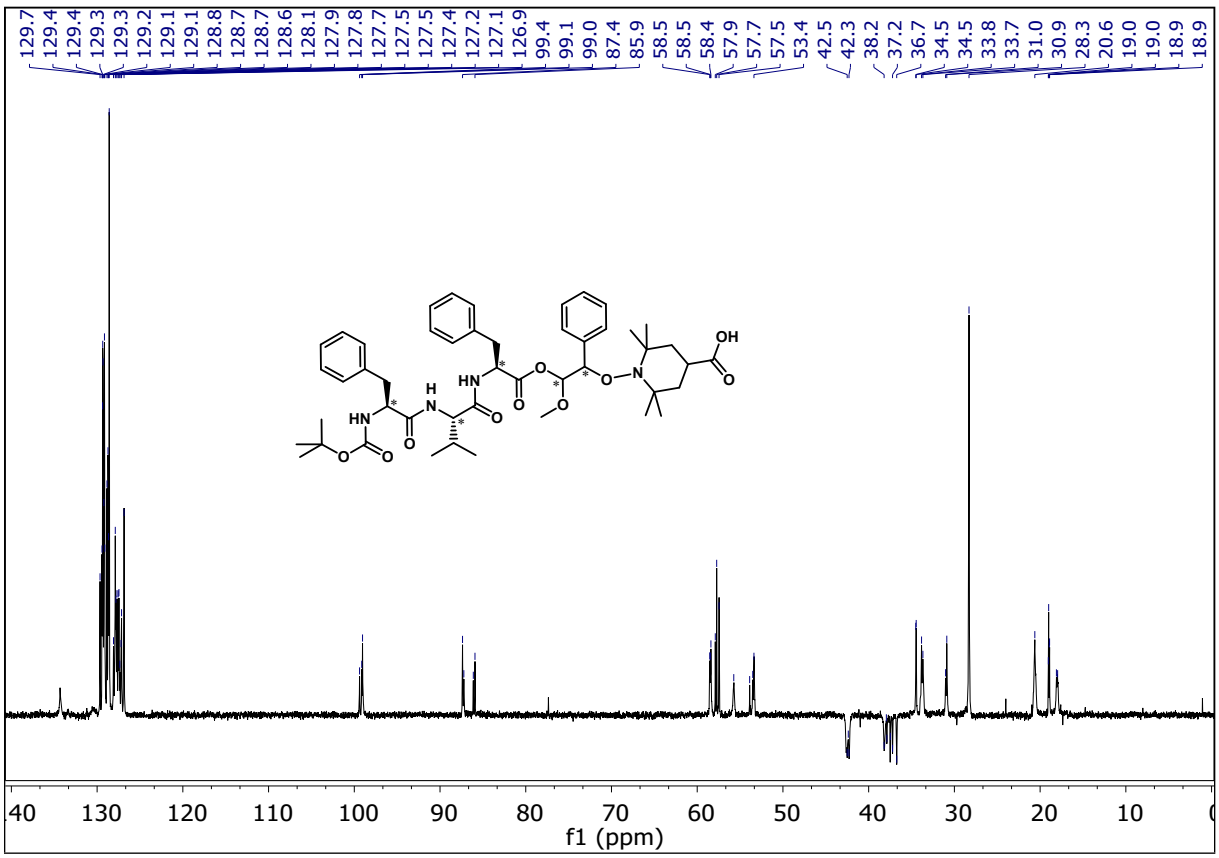
^{1}H NMR (400 MHz, $CDCl_3$)



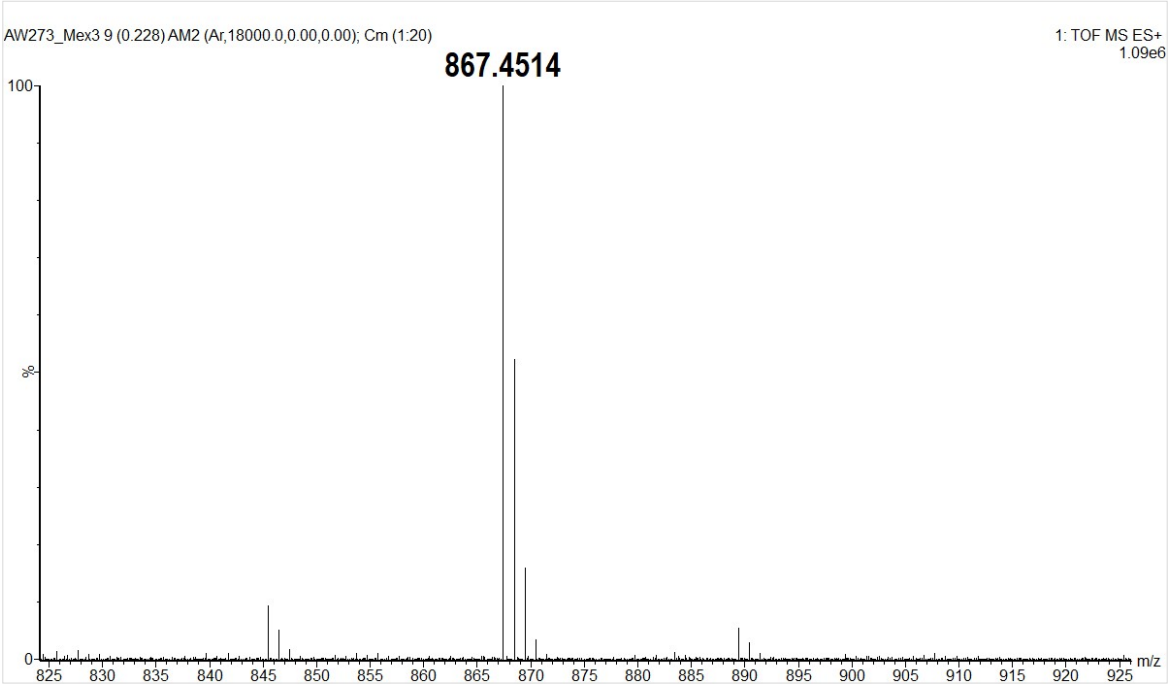
^{13}C NMR (101 MHz, $CDCl_3$)



DEPT 135 (101 MHz, $CDCl_3$)

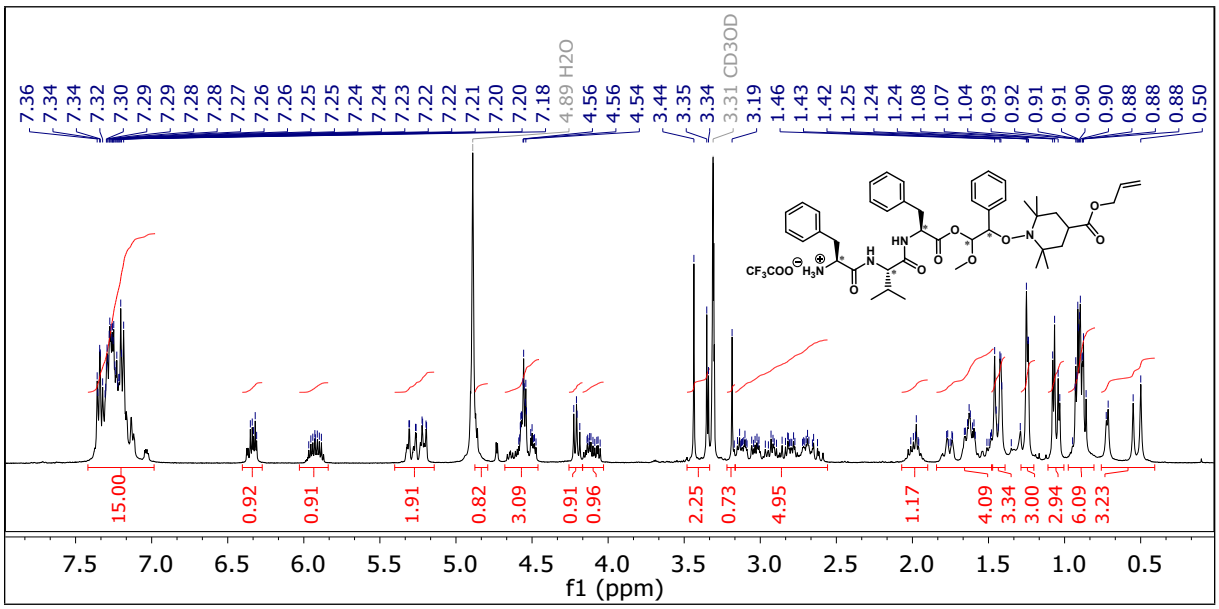


HRMS (ESI)

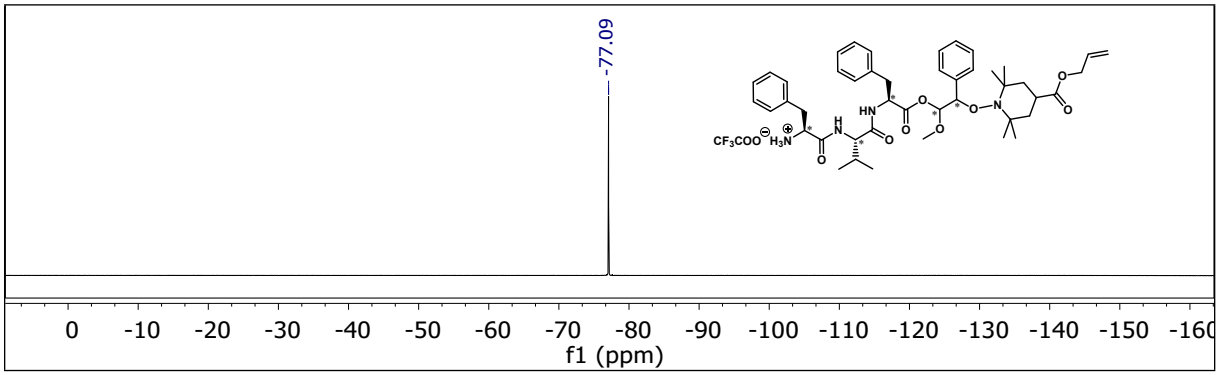


(6S,9S,12S)-3-(((4-((allyloxy)carbonyl)-2,2,6,6-tetramethylpiperidin-1-yl)oxy)(phenyl)methyl)-6-benzyl-9-isopropyl-5,8,11-trioxo-13-phenyl-2,4-dioxa-7,10-diazatridecan-12-aminium trifluoroacetate
(A14L)

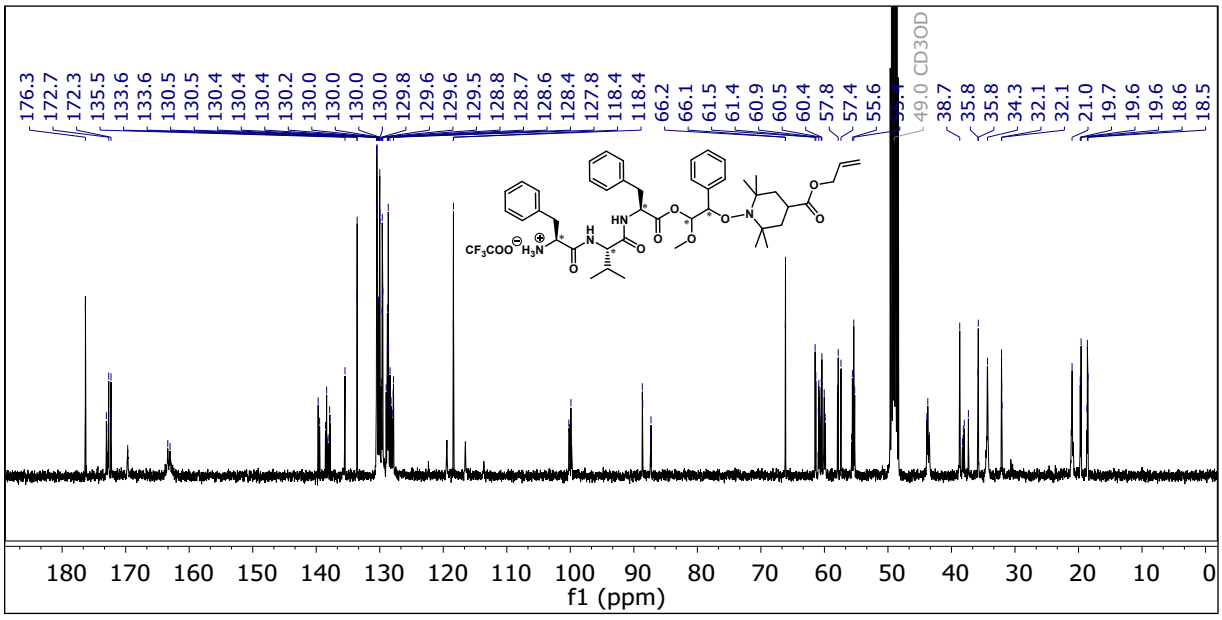
^{1}H NMR (400 MHz, MeOD)



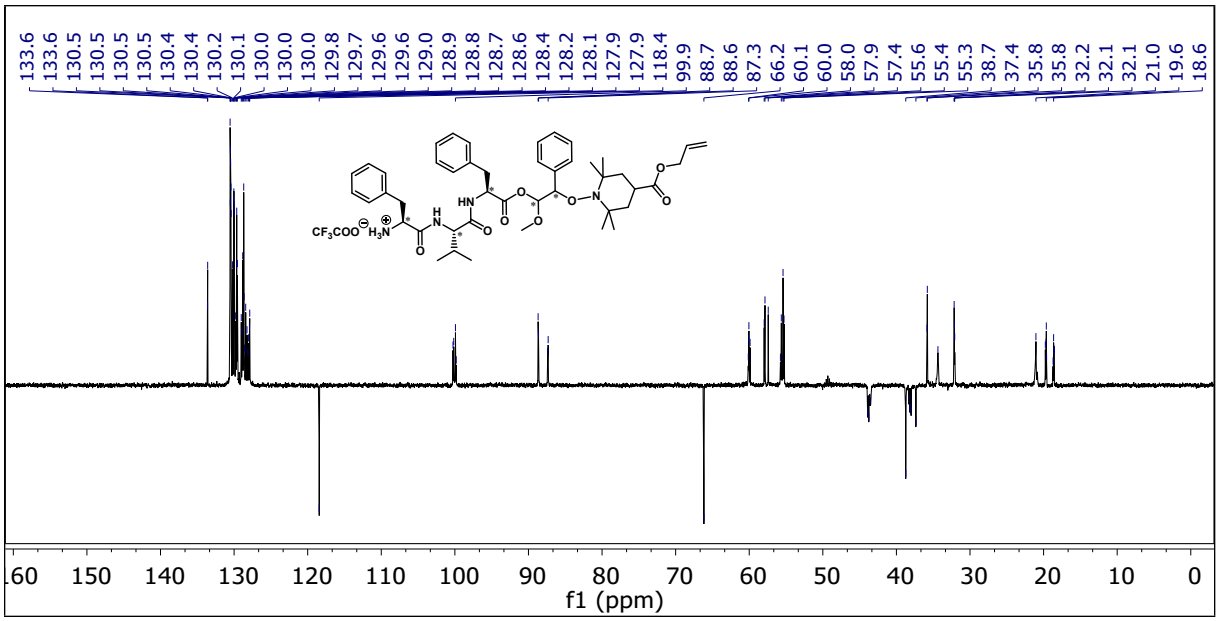
^{19}F NMR (376 MHz, MeOD)



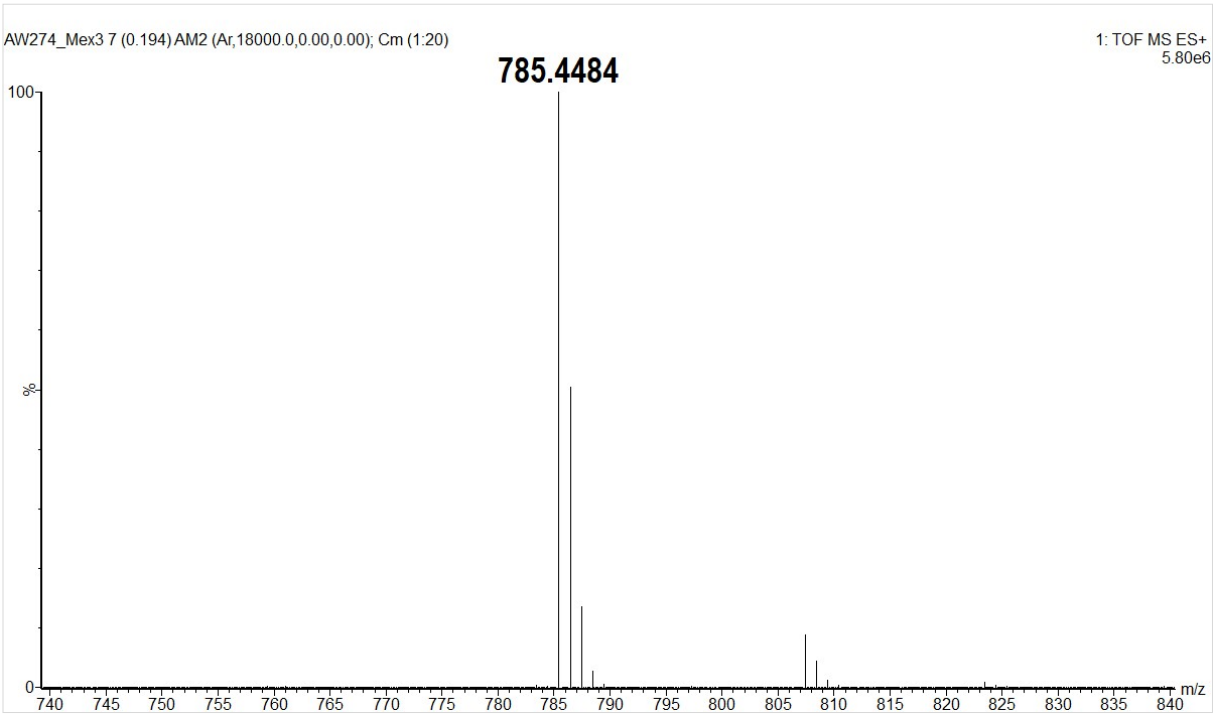
^{13}C NMR (101 MHz, MeOD)



DEPT 135 (101 MHz, MeOD)


133.6
133.6
130.5
130.5
130.5
130.5
130.4
130.4
130.2
130.1
130.0
130.0
130.0
129.8
129.7
129.6
129.6
129.0
128.9
128.8
128.7
128.6
128.4
128.2
128.1
127.9
127.9
118.4
99.9
88.7
88.6
87.3
66.2
60.1
60.0
58.0
57.9
57.4
55.6
55.4
55.3
38.7
37.4
35.8
35.8
32.2
32.1
32.1
21.0
19.6
18.6
CF3COO⊖ H3N⊕
HN
H
N
O
O
O
O
O
O
O
N
O
O
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

HRMS (ESI)


AW274_Mex3 7 (0.194) AM2 (Ar,18000.0,0.00,0.00); Cm (1:20)
1: TOF MS ES+
5.80e6
785.4484
100
%
0
740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840
m/z

Biological Testing: *Schistosoma* Raw Data

Molécule	[C] µg/mL	T0	1 H	2 H	3 H	4 H	5 H	6 H	7 H	8 H	24 H	48 H	72 H
A12L	100	24	24	24	24	24	24	24	24	24	24	24	24
	10	19	19	19	19	19	19	19	19	19	19	19	19
A13L	100	29	26	26	26	21	21	21	21	21	15	0	0
	10	31	31	31	31	31	31	31	31	31	31	31	31
A14L	100	24	24	24	24	24	24	24	24	24	24	24	24
	10	27	27	27	27	27	27	27	27	27	27	27	27
A8L	100	39	39	33	21	16	16	14	14	10	0	0	0
	10	41	41	41	41	41	38	38	31	29	15	0	0
A8DL	100	29	29	29	29	29	29	29	29	29	-	-	-
	10	25	25	25	25	25	25	25	25	25	-	-	-
A8D	100	45	45	33	6	0	0	0	0	0	0	0	0
	10	41	41	41	41	34	19	17	0	0	0	0	0

***Plasmodium* investigations**

The F32-ART (artemisinin-resistant) strain and the F32-TEM (artemisinin-sensitive strain)[1] were cultured according to Trager and Jensen[2] in RPMI 1640 medium (Fisher Scientific, Illkirch, France) supplemented with 5% human serum (Etablissement français du Sang, Toulouse, France) at 2% hematocrit with type O human blood (Etablissement français du Sang, Toulouse, France), 37 °C, and 5% CO_2 in a humidified atmosphere.

1. Biological Activity of New Compounds

IC_{50} values corresponding to 50% inhibition of *P. falciparum* growth were determined by SYBR Green assay, and the assessment of cytotoxicity was performed on Vero cells (cell line from the kidney of a normal adult African green monkey), as previously published.[3] The selectivity index corresponded to the cytotoxicity/activity ratio.

2. Quiescent Stage Survival Assay (QSA)

The QSA was performed according to Reyser et al.[4] Briefly, ART-resistant ring-stage parasites at 3% parasitemia were exposed for 6 h to 700 nM of DHA (dihydroartemisinin) to induce quiescence or were exposed to no drugs (control condition). After that, the compound to be tested was added in both conditions at 48 h. At the end of the treatment, the drugs were washed off with RPMI-1640, and parasites were replaced in drug-free conditions. Blood smears were performed to follow parasitemia until the day the cultures reached their starting parasitemia, defined as the recrudescence day. If after 30 days no parasite recrudescence was observed, then the data were censored. Data analysis was performed using Kaplan–Meier survival curves. Statistical significance was ascertained by using a log-rank (Mantel-Cox) test using GraphPad Prism 7 software (San Diego, CA, USA).

3. Microscopic Examination of Parasites upon Drug Exposure

The effect of the hybrid compound against *Plasmodium* parasites was assessed by the microscopic examination of parasite morphology upon drug exposure. For this purpose, parasites were synchronized at 0–24 h age by sorbitol treatment and treated by **A12L** at 5 μM. The parasite cycle progression for the treated condition was then compared to the untreated one (DMSO control).

Cytotoxicity experiments.

A non-cancer line of Vero cells was used to determine the cytotoxicity of the compound against mammalian cells. They were evaluated at a final DMSO concentration of 0.5%. The culture medium was MEM (Dutscher, Bernolsheim, France) supplemented with 10% fetal bovine serum (Fisher Scientific, Illkirch, France), 1X non-essential amino acids- (Fisher Scientific, Illkirch, France), 100 U/mL, 100 µg/mL penicillin/streptomycin (Fisher Scientific, Illkirch, France), and 2 mM L-glutamine (Fisher Scientific, Illkirch, France) at 37 °C in a humidified 5% CO_2 atmosphere.[5] Vero cells (100 µL of 105 cells/mL per well) were plated in 96-well plates for 24 h then treated with 100 µL of compound dilutions (in duplicate) during 48 h. All molecules were tested from 5 nM to 50 µM. Each well was then examined under the microscope to detect any precipitate formation before the supernatant was removed by flicking the plate. A volume of 100 µL of 0.5 mg/mL in MEM from a stock solution at 5 mg/mL of PBS-dissolved MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich/Merck, Darmstadt, Germany) was then added to each well.[6] After incubation for 1 h at 37 °C and 5% CO_2 the supernatant was removed and 100 µL of DMSO were added. Plates were gently shaken to dissolve formazan crystals resulting from the MTT reduction by living cells and read at 570 nm with the VICTOR Nivo plate reader (Perkin Elmer, Waltham, USA). CC_{50} values were determined using GraphPad Prism 7 software (San Diego, CA, USA).

Experiments on the viability of *Schistosoma mansoni* adult worms.

In vitro tests were performed on *Schistosoma mansoni* NMRI strains. This parasite is lab maintained on Biomphalaria glabrata (BRE strain) as intermediate mollusc host and Golden Hamster (Janvier Labs, Le Genest-Saint-Isle, France) as definitive vertebrate host. Methods, for molluscs' and hamsters' infection and for parasite recovery, were previously described.[7] Adult worms were recovered by hepatic perfusion technique between 42 and 47 days post hamster exposition to parasite larvae. Worms were carefully collected and disposed in 12-well Falcon. plate containing 2 mL of RPMI 1640 (supplemented with L-glutamine and Hepes 25 mM). The plates containing a minimum of 15 worms by well were then placed in an incubator chamber at 37 ◦C and 5% CO2. The worms sex ratio was almost balanced in each well. The drug was first dissolved in DMSO (Sigma-Aldrich) to give a 100 mg/mL mother solution. Then the stock solution was complemented with Tween 80 and RPMI to obtain the following final ratio dilution: RPMI 1640/Tween 80/DMSO and 1000/0.95/3.8, v/v/v. All molecules were tested at a final concentration of 100 µg/mL and 10 µg/mL. A negative control consisted in adding the same RPMI 1640/Tween 80/DMSO solution, but without any drug and a positive control consisted in adding praziquantel.[8] All molecules and control were performed in duplicates of well. The worm viability was checked after 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, and 72 h after adding the molecule. Parasites exhibiting no body contractions during a 30 s observation were considered as dead.

References

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