Targeting pathogenic *Acanthamoeba castellanii* using DNA minor groove binding agents

Hasan Y. Alniss[1,2]*, Sreedevi Sajeev[2], Ruqaiyyah Siddiqui[3,4], Meshal Daalah[5], Bader S. Alawfi[6], Hadeel M. Al-Jubeh[2], Anil Ravi[2], Naveed A. Khan[7,4]*.

[1]College of Pharmacy, Department of Medicinal Chemistry, University of Sharjah, 27272, Sharjah, United Arab Emirates; [2]Research Institute for Medical and Health Sciences, University of Sharjah, 27272, Sharjah, United Arab Emirates; [3]Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt University Edinburgh, EH14 4AS UK; [4]Microbiota Research Center, Istinye University, Istanbul, 34010, Turkey; [5]Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Health Sciences Center, Kuwait University, Jabriya, Kuwait; [6]Department of Medical Laboratories Technology, College of Applied Medical Sciences, Taibah University, Madinah 42353, Saudi Arabia; [7]School of Science, College of Science and Engineering, University of Derby, Derby, DE22 1GB, UK.

Short title: Antiamoebic properties of DNA minor groove binders

*Corresponding address: Naveed. A. Khan, School of Science, College of Science and Engineering, University of Derby, Derby, DE22 1GB, UK; Hasan Y. Alniss, College of Pharmacy, University of Sharjah, Sharjah, 27272, United Arab Emirates Arab Emirate. Tel: +(971)-6-5057427.

Abstract

DNA minor groove binders exhibit a high degree of sequence specificity and have a variety of biological actions including antiviral, anticancer, antibacterial, and anti-protozoal properties. Since it is the location of non-covalent interactions, the minor groove of double helical B-DNA is gaining significant interest as therapeutic targets. For the purpose of this investigation, the synthesis of five novel DNA minor groove binding agents was accomplished and antiparasitic efficacies were determined against *Acanthamoeba castellanii* of the T4 genotype *in vitro*. Using amoebicidal assays, the results revealed that all inhibitors tested showed significant killing of amoebae ($P<0.05$). Pre-treatment of amoebae with DNA minor groove binders inhibited parasite-mediated human cell death by measuring lactate dehydrogenase release using cytopathogenicity assays. Cytotoxicity assays revealed minimal effects on human cells. As phenotypic switching leads to infection recurrence, assays revealed that the inhibitors blocked amoebae transformation. These are promising findings and suggest that DNA minor groove binders may hold promise for further research in the effective eradication of pathogenic *A. castellanii*.

Key words: *Acanthamoeba*; Parasites; Cytopathogenicity; Cytotoxicity; DNA minor groove binders (MGBs); Treatment.

Introduction

A multitude of research endeavours have been dedicated to the precise targeting of specific DNA sequences using synthetic ligands, aiming to advance the development of medications and molecular probes for studying DNA polymorphism. The control of gene expression, or the selective blockage of transcription from specific regions by the specific targeting of a ligand, has become a particularly appealing and productive study subject (Bailly and Henichart, 1991; Nielsen, 1991; Verma et al., 2022). The need for novel anti-infective medications to treat bacterial, fungal, parasitic, and viral illnesses has been well-known medically and strategically. The rise of resistant strains of microorganisms has resulted in drug-resistant diseases globally, as classified by the WHO (O'Neill et al., 2019). A large number of non-covalent binding agents target the minor groove. DNA binding to specific sequences, primarily AT, is accomplished through a combination of specific hydrogen bonding to base pair edges, Van der Waals interactions with the walls of minor groove, and generalized electrostatic interactions with backbone of DNA (Alniss, 2019). The main advantage of this reversible binding mode of MGBs is that it avoids inducing permanent DNA damage, a common issue encountered with alkylating agents that form covalent bonds with DNA, leading to mutations and irreversible damage. Although there is growing evidence that most minor groove interacting molecules may function by directly reducing or preventing protein-DNA recognition, relatively little is documented about the molecular basis of their mode of actions (Lauria et al., 2007).

Pathogenic *Acanthamoeba* are free-living parasites that are abundant in the environment and cause opportunistic infections (Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007). These organisms cause granulomatous amoebic encephalitis (GAE), a rare disease that affects the central nervous system, particularly in immunocompromised people, and well-known to produce keratitis (AK), i.e., a corneal infection that can result in blindness (Clarke

and Niederkorn, 1999; Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007; Panjwani, 2010; Shing et al., 2021; Siddiqui et al., 2022a). Treatment is problematic and prognosis is poor despite advances in antimicrobial chemotherapy. Here, DNA minor groove binders were evaluated for their effects against amoebae.

Material and Methods

General Chemistry

We sourced chemical reagents through Sigma-Aldrich (Germany) as well as Santa Cruz Biotechnology Inc. (CA, USA) for our experiments, using them without additional purification. The 1H and 13C NMR experiments were conducted on a Bruker 500 MHz instrument, with chemical shifts provided in parts per million (ppm, δ-values) relative to the internal tetramethylsilane standard. NMR data analysis was performed uutilizing ACD NMR software. To confirm the structure of intermediate and final MGB compounds, NMR spectroscopy and mass spectrometry were employed. We conducted electrospray ionization–mass spectrometry (ESI–MS) experiments utilizing a mass spectrometer (MicromassW Quattro microTM, Waters Corp., USA) equipped with an electrospray ionization (ESI) interface, and it operated in the positive ionization mode. Full spectral characterizations of MGB compounds are provided in the Supplementary Information. For the purification of the MGB final products, we employed reverse-phase liquid chromatography (Agilent 1260 infinity HPLC, CA, USA) with a C18 column (5 μm; 25 cm × 9.4 mm i.d) utilizing gradient elution and a detection wavelength of 260 nm.

General experimental procedure for the synthesis MGB final products

The MGB compounds were synthesized using the standard protocol as previously described (Alniss et al., 2019; 2022). In brief, different carboxylic acid moieties (3a, 3b and

6) were coupled with nitro compounds (1a, 1b, and 4) to obtain the MGB final products. Equimolar quantities of (1a, 1b, and 4) were dissolved in a mixture of 3 mL methanol and 3.5 mL THF. The solution was then cooled to 0 ˚C, and 10%-Pd/C (0.1 g) was gradually added to the reaction flask. The flask underwent repeated evacuation and hydrogen refilling, the reaction was then left stirring under hydrogen for four hours. After Pd filtration, the solvent was removed under reduced pressure, resulting in an oily amine product. This amine was then mixed with DMF (1.5 mL) and added drop by drop to a flask containing equimolar amounts of the acid, HBTU, and triethylamine (5 equivalent), dissolved in 3 mL DMF. The reaction mixture was left to stir overnight under nitrogen gas at room temperature. Following the removal of the organic solvent under vacuum, 12 mL sodium bicarbonate solution was introduced, and the MGB product was extracted twice with ethyl acetate (2x15ml). The organic solvent was evaporated, and the crude MGB product underwent purification through reverse phase HPLC using gradient elution. The resulting product solution was freeze-dried to yield the desired MGB in the form of solid TFA salts.

Acanthamoeba Culturing

Acanthamoeba castellanii genotype T4 (ATCC 50492) were grown in 10 mL of PYG media (0.75% proteose peptone, 0.75% yeast extract, and 1.5% glucose) at 30ºC (Rodriguez-Exposito et al., 2022) in tissue culture flasks. Following 48 hours of incubation, amoebae cultures reached over 90% confluency and used for subsequent assays (Mungroo et al., 2020).

Human cerebral microvascular endothelial (HCME) cells

Human cerebral microvascular endothelial cells (HCME) cells were grown in RPMI supplemented with 1% penicillin-streptomycin (Pen-Strep), 1% minimum essential medium amino acids, 10% fetal bovine serum (FBS), and 1% L-glutamine in an incubator with 5% CO_2

with 95% humidity at 37°C. After the media was aseptically removed, the cells were enzymatically detached using 2 mL of trypsin-EDTA. The cells were then centrifuged for 5 minutes at 2500 x g and the cell pellet was reconstituted in the media and inoculated in 96-well plates, and subsequently used in assays (Mungroo et al., 2020).

Amoebicidal Assay

Assays were performed to determine the effects of DNA minor groove binders on the viability of amoebae. Briefly, 3 x 10^5 amoebae were incubated with DNA minor groove binders at various concentrations in 24-well plates to a final volume of 500 µL. Next, the plates were kept in a 30ºC incubator for 24 hours. Positive and negative controls were included by incubating amoebae with 25 µM chlorhexidine and RPMI respectively. After the incubation, amoebae were categorized as being alive or dead by the addition of 0.1% Trypan blue and enumeration using a haemocytometer.

Phenotypic switching assay

A. castellanii cysts were prepared by inoculating amoebae on non-nutrient bacteriological agar plates as described previously (Akbar et al., 2021). Plates were incubated in a 30ºC incubator for 14 days for complete transformation of cysts. Next, cysts were scraped off and centrifuged for 10 minutes at 3000 x g. Following this, 3 x 10^5 cysts were treated with 100 µM of various inhibitors in PYG. For controls, amoebae cysts were incubated in PYG alone and emerging viable trophozoites were enumerated using a haemocytometer (Lorenzo-Morales et al., 2010).

Cytotoxicity Assays

To assess compound toxicity on HCME cells, cytotoxicity experiments were conducted. HCME cells were cultivated in 96-well plates and exposed to the inhibitors. Plates were placed in a 37°C incubator with 95% humidity and 5% CO2 for 24 hours. Next, supernatants were collected, and the compound cytotoxicity was assessed using a cytotoxicity detection kit that measures lactate dehydrogenase (LDH) release (Jeyamogan et al., 2018). Untreated HCME cells were negative controls, while HCME cells treated with 1% Triton X-100, inducing 100% cell death, served as positive controls (Kim et al., 2021; Siddiqui et al., 2022b).

Cytopathogenicity assay

Cytopathogenicity assays were accomplished as previously described by (Ahmed et al., 2022), measuring LDH release. Trophozoites (3 x 10^5) were treated with inhibitors at 100 µM in RPMI-1640 for 2 hours at 30°C in a 24-well plate. After centrifugation at 2500 x g for 10 minutes, the amoebae were resuspended in RPMI-1640, and 200µl of treated *Acanthamoeba* were inoculated into a 96-well plate containing human cell monolayers. Negative controls consisted of untreated cells in RPMI-1640, while positive controls were cells treated with 1% Triton X-100. The extent of cell damage was determined by measuring LDH release. Absorbance at 490 nm was quantified using a spectrophotometer, and the percentage of cytopathogenicity was calculated using the cytotoxicity formula, as previously described (Anwar et al., 2019).

Results

Chemical synthesis

The MGB intermediates and final products were synthesized using the established protocol described earlier (Alniss et al., 2019; 2022). In brief, the nitro compounds **1a**, **1b**,

previously reported by our group (Alniss et al., 2022), were reduced by catalytic hydrogenation with 10%-Pd/C to afford amine intermediates **2a**, **2b** and then followed by direct coupling with acids **3a**, **3b** using HBTU in presence of basic conditions to obtain the final products **MGB25**, **MGB27**, **MGB29** and **MGB31** in 49%, 83.3%, 28.8% and 91.7% yield, respectively (Scheme 1, A). The nitro group of intermediate **4,** previously reported by our group (Alniss et al., 2022), was reduced under catalytic hydrogenation conditions using 10%-Pd/C in a similar manner of previous reaction to give the amine intermediate **5**, which was then coupled with the carboxylic acid **6** to afford the final product **MGB33** in 19.7% yield (Scheme 1, B). These final yields were reported after HPLC purification and lyophilization. The MGB compounds evaluated in this study exhibit purity levels above 95%, as determined by reverse-phase HPLC analysis (S1). Full spectral characterization of MGB compounds is provided in the Supplementary Information (S1).

DNA minor groove binders exhibited significant amoebicidal effects against A. castellanii

The amoebicidal assay was used to evaluate the effects of DNA minor groove binders on *A. castellanii* viability. At micromolar concentration, the results showed that all drugs tested demonstrated significant killing against *A. castellanii* as compared with the solvent control (Fig. 1). Importantly, the derivative MGB29 demonstrated the most significant activity and treatment resulted in 79 % reduction in amoebae viability. In addition to MGB29, MGB31 also reduced amoebae viability. While MGB25, MGB27, and MGB33 showed significant anti-amoebic activity and reduced viability by 53%, 67%, and 40% respectively (Fig. 1).

A. castellanii phenotypic switching was significantly suppressed by DNA minor groove binders

Cysts are often resistant to chemotherapeutic agents and they are the major cause of infection recurrence. When challenged with pre-formed mature *A. castellanii* cysts, all DNA minor groove binders tested inhibited phenotypic switching at micromolar concentrations. Of note, the derivatives MGB27, MGB29 and MGB31 demonstrated potent activity and abolished up to 90% transformation. Overall, the derivatives MGB25, MGB27, MGB29, MGB31 and MGB33 inhibited cellular differentiation of amoebae by 70%, 89%, 85%, 88% and 73% (Fig. 2).

DNA minor groove binding agents depict minimal toxicity against human cells

The cytotoxic effects of DNA minor groove binder derivatives on human cells were investigated using LDH release assays. Cytotoxicity assays revealed that all derivatives tested exhibited negligible cytotoxicity. MGB25, MGB27, MGB29, MGB31 and MGB33 exhibited cytotoxicity at 32%, 11%, 22%, 16%, and 24%, respectively (Fig. 3).

Amoeba-mediated host cell death was inhibited by pre-treatment of parasites with DNA minor groove binders

To determine whether pre-treatment of amoebae with DNA minor groove binders can inhibit amoebae-mediated human cell death, cytopathogenicity assays were conducted. The results demonstrated that all derivatives tested significantly reduced *A. castellanii*-mediated human cell toxicity as determined by estimation of LDH release. Among various derivatives tested, cytopathogenicity was reduced by MGB25 to 78%, MGB27 to 57%, MGB29 to 72%, MGB31 to 61%, and MGB33 to 47% at micromolar concentrations tested.

Discussion

Minor groove binders have the ability to bind reversibly to the minor groove of DNA, with a significant affinity (Barrett et al., 2013; Wittayanarakul et al., 2010). For many years, numerous types of natural products with minor groove binding characteristics have been recognized. Polyamides, netropsin, and distamycin are among a few examples (Arcamone et al., 1989). The physical properties of the ligand and its ability to cross cellular membranes are also important factors in its activity. While certain polar minor groove binders exhibit DNA interaction capabilities, their inability to penetrate lipophilic membranes renders them inactive. (Alniss et al., 2019; 2022).

A substantial amount of research has been published in the discovery of novel and effective chemotherapeutic agents in the treatment of *Acanthamoeba* infections and/or uncovering novel targets. Among these, the DNA minor groove has been identified as a valuable target, and a variety of intriguing chemicals have been noted. In a recent study, Rahman et al., (2011) showed that polyamides are employed in antiparasitic therapy in addition to being antibacterial and antifungal medications. Similarly, Arafa et al., (2011) developed and examined a series of amidine-related dicationic flexible triaryl bis-guanidines as antiparasitic DNA minor groove binders. For the treatment of leishmaniasis, malaria, and human African trypanosomiasis, amidine-like compounds are employed as DNA minor groove binders in numerous trials (Barrett et al., 2013; Basselin et al., 2002; Rahman et al., 2019; Wang et al., 2006). However, successful treatment of *Acanthamoeba* infections remains an unmet challenge (Siddiqui et al., 2022a). In this study, a range of DNA minor groove binding agents with enhanced lipophilicity were synthesized, characterised, and evaluated for anti-amoebic properties using a range of assays *in vitro*. Our findings showed clearly that MGB25, MGB27, MGB29, MGB31, and MGB33 have considerable anti-amoebic activities and inhibited parasite-mediated human cell death. The compounds tested were also discovered to have limited cytotoxic effects against human cells tested. These findings are consistent with recent studies

which showed that selected S-MGBs exhibit activity against *A. castellanii* (Mcgee et al., 2024).

The MGB compounds presented in this study are derivatives of the MGB compounds previously reported by our group in an earlier study (Table 1) (Alniss et al., 2022). Among the tested compounds, MGB29 displayed the highest amoebicidal activity against *Acanthamoeba castellanii*, while MGB33 showed the weakest amoebicidal activity. Considering the ligand's structural and physical properties, these results suggest that the lipophilicity of MGBs may play a pivotal role in their anti-amoebic attributes. This is consistent with the fact that MGB33 has the lowest LogD (determined by Marvin, https://www.chemaxon .com) among the investigated MGBs (Table 1). Furthermore, the new MGBs (25, 27, 29 & 31), exhibit stronger amoebicidal activity against *Acanthamoeba castellanii* compared the previously reported MGBs (3,5, 6, 16, 22). This also could be attributed the ligand's lipophilicity, since the MGBs described in this study possess higher logD values compared to the previous MGBs (Table 1). The lipophilicity of the new MGBs was enhanced by introducing lipophilic moieties into the MGB structure, such as thiazole or benzene instead of pyrrole rings, and double or triple bond instead of the amide links. The ligand's ability to penetrate the cell membrane and reach its molecular target within the nucleus is influenced by its lipophilicity. This conclusion is consistent with the calculated cell permeability using the computational permeability tool, PerMM (Lomize et al., 2019) (Table 2). Among the tested compounds, MGB29, which demonstrated the highest amoebicidal activity, also displayed the highest cell permeability. In comparison to MGB33, MGB29 exhibited a 10-fold higher cell permeability in the Caco-2 model and a 3,890-fold increase in permeability in the PAMPA model (Table 2). Moreover, when compared to MGB3 (from the previous series), MGB29 demonstrated a 31-fold higher cell permeability in the Caco-2 model and a remarkable 229,086-fold higher permeability in the PAMPA model. These findings correlate with the observed amoebicidal activity of MGB compounds and highlight the crucial role of lipophilicity in their biological activity. A similar correlation between

lipophilicity and lung tumour potency has been previously reported for another structural class of MGBs (Scott et al., 2016). Apart from differences in lipophilicity, the variations in activity against *A. castellanii* among the tested MGBs may be ascribed to their capacity to bind to distinct DNA sequences with varying affinities. MGBs featuring imidazole/thiazole rings demonstrate a preference for GC-rich sites, whereas those containing N-methylpyrrole tend to bind to AT-rich sequences (Alniss et al., 2014; Gottesfeld et al., 2001; Salvia et al., 2013). Moreover, the binding affinity between different MGBs and DNA is expected to be unequal; even minor structural variations may substantially modify the binding affinity, thereby influencing the ligand's bioactivity. The MGBs formulated in this study possess notable structural features that facilitate their interaction with DNA. These compounds bind reversibly to the minor groove via non-covalent hydrogen bonds between the amide NH groups/ N of thiazole and the DNA bases within the floor of the minor groove. This reversible binding mode prevents the induction of permanent DNA damage, thereby minimizing toxic effects. The MGBs also possess a crescent shape, an essential feature allowing them to match the curvature of the minor groove. Consequently, structural modifications to the MGBs, including the addition of double or triple bonds, were carried out at the terminal position of the ligand structure to preserve the MGB crescent shape. The results of this study indicate that alkene-linked MGBs demonstrate greater activity compared to alkyne-linked MGBs, and the lipophilicity of MGBs may play an important role in their amoebicidal attributes.

Funding: This study received funding through a grant from the Research Funding Department at the University of Sharjah-UAE (grant number: 2101110149 & 2401110195). Ruqaiyyah Siddiqui and Naveed Ahmed Khan are supported by the Air Force Office of Scientific Research (AFOSR), USA.

Authors contributions: HYA and NAK conceived the study amid discussions with RS. HYA performed the synthesis of compounds. HMA, AR, SS, MD and BSA designed the studies, and carried out all experimentation under the supervision of HYA, RS, and NAK. HYA and NAK prepared the first draft of the manuscript. All authors corrected and approved the final manuscript.

Competing interests*:* Hasan Alniss has a patent pending for MGB compounds. The remaining authors have no conflicts of interest to declare.

Appendix. Supplementary information

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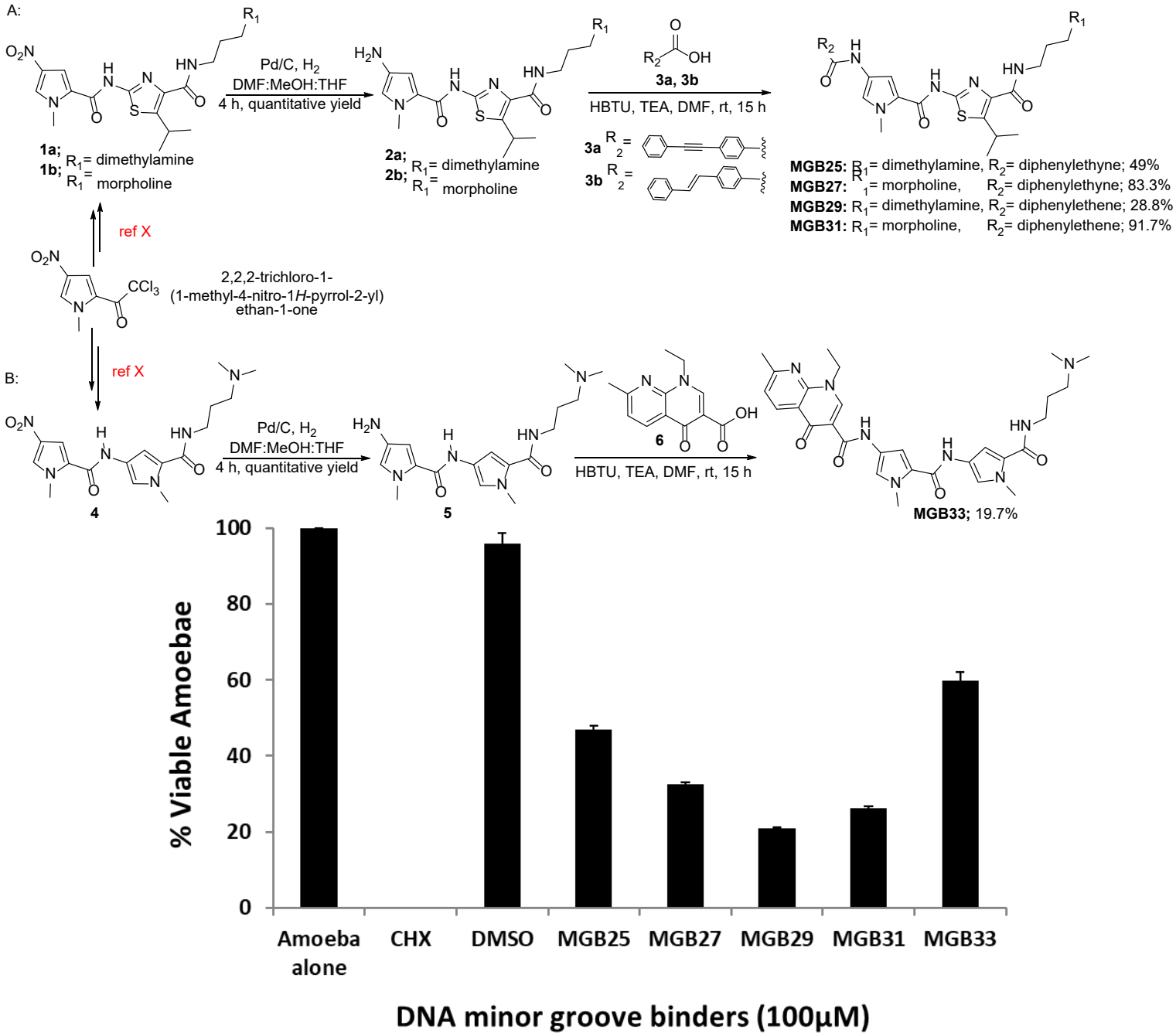
Scheme 1: Synthesis of MGB final products**.**

Figure 1: MGB derivatives demonstrated significant amoebicidal activity against *Acanthamoeba castellanii.* Briefly, $3X10^5$ of *A. castellanii* were treated with MGB derivatives at a concentration of 100 µM for 24 hours at 30°C. Data are presented as the mean ± standard error and are representative of at least three independent experiments performed in duplicate. Additionally, p-values were calculated using a two-sample t-test with a two-tailed distribution; * is $p \leq 0.05$.

Figure 2: Excystation effect of MGB derivatives against *A. castellanii.* Data are presented as the mean ± standard error and are representative of at least three independent experiments performed in duplicate. Additionally, p-values were calculated using a two-sample t-test with a two-tailed distribution; * is $p \leq 0.05$.

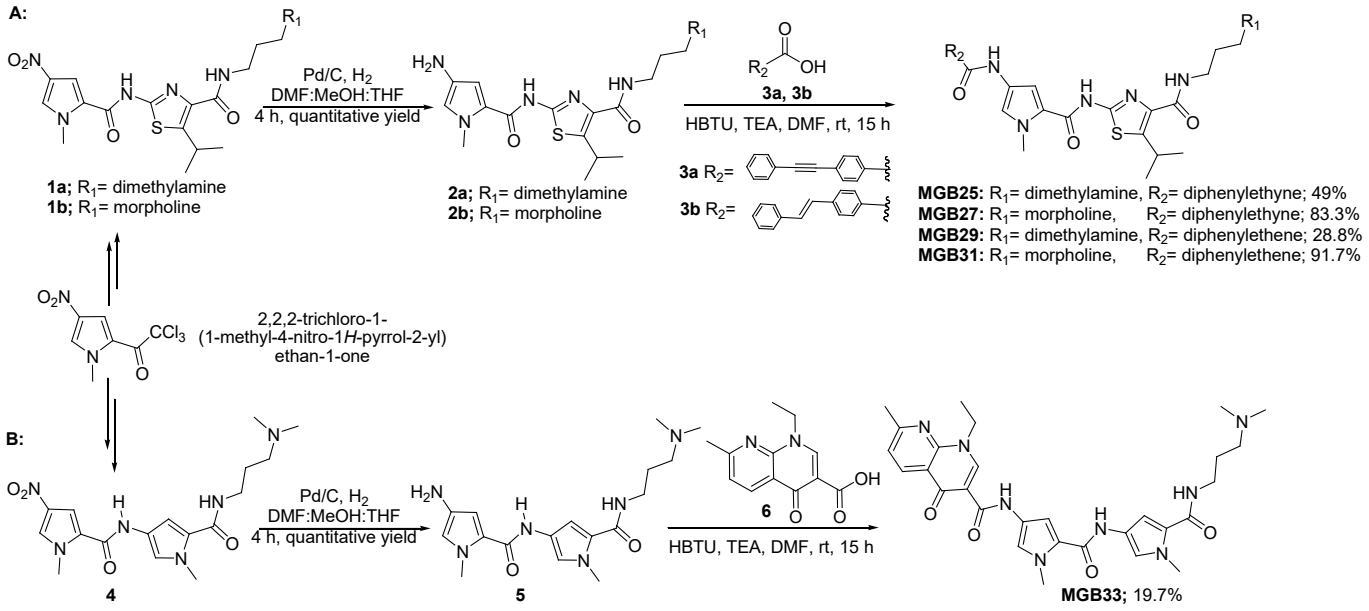
Figure 3: A) Cytotoxicity assays were performed to determine the toxicity of MGB derivatives towards human cells as described in Materials and Methods. The results are presented as the mean ± standard error.

Figure 4: MGB derivatives protected human cells against amoebae. After being treated with 100μM compounds to $3x10^5$ amoebae for 2 hours, the compounds showed varying degrees of reduction in amoebae-mediated host-cell death. *$p \leq 0.05$.


A:
Pd/C, H2
DMF:MeOH:THF
4 h, quantitative yield
HBTU, TEA, DMF, rt, 15 h
3a, 3b
1a; R1= dimethylamine
1b; R1= morpholine
2a; R1= dimethylamine
2b; R1= morpholine
3a R2 =
3b R2 =
MGB25: R1= dimethylamine, R2= diphenylethyne; 49%
MGB27: R1= morpholine, R2= diphenylethyne; 83.3%
MGB29: R1= dimethylamine, R2= diphenylethene; 28.8%
MGB31: R1= morpholine, R2= diphenylethene; 91.7%
ref X
2,2,2-trichloro-1-
(1-methyl-4-nitro-1H-pyrrol-2-yl)
ethan-1-one
B:
ref X
Pd/C, H2
DMF:MeOH:THF
4 h, quantitative yield
HBTU, TEA, DMF, rt, 15 h
6
4
5
MGB33; 19.7%
% Viable Amoebae
100
80
60
40
20
0
Amoeba alone
CHX
DMSO
MGB25
MGB27
MGB29
MGB31
MGB33
DNA minor groove binders (100µM)

Highlights

- Minor groove of double helical B-DNA is gaining interest as therapeutic targets.
- Here, five novel DNA minor groove binding agents were synthesized.
- Inhibitors showed significant killing of amoebae and blocked differentiation.
- Pre-treatment of amoebae inhibited parasite-mediated human cell death
- Inhibitors blocked amoebae transformation.
- DNA minor groove binders hold promise in the effective eradication of amoebae.



Scheme 1: synthesis of MGB final products

Fig. 1

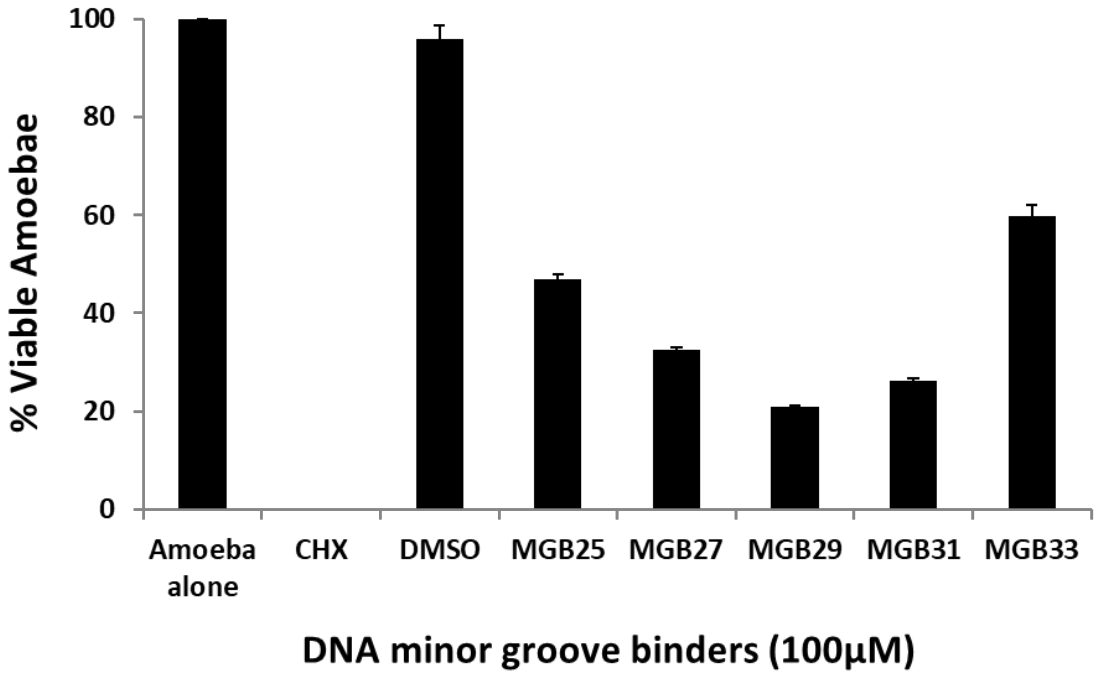

% Viable Amoebae
100
80
60
40
20
0
Amoeba alone
CHX
DMSO
MGB25
MGB27
MGB29
MGB31
MGB33
DNA minor groove binders (100µM)

Fig. 2

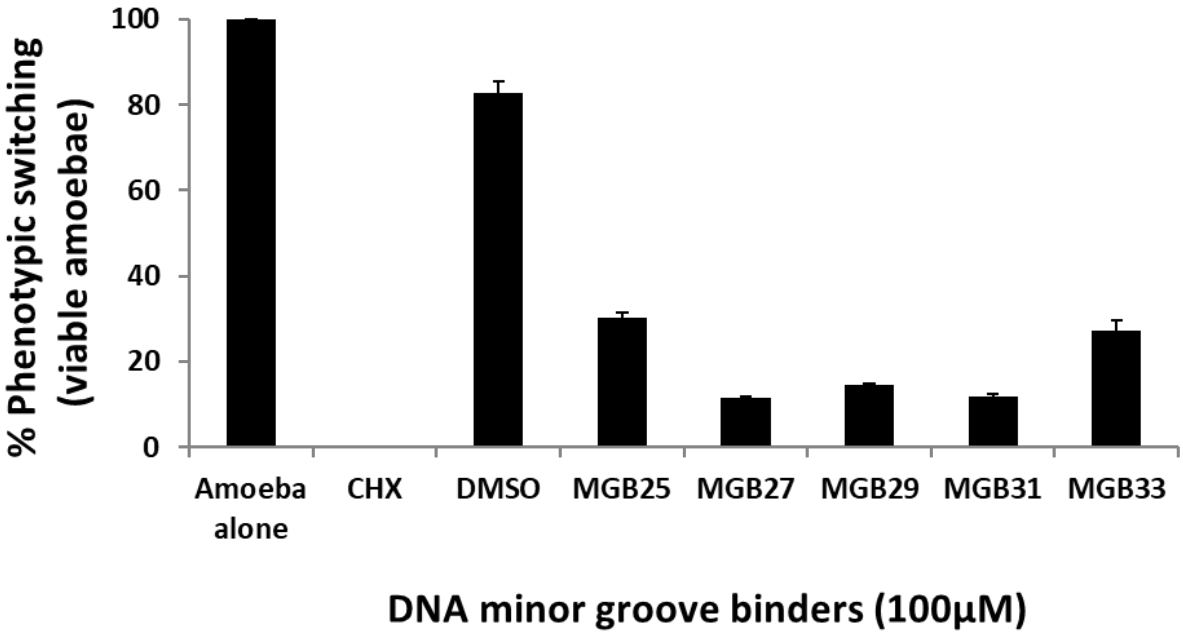

% Phenotypic switching (viable amoebae)
100
80
60
40
20
0
Amoeba alone
CHX
DMSO
MGB25
MGB27
MGB29
MGB31
MGB33
DNA minor groove binders (100µM)

Fig. 3

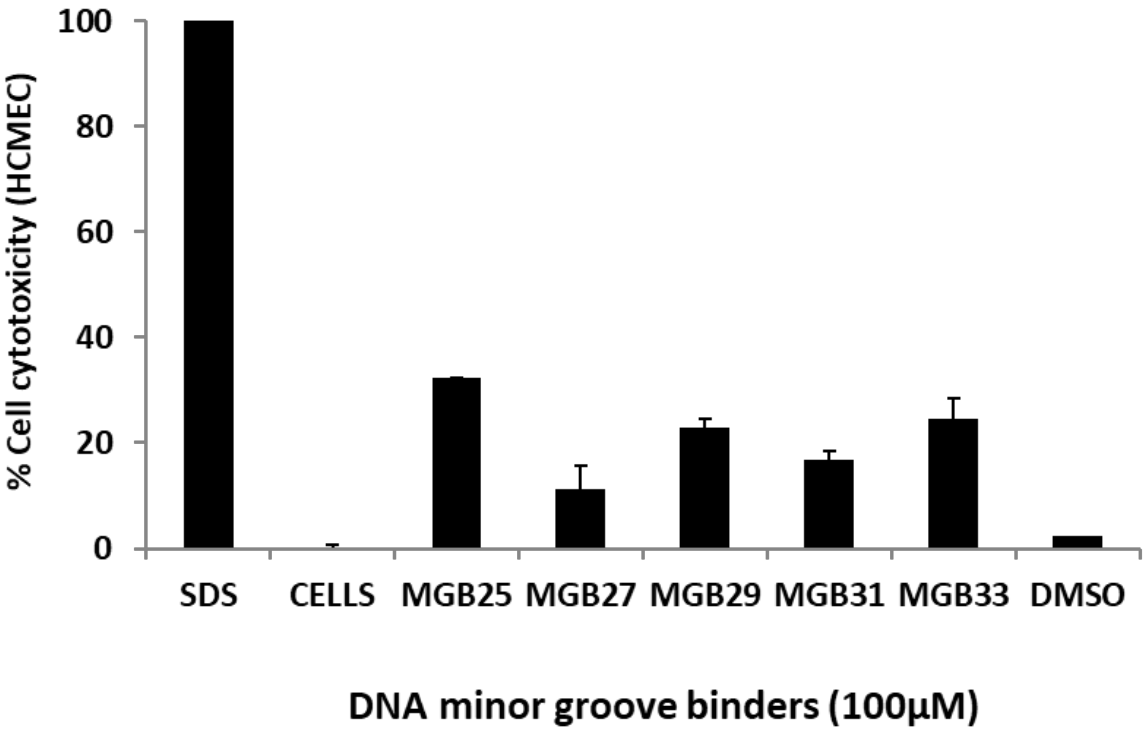
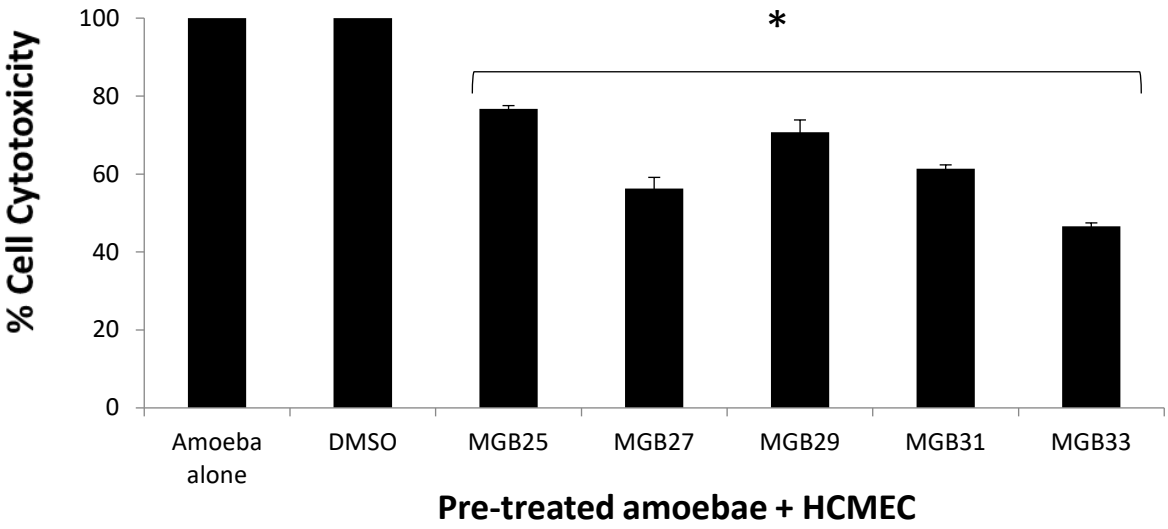

% Cell cytotoxicity (HCMEC)
100
80
60
40
20
0
SDS
CELLS
MGB25
MGB27
MGB29
MGB31
MGB33
DMSO
DNA minor groove binders (100µM)

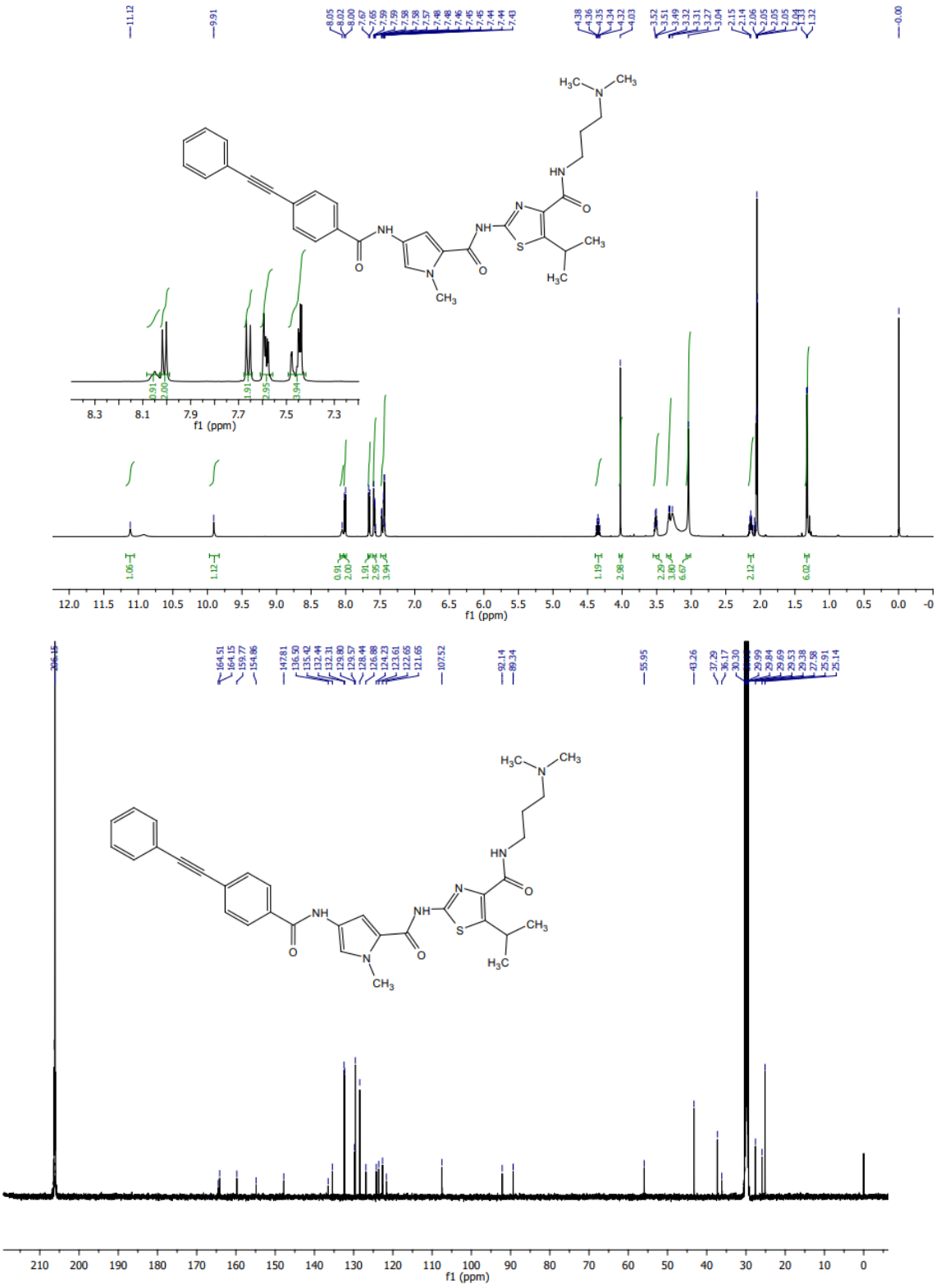
Fig. 4


% Cell Cytotoxicity
100
80
60
40
20
0
*
Amoeba alone
DMSO
MGB25
MGB27
MGB29
MGB31
MGB33
Pre-treated amoebae + HCMEC

List of Contents:

S1: Spectral characterization of MGB compounds

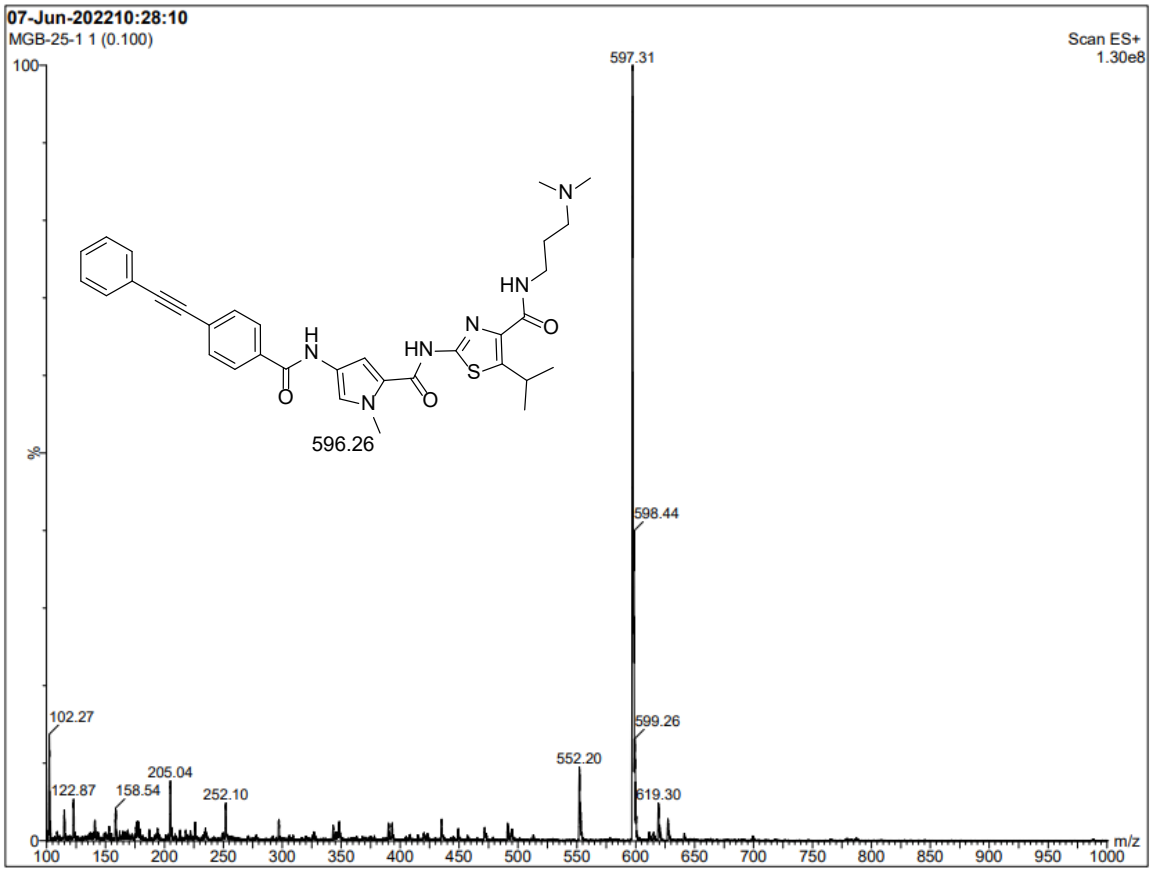
^{1}HNMR & APT (^{13}CNMR) Spectra



^{1}H NMR (500 MHz, Acetone-d_6) &^{13}C {^{1}H} APT NMR (126 MHz, Acetone-d_6) spectrum of **MGB25.**

S1: Spectral characterization of MGB compounds

1H NMR (500 MHz, Acetone-d6) δ 11.12 (s, 1H), 9.91 (s, 1H), 8.05 (s, 1H), 8.01 (d, J = 8.3 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H), 7.61 – 7.56 (m, 3H), 7.49 – 7.42 (m, 4H), 4.35 (sep, J = 6.9 Hz, 1H, (CH3)2CH-), 4.03 (s, 3H, -NCH3), 3.54-3.48 (m, 2H, -NCH2CH2CH2N-), 3.36 – 3.30 (m, 2H, -NCH2CH2CH2N-), 3.04 (s, 6H, -N(CH3)2), 2.17 – 2.10 (m, 2H, -NCH2CH2CH2N-), 1.32 (d, J = 6.9 Hz, 6H, (CH3)2CH-). 13C {1H} NMR (126 MHz, Acetone-d6) δ 164.5, 164.2, 159.7, 154.8, 147.8, 136.5, 135.4, 132.4, 132.3, 129.8, 129.6, 128.4, 126.8, 124.2, 123.6, 122.6, 121.6, 107.5, 92.1, 89.3, 55.9, 43.3, 37.3, 36.2, 27.6, 25.9, 25.1. ESI-MS: m/z calcd for C33H36N6O3S, 596.26 found 597.31 [M + H]+.



ESI-MS for MGB25: m/z calcd for $C_{33}H_{36}N_6O_3S$, 596.26 found 597.31 $[M + H]^+$.

S1: Spectral characterization of MGB compounds

```
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Sample Name: MGB 25

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                                                    Inj Volume : 10.000 µl
    Acq. Method     : C:\CHEM32\1\METHODS\FINAL MGB.M
    Last changed    : 10/4/2023 12:21:39 PM by hadeel
                      (modified after loading)
    Analysis Method : C:\CHEM32\1\METHODS\MGB3Y.M
    Last changed    : 10/4/2023 4:18:39 PM by hadeel
                      (modified after loading)
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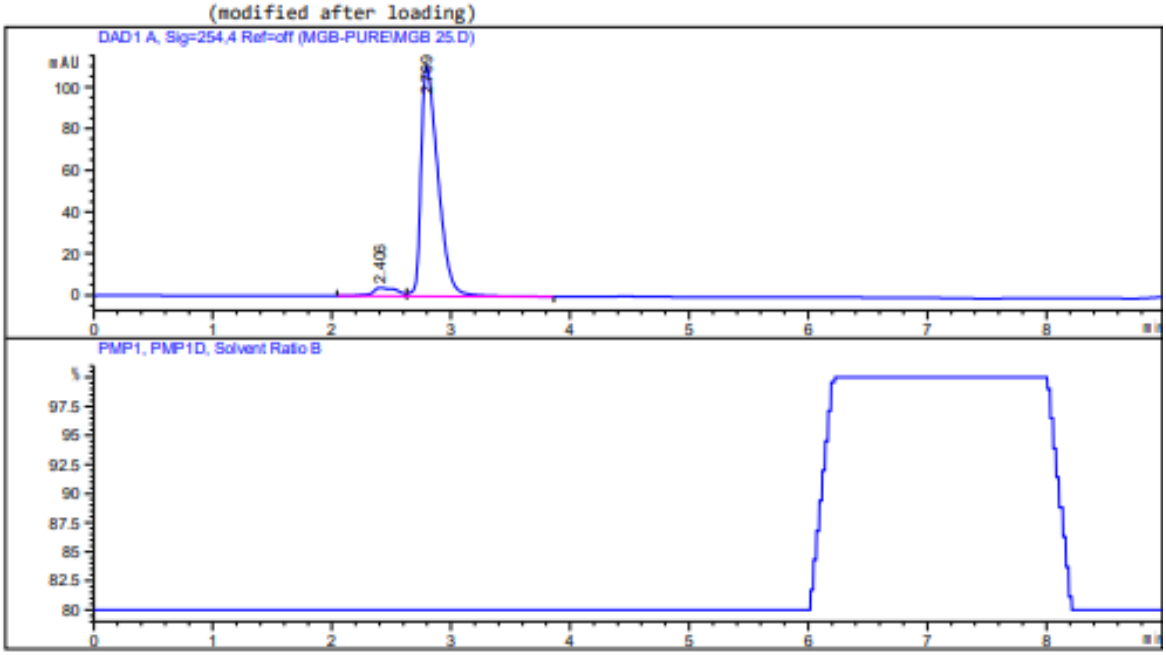


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    ======================================================================
                            Area Percent Report
    ======================================================================

    Sorted By             :      Signal
    Multiplier:                   :      1.0000
    Dilution:                     :      1.0000
    Use Multiplier & Dilution Factor with ISTDs


    Signal 1: DAD1 A, Sig=254,4 Ref=off

    Peak RetTime Type  Width     Area      Height     Area
      #   [min]        [min]   [mAU*s]     [mAU]       %
    ----|-------|----|-------|----------|----------|--------|
       1   2.406 BV    0.1882   57.20536    4.01581   4.9833
       2   2.799 VB    0.1530 1090.73889  110.61087  95.0167

    Totals :                  1147.94425  114.62668
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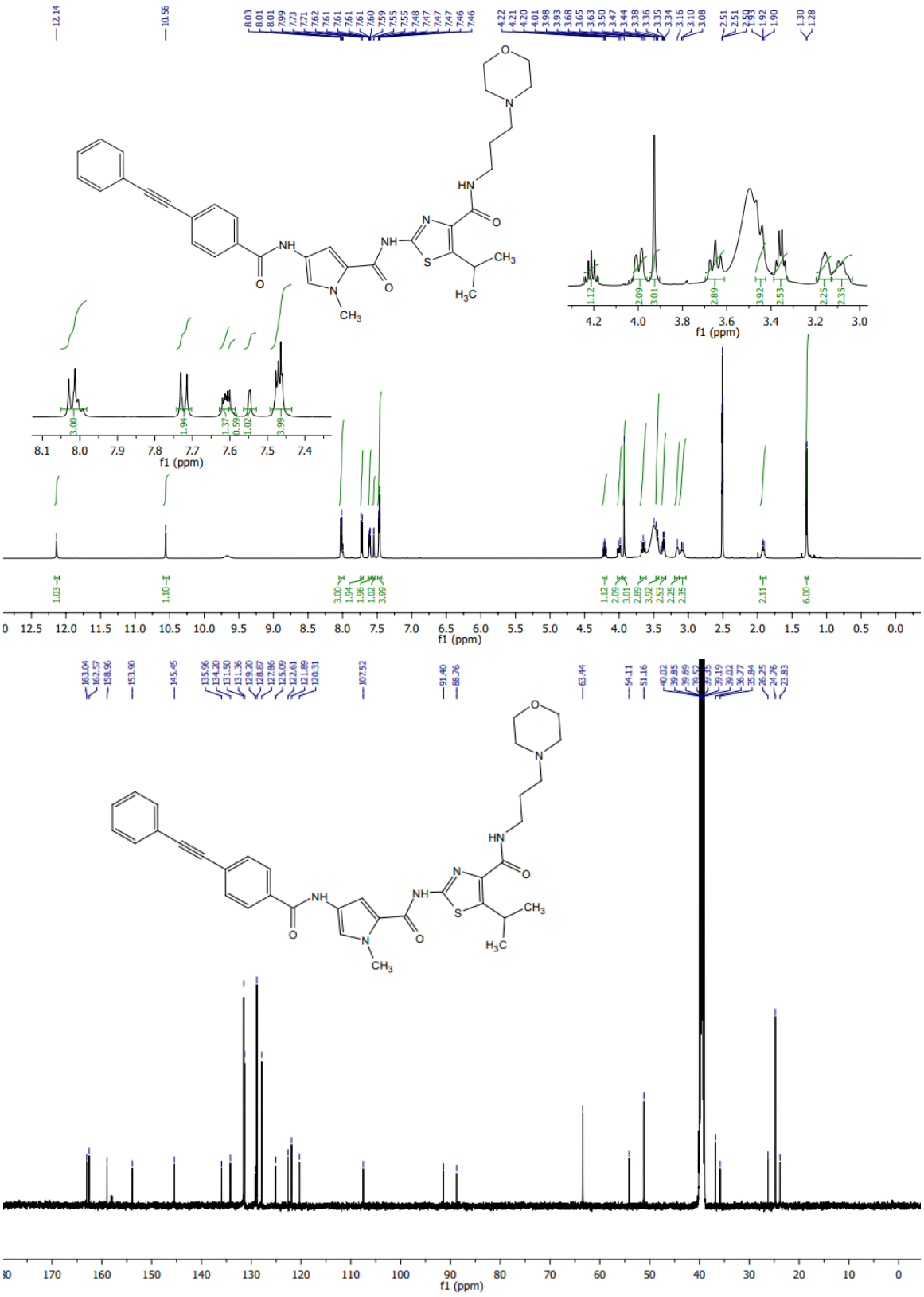
Purity determined by HPLC for MGB25

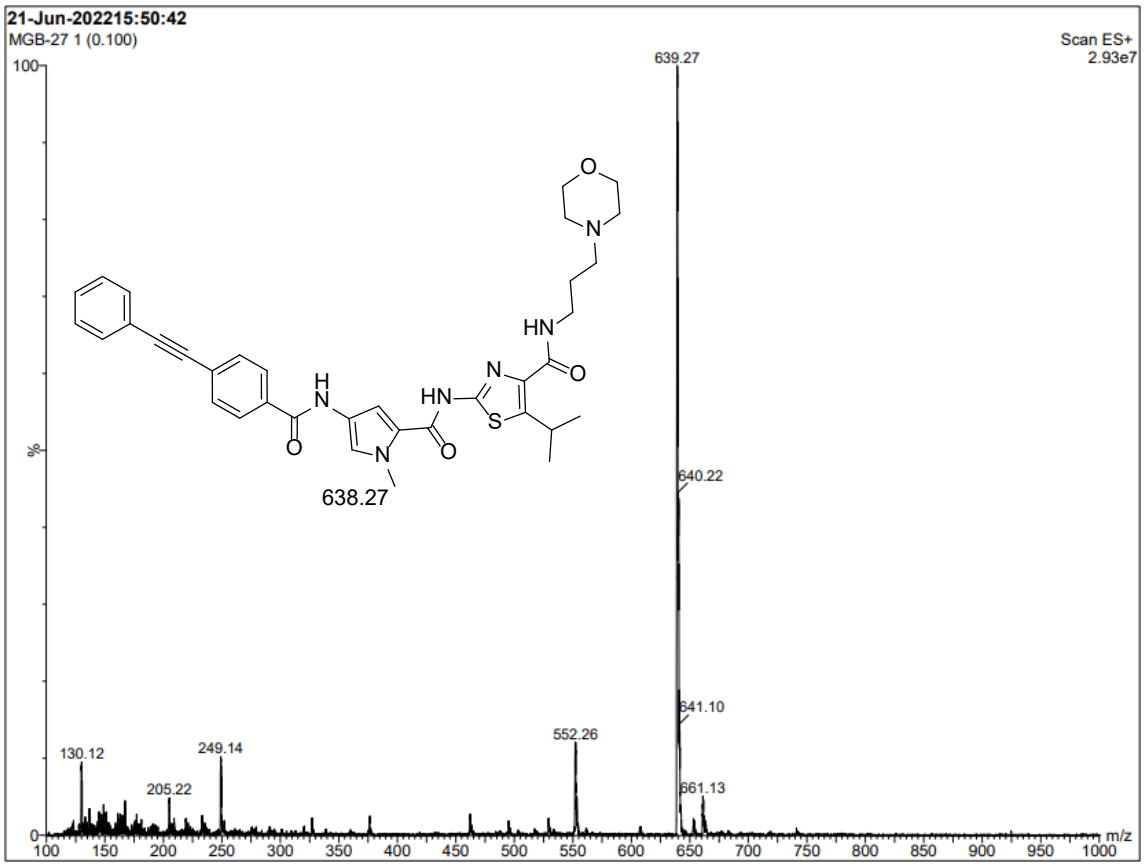
S1: Spectral characterization of MGB compounds



^{1}H NMR (500 MHz, DMSO-d_6) &^{13}C {^{1}H} APT NMR (126 MHz, DMSO-d_6) spectrum of **MGB27.**

S1: Spectral characterization of MGB compounds

MGB27:1H NMR (500 MHz, DMSO-d6) δ 12.14 (s, 1H), 10.56 (s, 1H), 8.05-7.98 (m, 3H), 7.72 (d, J = 8.4 Hz, 2H), 7.63 – 7.59 (m, 2H), 7.55 (d, J = 1.2 Hz, 1H), 7.50 – 7.44 (m, 4H), 4.20 (sep, J = 6.9 Hz, 1H, (CH3)2CH-), 4.03 – 3.96 (m, 2H, -NCH2CH2O), 3.93 (s, 3H, -NCH3), 3.65 (t, J = 12.1 Hz, 2H, -NCH2CH2O), 3.48 – 3.40 (m, 2H, -NCH2CH2CH2N-), 3.36 (dd, J = 12.9, 6.5 Hz, 2H, -NCH2CH2CH2N-), 3.19-3.13 (m, 2H, -NCH2CH2O), 3.12-3.03 (m, 2H, -NCH2CH2O), 1.96 – 1.88 (m, 2H, -NCH2CH2CH2N-), 1.29 (d, J = 6.9 Hz, 6H, (CH3)2CH-). 13C {1H} NMR (126 MHz, DMSO-d6) δ 163.0, 162.5, 158.9, 153.9, 145.4, 135.9, 134.2, 131.5, 131.36, 129.2, 128.8, 127.8, 125.1, 122.6, 121.8, 120.3, 107.5, 91.4, 88.7, 63.4, 54.1, 51.2, 36.7, 35.8, 26.3, 24.7, 23.8. ESI-MS: m/z calcd for C35H38N6O4S, 638.27 found 639.27 [M + H]+.



ESI-MS for **MGB27**: m/z calcd for $C_{35}H_{38}N_6O_4S$, 638.27 found 639.27 $[M + H]^+$.

S1: Spectral characterization of MGB compounds

```
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Sample Name: MGB 27

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Acq. Instrument : Instrument 1                    Location : Vial 1
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                                                  Inj Volume : 10.000 µl
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Analysis Method : C:\CHEM32\1\METHODS\MGB3Y.M
Last changed    : 10/4/2023 4:22:11 PM by hadeel
                  (modified after loading)
Additional Info : Peak(s) manually integrated
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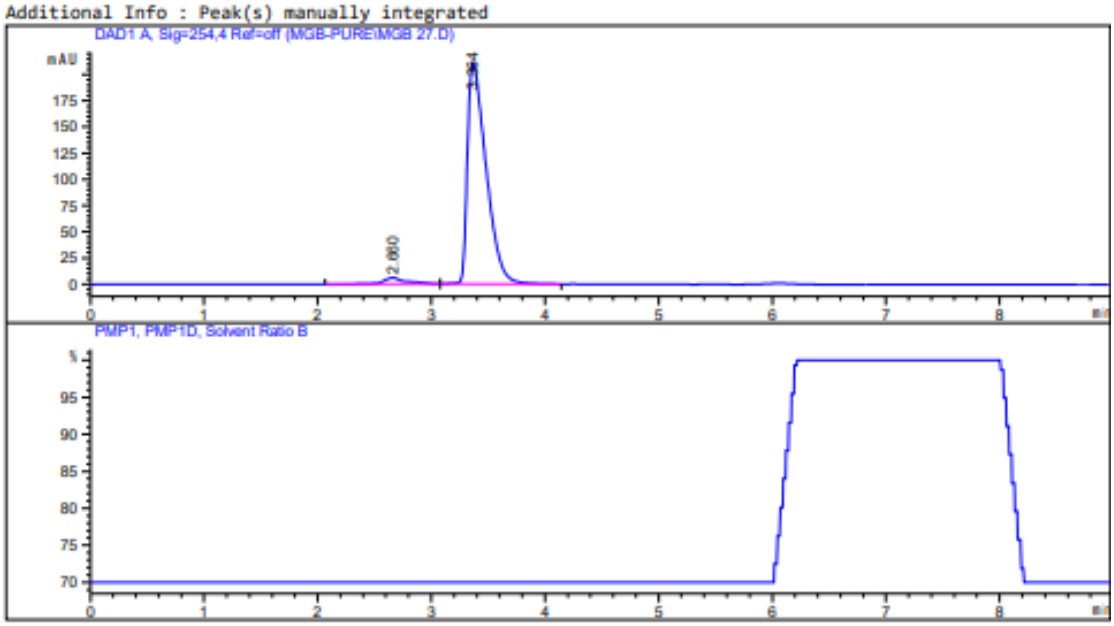


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======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.660 BB    0.2010   83.49950    5.97587   3.3685
   2   3.364 BB    0.1726 2395.30322  210.71802  96.6315

Totals :                  2478.80273  216.69389
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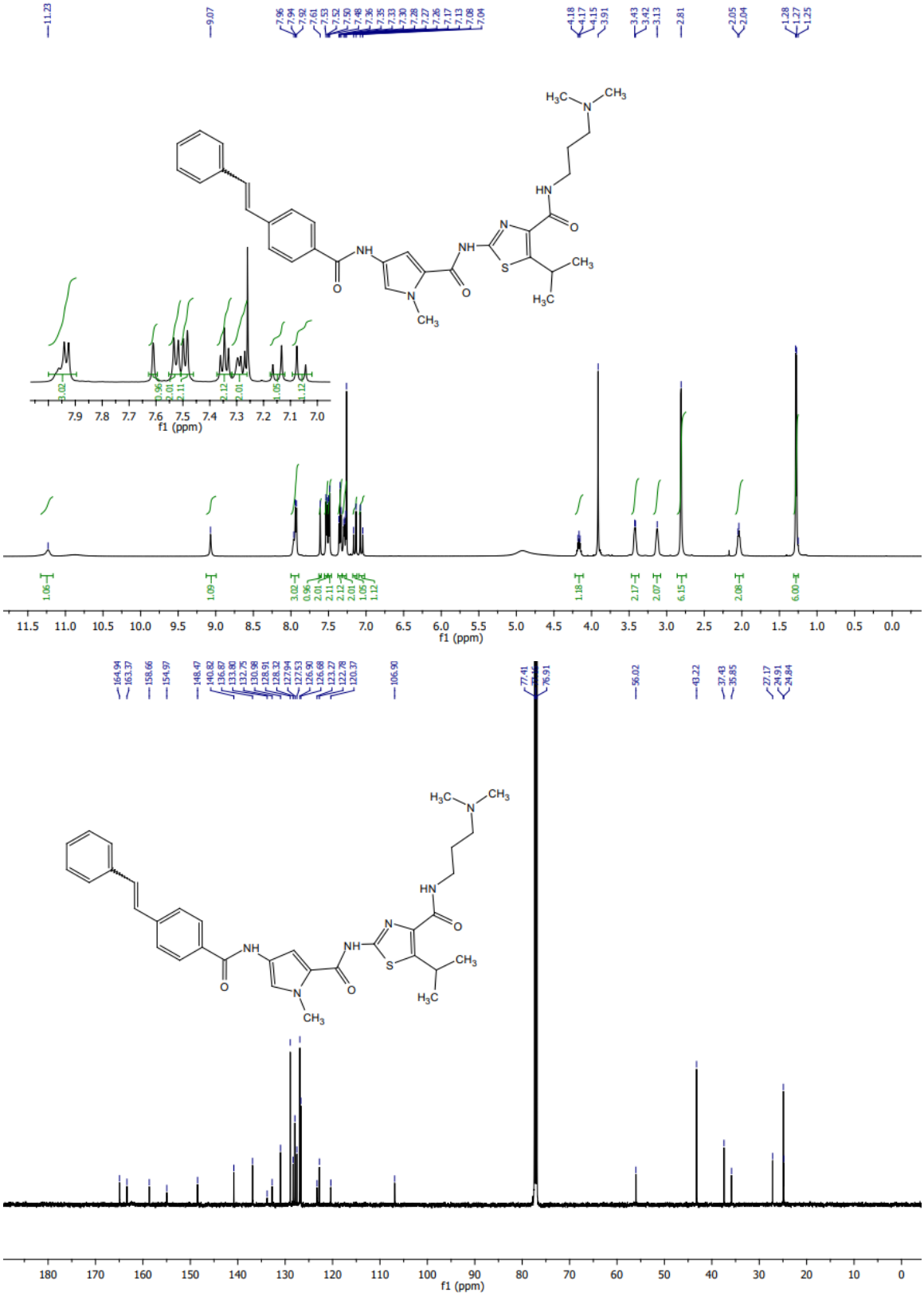
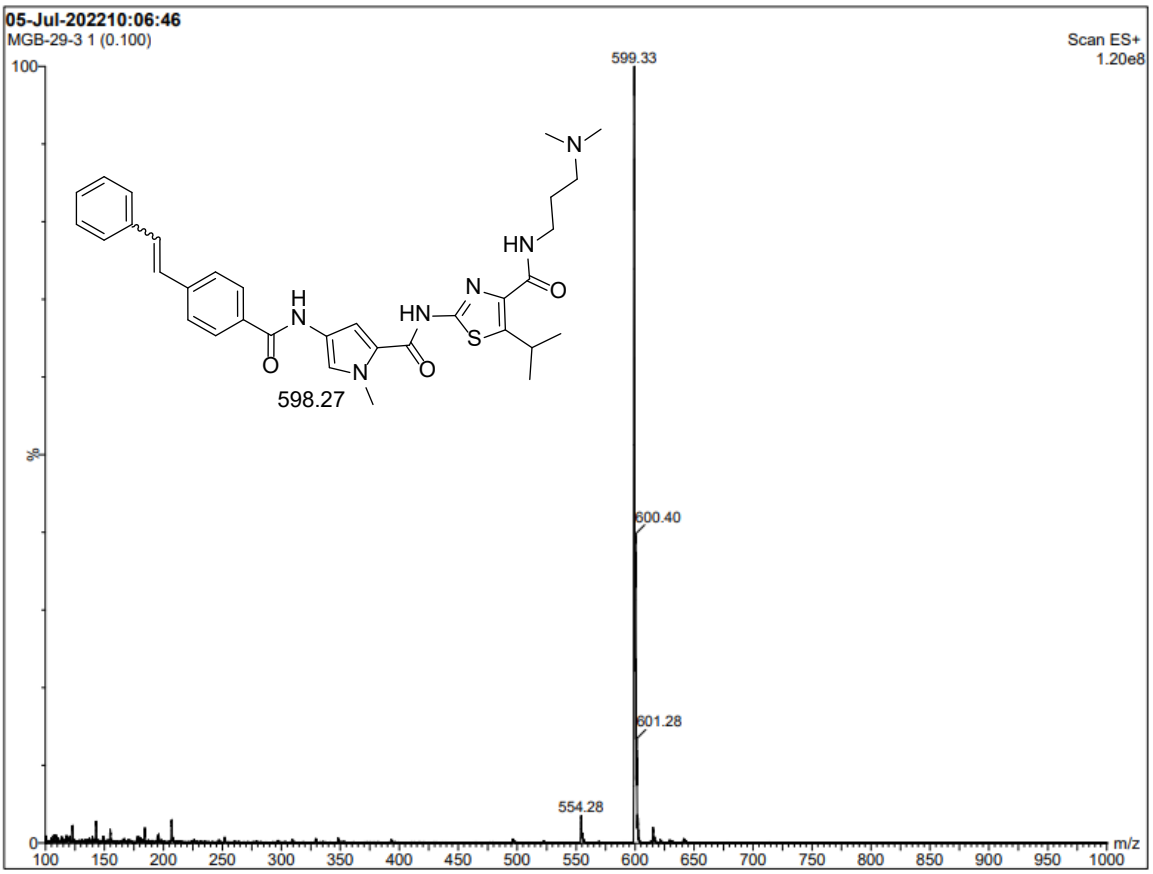
Purity determined by HPLC for MGB27

S1: Spectral characterization of MGB compounds



^{1}H NMR (500 MHz, $CDCl_3$) &^{13}C {^{1}H} APT NMR (126 MHz, $CDCl_3$) spectrum of **MGB29.**

MGB29: 1H NMR (500 MHz, CDCl3) δ 11.23 (s, 1H), 9.07 (s, 1H), 8.03 – 7.89 (m, 3H), 7.61 (s, 1H), 7.53 (d, J = 8.2 Hz, 2H), 7.49 (d, J = 7.4 Hz, 2H), 7.35 (t, J = 7.5 Hz, 2H), 7.32 – 7.26 (m, 2H), 7.15 (d, J = 16.3 Hz, 1H), 7.06 (d, J = 16.3 Hz, 1H), 4.17 (sep, J = 6.9 Hz, 1H, (CH3)2CH-), 3.91 (s, 3H, -NCH3), 3.46-3.36 (m, 2H, -NCH2CH2CH2N-), 3.17-3.08 (m, 2H, -NCH2CH2CH2N-), 2.81 (s, 6H, -N(CH3)2), 2.13-2.03 (m, 2H, -NCH2CH2CH2N-), 1.28 (d, J = 6.9 Hz, 6H, (CH3)2CH-). 13C {1H} NMR (126 MHz, CDCl3) δ 164.9, 163.4, 158.6, 154.9, 148.5, 140.8, 136.8, 133.8, 132.7, 130.9, 128.9, 128.3, 127.9, 127.5, 126.9, 126.6, 123.3, 122.7, 120.4, 106.9, 56.0, 43.2, 37.4, 35.8, 27.2, 24.9, 24.8. ESI-MS: m/z calcd for C33H38N6O3S, 598.27 found 599.33 [M + H]+.



ESI-MS for **MGB29**: m/z calcd for $C_{33}H_{38}N_6O_3S$, 598.27 found 599.33 $[M + H]^+$.

S1: Spectral characterization of MGB compounds

Data File C:\CHEM32\1\DATA\MGB-PURE\MGB 29.D
Sample Name: MGB 29

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Acq. Instrument : Instrument 1                    Location : Vial 1
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                                                  Inj Volume : 10.000 µl
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Analysis Method : C:\CHEM32\1\METHODS\MGB3Y.M
Last changed    : 10/4/2023 4:23:55 PM by hadeel
                  (modified after loading)
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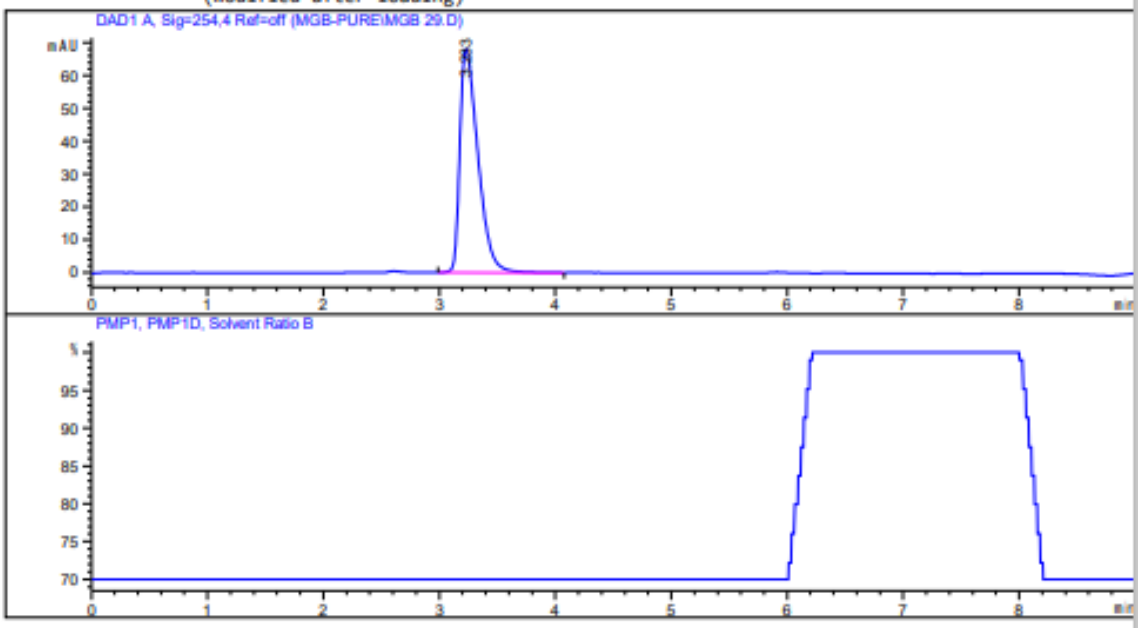


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                        Area Percent Report

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   3.233 BB    0.1666  739.72644   68.15727 100.0000

Totals :                   739.72644   68.15727
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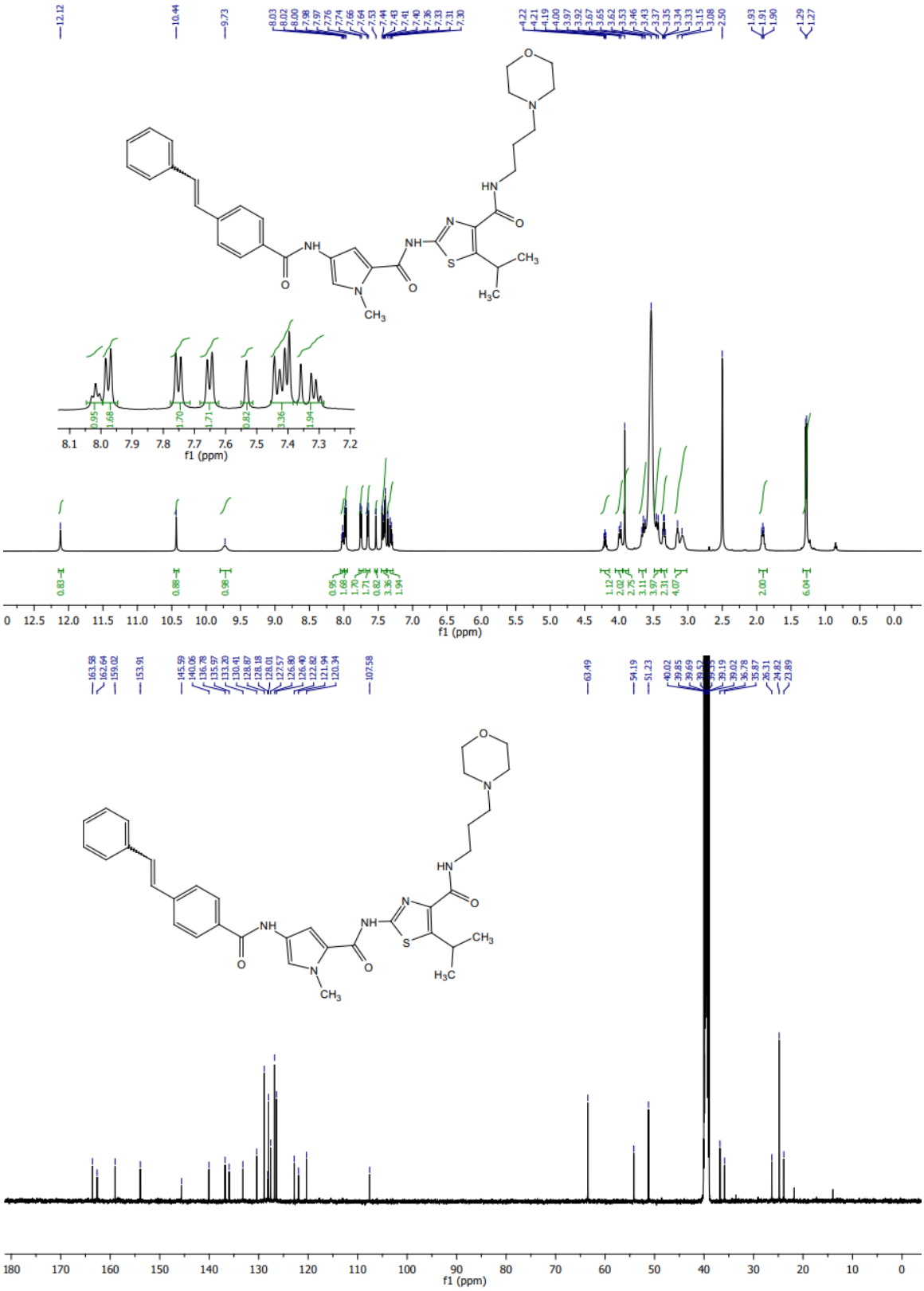
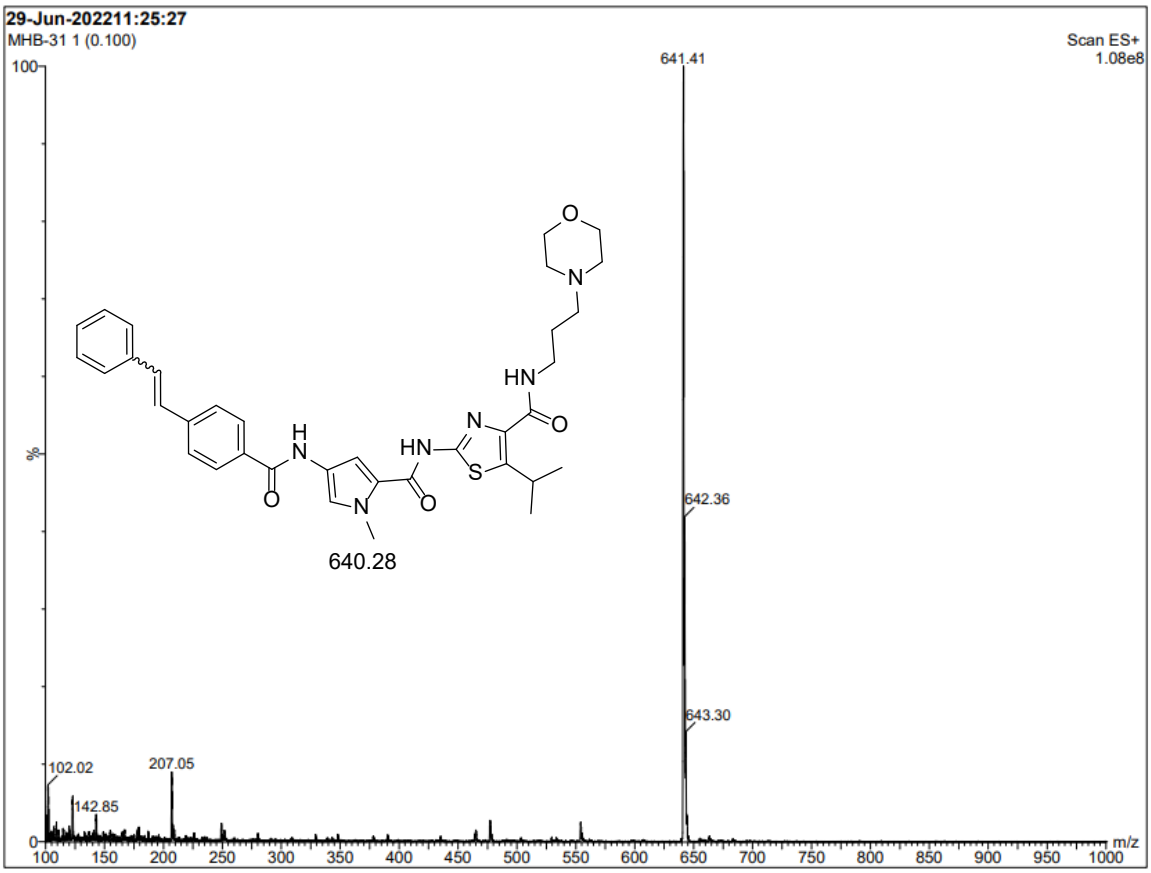
Purity determined by HPLC for MGB29



^{1}H NMR (500 MHz, DMSO-d_6) &^{13}C {^{1}H} APT NMR (126 MHz, DMSO-d_6) spectrum of **MGB31.**

MGB31: 1H NMR (500 MHz, DMSO-d6) δ 12.12 (s, 1H), 10.44 (s, 1H), 9.73 (s, 1H), 8.02 (t, J = 6.0 Hz, 1H), 7.98 (d, J = 8.0 Hz, 2H), 7.75 (d, J = 8.0 Hz, 2H), 7.65 (d, J = 7.7 Hz, 2H), 7.53 (s, 1H), 7.46-7.38 (m, 3H), 7.37-=7.28 (m, 2H), 4.21 (sep, J = 6.8 Hz, 1H, (CH3)2CH-), 3.99 (m, J = 11.8 Hz, 2H), 3.92 (s, 3H, -NCH3), 3.70-3.60 (m, 2H), 3.48-3.39 (m, 2H), 3.39-3.29 (m, 2H), 3.20-3.00 (m, 4H, NCH2CH2O), 1.97-1.85 (m, 2H, -NCH2CH2CH2N-), 1.28 (d, J = 6.8 Hz, 6H, (CH3)2CH-).13C {1H} NMR (126 MHz, DMSO-d6) δ 163.5, 162.6, 159.0, 153.9, 145.6, 140.0, 136.7, 135.9, 133.2, 130.4, 128.9, 128.2, 128.0, 127.5, 126.8, 126.4, 122.8, 121.9, 120.3, 107.5, 63.4, 54.1, 51.2, 36.7, 35.8, 26.3, 24.8, 23.9. ESI-MS: m/z calcd for C35H40N6O4S, 640.28 found 641.41 [M + H]+.



ESI-MS for **MGB31**: m/z calcd for $C_{35}H_{40}N_6O_4S$, 640.28 found 641.41 $[M + H]^+$.

S1: Spectral characterization of MGB compounds

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                                                     Inj Volume : 10.000 µl
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                       (modified after loading)
    Analysis Method : C:\CHEM32\1\METHODS\MGB3Y.M
    Last changed     : 10/4/2023 4:25:42 PM by hadeel
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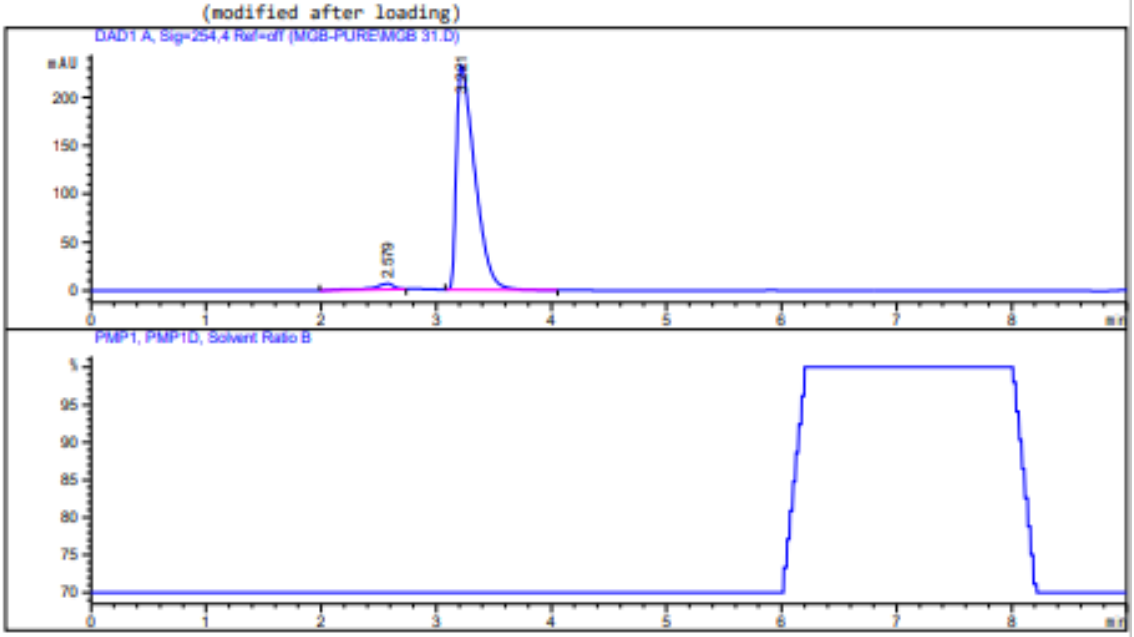


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                            Area Percent Report
    ======================================================================

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    Multiplier:                   :      1.0000
    Dilution:                     :      1.0000
    Use Multiplier & Dilution Factor with ISTDs

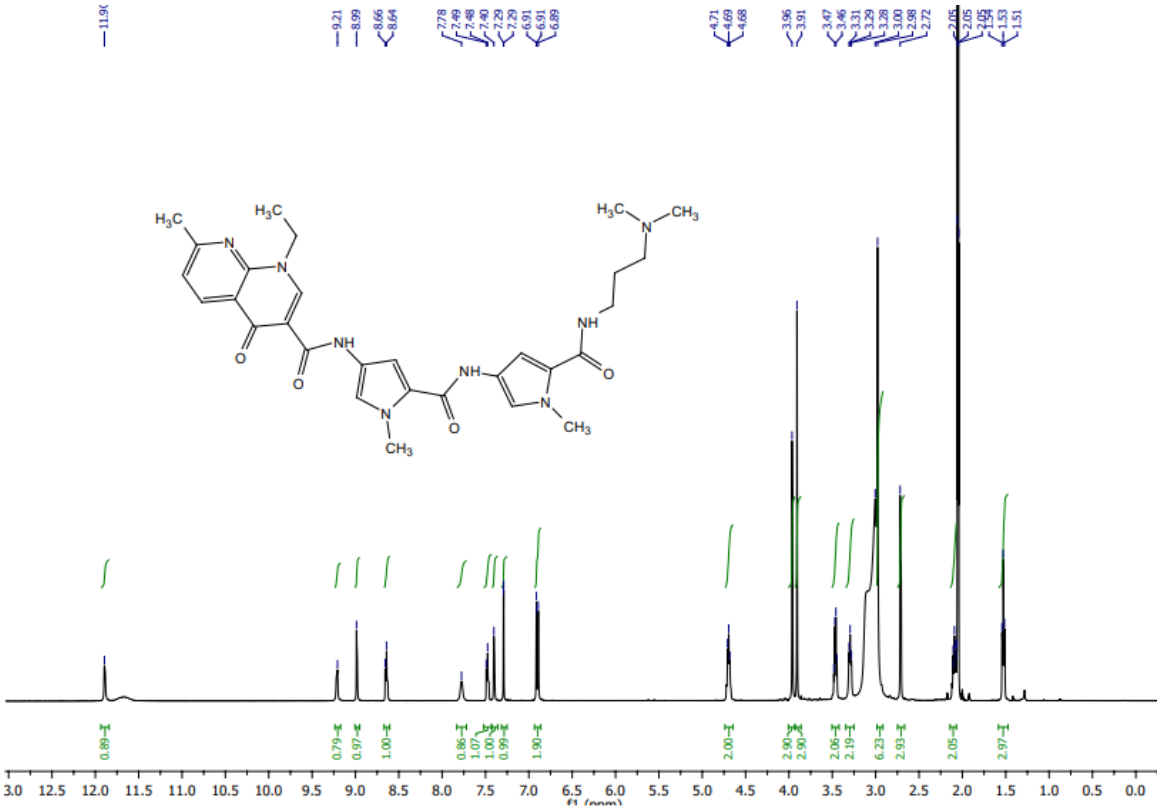

    Signal 1: DAD1 A, Sig=254,4 Ref=off

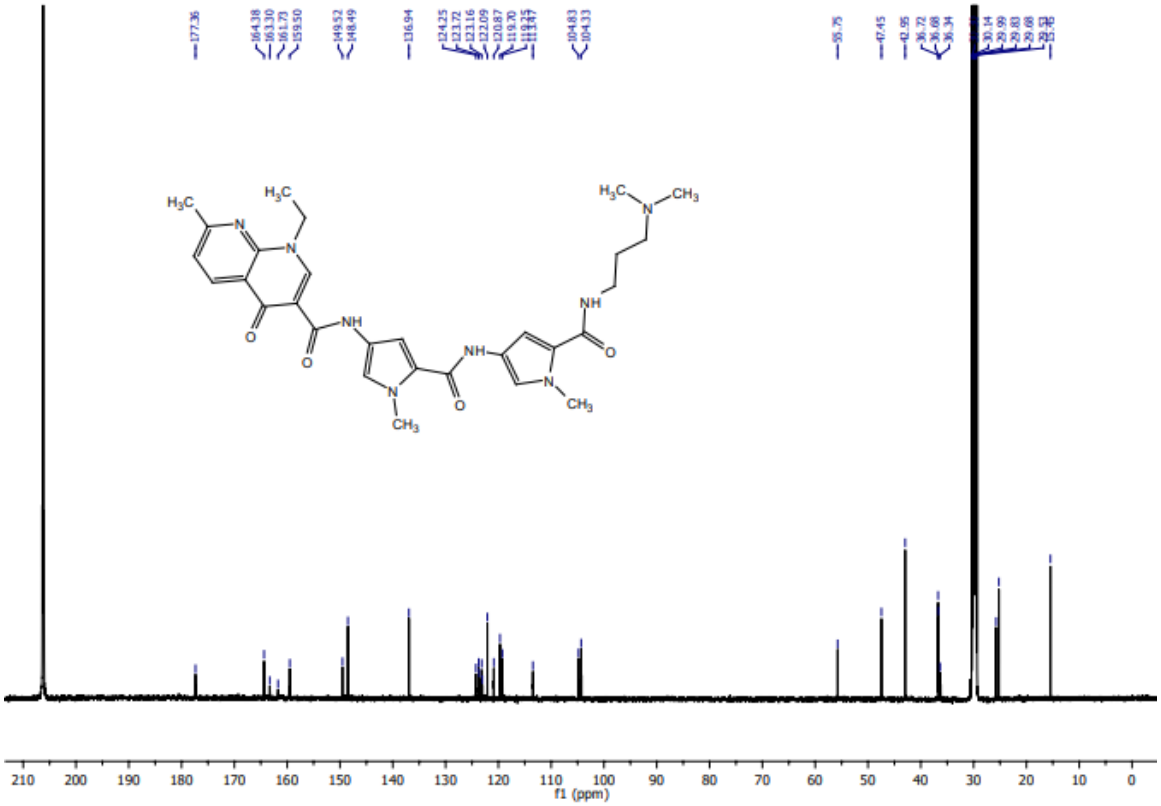
    Peak RetTime Type  Width     Area      Height     Area
      #   [min]        [min]   [mAU*s]     [mAU]       %
    ----|-------|----|-------|----------|----------|--------|
       1   2.579 BB    0.1714   69.02150    5.86149   2.6083
       2   3.221 BB    0.1697 2577.18237  231.87350  97.3917

    Totals :                  2646.20387  237.73499
```

Purity determined by HPLC for MGB31

S1: Spectral characterization of MGB compounds

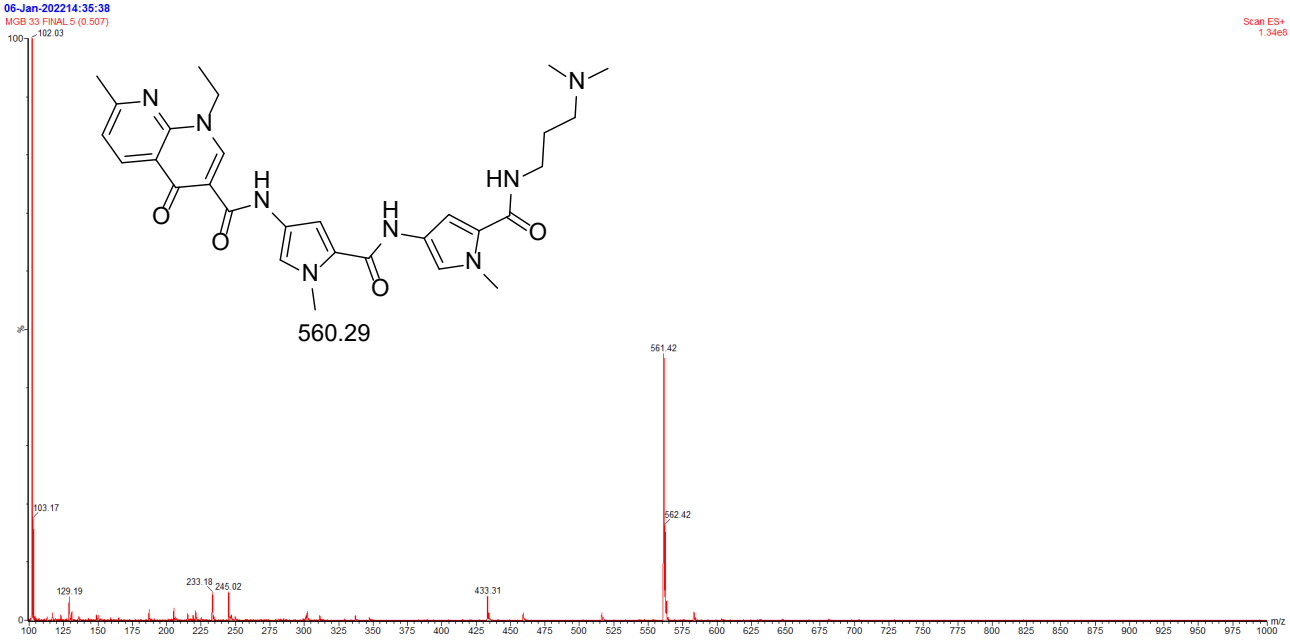




^{1}H NMR (500 MHz, Acetone-d_6) &^{13}C {^{1}H} APT NMR (126 MHz, Acetone-d_6) spectrum of **MGB33.**

S1: Spectral characterization of MGB compounds

MGB33: 1H NMR (500 MHz, Acetone-d6) δ 11.90 (s, 1H), 9.21 (d, J = 6.0 Hz, 1H), 8.99 (s, 1H), 8.67-8.62 (m, 1H), 7.78 (bs, 1H), 7.50-7.45 (m, 1H), 7.40 (bs, 1H), 7.29 (d, J = 1.5 Hz, 1H), 6.91 (s, 1H), 6.89 (s, 1H), 4.74-4.65 (m, 2H, -NCH2CH3), 3.96 (s, 3H, -NCH3), 3.91 (s, 3H, -NCH3), 3.50-3.40 (m, 2H, -NCH2CH2CH2N-), 3.34 – 3.25 (m, 2H, -NCH2CH2CH2N-), 2.98 (s, 6H, -NCH3CH3), 2.72 (s, 3H, -NCH3), 2.14-2.06 (m, 2H, -NCH2CH2CH2N-), 1.53 (t, J = 7.1 Hz, 3H, -NCH2CH3).13C {1H} NMR (126 MHz, Acetone-d6) δ 177.3, 164.4, 163.3, 161.7, 159.5, 149.5, 148.5, 136.9, 124.3, 123.7, 123.6, 123.2, 123.0, 122.1, 120.9, 119.7, 119.3, 113.5, 104.8, 104.3, 55.7, 47.4, 42.9, 36.7, 36.7, 36.3, 25.7, 25.2, 15.4. ESI-MS: m/z calcd for C29H36N8O4, 560.29 found 561.42 [M + H]+.



ESI-MS for **MGB33**: m/z calcd for $C_{29}H_{36}N_8O_4$, 560.29 found 561.42 $[M + H]^+$.

S1: Spectral characterization of MGB compounds

Data File C:\CHEM32\1\DATA\MGB-PURE\MGB 33.D
Sample Name: MGB 33

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======================================================================
Acq. Operator   : hadeel
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 10/4/2023 11:57:10 AM
                                                  Inj Volume : 10.000 µl
Acq. Method     : C:\CHEM32\1\METHODS\FINAL MGB.M
Last changed    : 10/4/2023 11:55:02 AM by hadeel
Analysis Method : C:\CHEM32\1\METHODS\MGB3Y.M
Last changed    : 10/4/2023 4:26:35 PM by hadeel
                  (modified after loading)
Additional Info : Peak(s) manually integrated
```

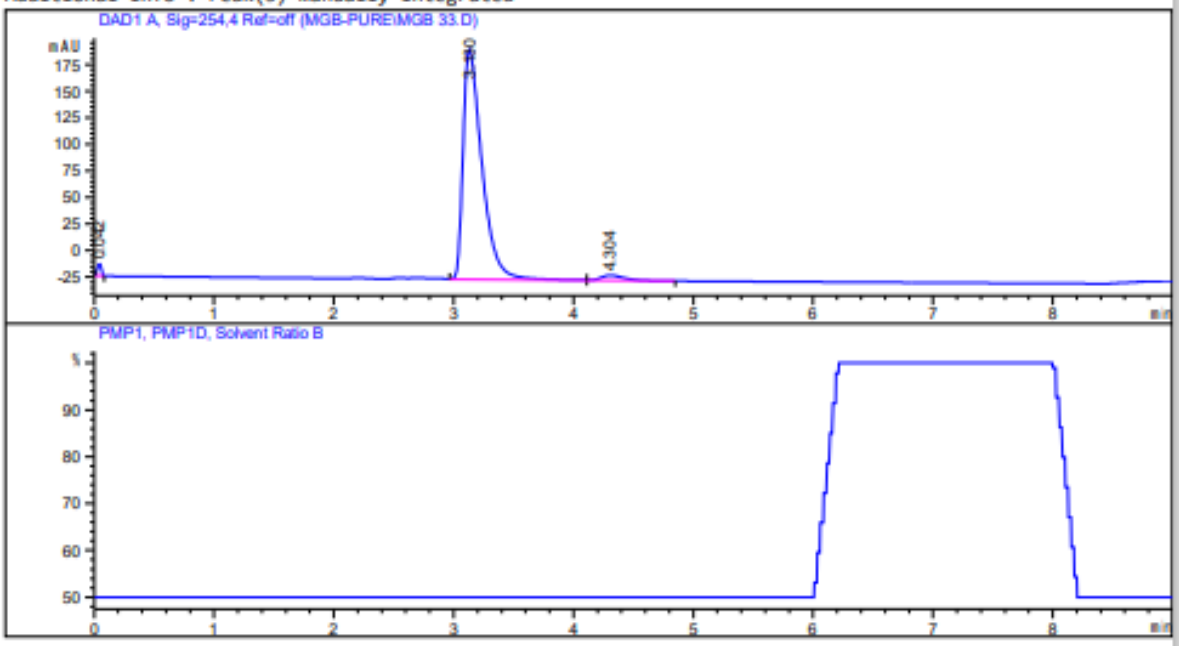


```
======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   0.042 BB    0.0400   26.28660   10.94290   1.0686
   2   3.130 VV    0.1658 2368.51001  216.20441  96.2817
   3   4.304 VB    0.1982   65.18322    4.93130   2.6497

Totals :                  2459.97983  232.07861
```

Purity determined by HPLC for MGB33