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α-Galactosyl Phytosphingosine 2,6'-Diamide as an Inducer of Invariant Natural Killer T Cell

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ABSTRACT

Four α-galactosyl phytosphingosine 2,6'-diamide analogs were prepared from 2,6'-diamino α-galactosylphytosphingosine and the aromatic-bearing carboxylic acids. After purification with High Performance Liquid Chromatography, a flowcytometry for the four compounds for stimulation of human Vα24+/Vβ11+ NKT cell populations was carried out. Additional keto groups on the acyl chains of the 2,6'-diamide compound were associated with the enhanced stimulating effect.

Keywords: Phytosphingosine; HPLC; Keto; Flow Cytometry; Simulation

1. Introduction

α-galactosyl ceramide (α-GalCer) [1,2] has been associated with the treatment of immunological disorders such as certain tumors, infections and autoimmune diseases (**Figure 1**) [3-6]. Its immune-stimulation potency is implicated in the activation of the invariant natural killer T cells (iNKT cells) to trigger the release of cytokines. The semi-invariant T cell receptor (TCR) which is encoded by an invariant Vα24-Jα18 chain in humans recognizes the complex formed from glycolipid antigen and MHC class I-like protein CD1d. After binding, iNKT cells could be induced, thereby leading to a rapid release of the relevant proinflammatory cytokines such as IFN-γ and IL-4 which are correlated with Th1 and Th2 pathway, respectively. Because of the opposing activities affected by the simultaneously secreted Th1 and Th2 cytokines that may counteract the therapeutic applications of α-GalCer, [7] endeavor has been continuously devoted to the development of potent α-GalCer analogues with a biased Th1 and Th2 profile [8-12].

We have recently reported a chemical preparation of 2,6'-diamino α-galactosyl phytosphingosine (α-GalSph) analogue that could be coupled with carboxylic acids to generate a library consisting of 40 members of 2,6'-diamide α-GalSph analogues. [13] Among them, the compound that exerted minimal cytotoxicity could induce a moderate proliferation of iNKT cells (**Figure 2**). Because the potential compound bears two aromatic groups, a further study based on this structural feature is therefore pursued. The present work is aimed to prepare the four diamide compounds **1-4** with structural variation on the linker or the aromatic ring (**Figure 3**). The bioactivities of the four compounds were also addressed to correlate with their structures (SAR).

2. Results and Discussion

Preparation of 6-amino α-galactosyl phytosphingosine **5** could be found in the relevant work as described before. [13] Conjugation of 6-amino α-galactosyl phytosphingosine **5** with four carboxylic acids furnished the diamide products **1-4** with a fair yield (27% - 46%). Whereas a number of syntheses of ceramide analogs have been reported, the concern about purity has been rarely addressed. [14] In our case, the four products were still insufficiently pure after flash chromatography. Further purification with High Performance Liquid Chromatography

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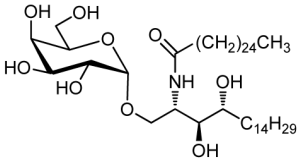

Figure 1. Structure of α-GalCer.

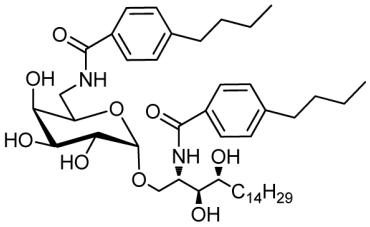

Figure 2. 2,6'-di-4-butylphenyl α-GalSph amide discovered from parallel solution phase synthesis exhibited moderate iNKT cell proliferation.

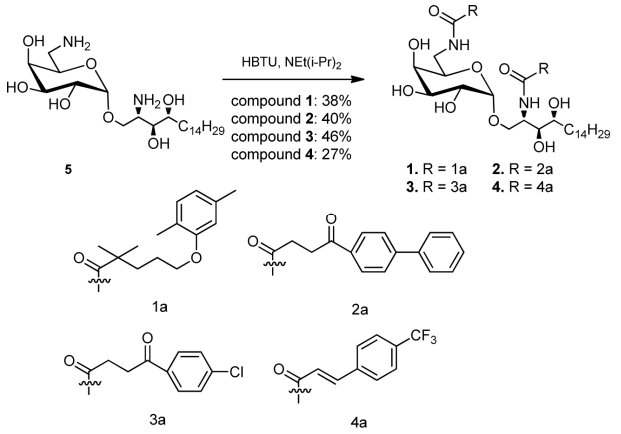

Figure 3. Structures of the four diamide compounds 1-4.

(HPLC) was needed. Above all, compound **4** after HPLC purification was additionally purified by recrystallization. Whereas the spectra for 1H- and 13C-NMR of the four compounds were satisfactory, some unknown peaks related to compound **4** were marked. For example, two peak clusters were found in ^{19}F-NMR, *i.e.* cluster 1:δ = −210.26 and −210.28 ppm and cluster 2:δ = −220.17 and −221.67 ppm. The peak cluster 1 was unlikely due to the residual carboxylic acids because both the HPLC chromatograms of the crude and purified compounds showed solely one peak. Furthermore, the underestimated integrals from 7.6 to 8.0 ppm in ^{1}H-NMR that corresponded to aromatic protons precluded the presence of an unreacted aromatic-containing carboxylic acid. Hence, the presence of a conformer was speculated to account for the present observation.

Compared to compound **1** and **4**, which exhibited limited activity, compound **2** and compound **3** both induced meaningful cellular populations (**Figure 4**). This could be rationalized by the contribution of the keto group. In contrast, other structural alterations did not significantly affect the bioactivity. For example, the bioactivity of compound **2** was tolerated by the introduction of the biphenyl group. Furthermore, the presence of the germinal dimethyl groups or a double bond did not enhance the activity. The role played by the keto group was therefore elucidated by molecular docking (**Figure 5**). The binding site for docking was defined by employing the crystallo0 graphic data for a complex of α-GalCer with Vα24^{+} chain of iNKT cells (PDB:4EN3). [15] Interestingly, all compounds (**1-4**) failed to well dock to the site. Only when adopting a site defined by a complex of β-GalCer (PDB code: 3SDX) [16] and using a truncated compound 3, in which the aryl group on the amide chain of the sugar moiety was removed deliberately while the rest groups including the keto group and the other amide residue on backbone were retained, an acceptable docking result arose with a score of 65 points. The two hydroxy groups of the sugar moiety *i.e.* 2-OH and 3-OH could act as hydrogen bond acceptors with Arg94. In addition, 3-OH could act as hydrogen bond donor with ASP93. The 6-amido group of the sugar moiety did not form intermolecular hydrogen bond with the neighboring amino acids but form intramolecular hydrogen bonding with 4-OH. In spite of these stabilizations gained by hydrogen bondings between the sugar moiety and the surrounding amino acid residues, an inherent difference in stereochemistry between α-GalPhy and β-GalCer could not be overlooked. In brief, the present α-GalSph analogs **2** and **3** with an additional keto group on the amide linkage of sugar moiety and backbone warrants a further study.

3. Experimental

3.1. General

All reagents and solvents were purchased from Sigma-Aldrich, Malingkrodt, Acros, Alfa, Tedia, or Fluka. All preparations of compounds were routinely conducted in dried glassware under a positive pressure of nitrogen at room temperature unless otherwise noted. CH_2Cl_2 was dried over CaH_2. DMF and NEt_3 were distilled under reduced pressure prior use. Reagents and solvents were of reagent grade. The eluents for chromatography including MeOH and $CHCl_3$ were reagent grade and used without further purification. NMR spectroscopy including ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz, DEPT-135) was measured on Varian UnityInova 500 MHz. Low-resolution mass spectrometry (LRMS) was performed on an ESI-MS spectrometry employing VARIAN 901-MS Liquid Chromatography Tandem Mass Q-Tof Spectrometer at the Department of Chemistry of National Tsing-Hua University (NTHU). High-Resolution Mass Spectrometry (HRMS) was performed using a

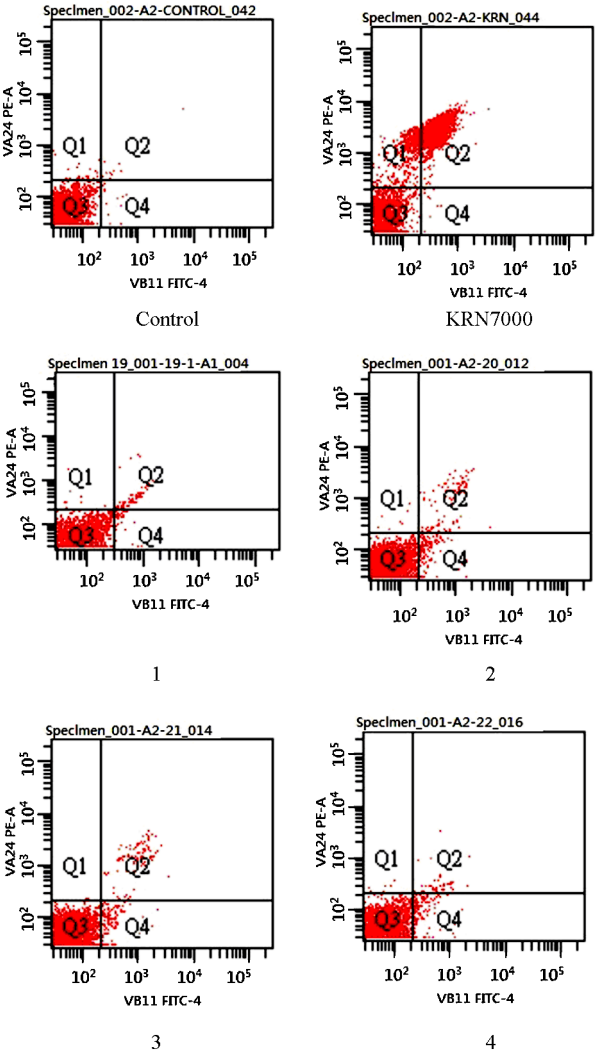

Figure 4. Potencies of analogs 1-4 for stimulation of human Vα24$^+$/Vβ11$^+$ NKT cell populations. Peripheral blood mononuclear cells (PBMC) from a normal healthy donor were incubated with each individual compound at a final concentration of 100 nM. After 14 days of culture, NKT cell frequencies were determined by flow cytometry. NKT cell frequencies were defined as the percentage of Vα24$^+$/Vβ11$^+$ NKT cells among gated lymphocytes in the upper right (UR) corner (Q2) for each case. Shown here are the profiles of PBMC harvested from 14-day cultures containing (control) vehicle alone (DMSO, UN) and the compounds 1-4 tested.

varian HPLC (prostar series ESI/APCI) coupled mass detector of Varian 901-MS (FT-ICR Mass) and triple quadrapole. Flash chromatography was performed using Geduran Si 60 silica gel (230 - 400 mesh). The final conjugation products were further purified and analyzed by HPLC, consisting of an Angilent 1100 pump and a linear UVIS detector (254 nm). A ZORBAX SILcolumn (250 mm × 9.4 mm, 5 μm, Si-100) was used as the stationary phase and the eluents of a combination of MeOH and CH_2Cl_2 with a flow rate of 3 mL/min were used as

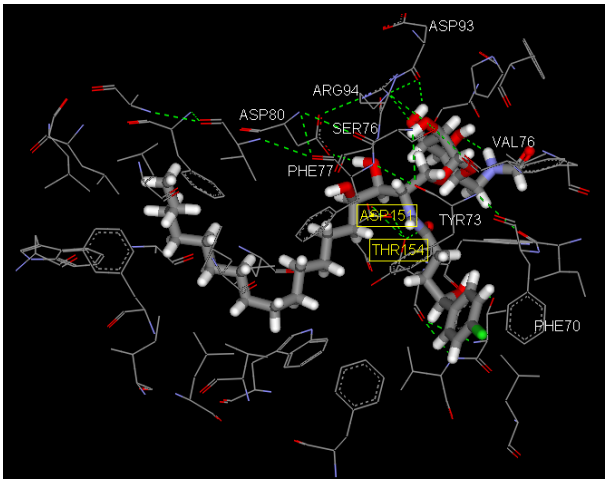

Figure 5. Molecular docking of truncated compound 3 onto the complex of CD1d and Vα24$^+$ chain. Software: Discovery Studio 2.1 with Ligandfit. Active site was defined by using Auto-Vα24-CD1d-β-GalCer complex (PDB entry of 3SDX).

the mobile phase.

3.2. General Procedure for Preparation of the Four Amide Products

5-(2,5-dimethylphenoxy)-N-(((2R,3R,4S,5R,6S)-6-(((2S,3S,4R)-2-(5-(2,5-dimethylphenoxy)-2,2-dimethylpentanamido)-3,4-dihydroxyoctadecyl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)methyl)-2,2-dimethylpentanamide (**1**).

The conjugating procedure adopted the protocol as reported before [9].

To a mixture of Gemfibrozil (4 eq., 42 mg, 0.167 mmol), HBTU (4.0 equiv, 64 mg, 0.167 mmol) and DMF (1 mL) was added diisopropylethylamine (8.0 equiv, 60 μL, 0.334 mmol) under N_2. After stirring for 30 min, TLC (EtOAc:*n*-hexane = 2.5:7.5) indicated the formation of the ester intermediate (R_f = 0.73) and consumption of the starting Gemfibrozil (R_f = 0.12). To this mixture was added the solution of compound **5** ((1 equiv, 20 mg, 41.8 μmol) in DMF (1 mL). After stirring for 24 h, TLC (MeOH/CH_2Cl_2 = 1:8) indicated the formation of the product **1** (R_f = 0.80) and consumption of the active ester (R_f = 0.90). The mixture was concentrated under reduced pressure. The residue obtained was purified with HPLC using column chromatography (MeOH:CH_2Cl_2 = 1:10 → MeOH:CH_2Cl_2 = 1:9) to afford the crude product **1** in 38% yield (15 mg). The sample was further purified using MeOH/CH_2Cl_2 = 1:9 as eluent at a flow rate of 3 mL/min to afford an oil (7 mg) which was recorded as retention time at t_R = 5.79 min.

Anal. $C_{54}H_{90}N_2O_{11}$, M (calcd.) = 942.6 (*m/z*), ESI + Q-TOF:M = 798.6 (*m/z*), $[M + H]^+$ = 943.9 (100%), $[M + Na]^+$ = 965.9 (50%); HRMS (ESI) calcd. $[M + H]^+$ = 943.66229 (*m/z*), found $[M + H]^+$ = 943.66552 (*m/z*), Δ =

3.42 ppm; ^{1}H-NMR (500 MHz, CD_3OD):δ 0.88 (t, 3H, $H_{aliphatic}$), 1.18 - 1.20 (m, 12H, $H_{aliphatic}$), 1.25 - 1.39 (m, 24H, $H_{aliphatic}$), 1.54 - 1.72 (m, 10H, $H_{aliphatic}$), 2.12 (s, 6H, 2 × OPh<u>Me</u>), 2.26 (s, 6H, 2 × OPh<u>Me</u>), 3.24 - 3.29 (m, 1H), 3.50 - 3.57 (m, 2H), 3.60 - 3.71 (m, 3H), 3.74 - 3.84 (m, 4H), 3.88 - 3.89 (m, 4H, PhO-<u>CH_2</u>), 4.18 - 4.22 (m, 1H), 4.88 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 6.59 (d, 2H, J = 7.5 Hz, ArH), 6.64 (s, 2H, ArH), 6.93 (d, 2H, J = 7.5 Hz, ArH); ^{13}C-NMR (125 MHz, CD_3OD):δ 14.44 (CH_3, $C_{aliphatic}$), 16.08 (CH_3), 16.12 (CH_3), 21.50 (CH_3), 23.74 (CH_2), 25.68 (CH_3), 25.86 (CH_3), 25.95 (CH_3), 26.02 (CH_3), 26.28 (CH_2), 26.34 (CH_2), 27.04 (CH_2), 30.48 (CH_2), 30.77 (CH_2), 30.81 (CH_2), 30.85 (CH_2), 30.93 (CH_2), 33.08 (CH_2), 38.64 (CH_2), 41.39 (CH_2), 43.02 (C), 43.10 (C), 51.97 (CH), 68.09 (CH_2), 69.13 (CH_2), 69.23 (CH_2), 70.03 (CH), 70.43 (CH), 71.25 (CH), 71.37 (CH), 72.93 (CH), 75.55 (CH), 101.29 (CH, C-1), 113.09 (CH, aromatic), 121.78 (CH, aromatic), 121.83 (CH, aromatic), 124.42 (C, aromatic), 131.20 (CH, aromatic), 131.23 (CH, aromatic), 137.61 (C, aromatic), 158.33 (C, aromatic), 179.65 (C, amide), 180.91 (C, amide).

4-([1,1'-biphenyl]-4-yl)-N-(((2R,3R,4S,5R,6S)-6-(((2S,3S,4R)-2-(4-([1,1'-biphenyl]-4-yl)-4-oxobutanamido)-3,4-dihydroxyoctadecyl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)methyl)-4-oxobutanamide (**2**).

The same procedure as that for amide coupling of compound **1** was used. Fenbufen (42 mg, 0.167 mmol) was used as the carboxylic acid for the conjugation. The required reagents were the same as that described above. Column chromatography was performed by using eluents of MeOH:CH_2Cl_2 = 1:11 to provide the white crude product **2** in 40% yield (16 mg). After further purification with HPLC using eluents of MeOH:CH_2Cl_2 = 1:11, a white solid was obtained in an overall yield of 15% (6 mg). t_R = 9.04 min. Anal. $C_{56}H_{74}N_2O_{11}$, HRMS (ESI) M (calcd.) = 950.5293 (*m/z*), $[M + H]^+$ = 951.5371 m/z, $[M - H]^-$ = 949.5214 m/z; found $[M + H]^+$ = 951.5289 (m/z), Δ = 8.62 ppm; $[M - H]^-$ = 949.5216 (m/z), Δ = 0.21 ppm; ^{1}H-NMR (600 MHz, CD_3OD):δ 0.79 (t, 3H, J = 7.2 Hz, $H_{aliphatic}$), 1.15 - 1.34 (m, 24 H, $H_{aliphatic}$), 1.44 - 1.46 (m, 1H, $H_{aliphatic}$), 1.57 - 1.60 (m, 1H, $H_{aliphatic}$), 2.54 - 2.62 (m, 4H $_{aliphatic}$), 3.18 - 3.36 (m, 6H), 3.50 - 3.65 (m, 4H), 3.72-3.78 (m, 3H), 3.85 (dd, 1H, $J_{2,3}$ = 10.5, $J_{2,1}$ = 3.5 Hz, H-2), 4.15 - 4.16 (m, 1H), 4.82 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 7.38 - 7.97 (m, 18H, Ph_{amide}); ^{13}C-NMR (150 MHz, CD_3OD):δ 20.50 (C), 29.17 (CH_2), 32.35 (CH_2), 35.86 (CH_2), 36.16 (CH_2), 36.22 (CH_2), 36.26 (CH_2), 36.31 (CH_2), 38.42 (CH_2), 39.27 (CH_2), 40.26 (CH_2), 40.32 (CH_2), 46.15 (CH_2), 55.81 (CH_2), 73.55 (CH_2), 75.55 (CH), 76.44 (CH), 78.64 (CH), 81.04 (CH), 105.94 (CH, C-1), 133.72 (CH, aromatic), 134.82 (CH, aromatic), 135.25 (CH, aromatic), 135.48 (CH, aromatic), 141.61 (C, aromatic), 146.17 (C, aromatic), 146.22 (C, aromatic), 152.59 (C, aromatic), 152.67 (C, aromatic), 179.20 (C, CO), 180.63 (C, CO), 205.60 (C, CO), 206.17 (C, CO)

4-(4-chlorophenyl)-N-(((2R,3R,4S,5R,6S)-6-(((2S,3S,4R)-2-(4-(4-chlorophenyl)-4-oxobutanamido)-3,4-dihydroxyoctadecyl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)methyl)-4-oxobutanamide (**3**).

3-(4-Chlorobenzoyl)propionic acid (53 mg, 0.251 mmol) was used as the carboxylic acid for the conjugation. Column chromatography was performed by using eluents of MeOH:CH_2Cl_2 = 1:9 to provide the white crude product **3** in 46% yield (25 mg). After further purification with HPLC using eluents of MeOH:CH_2Cl_2 = 1:11, a white solid was obtained in an overall yield of 28% (15 mg). t_R = 10.94 min. Anal. $C_{44}H_{64}Cl_2N_2O_{11}$, M (calcd.) = 866.4 (*m/z*); found: ESI + Q-TOF:$[M + Na]^+$ = 889.5 (47.1%); HRMS (ESI) M (calcd.) = 866.38872 (*m/z*), $[M + H]^+$ = 867.39654 amu; found $[M + H]^+$ = 867.39501, Δ = 1.76 ppm; ^{1}H-NMR (600 MHz, CD_3OD): δ 0.89 (t, 3H, J = 7.2 Hz, $H_{aliphatic}$), 1.23-1.38 (m, 24H, $H_{aliphatic}$), 1.51 (m, 1H, $H_{aliphatic}$), 1.60 - 1.62 (m, 1H, $H_{aliphatic}$), 2.58 - 2.64 (m, 4H $_{aliphatic}$), 3.26 - 3.29 (m, 2H), 3.30 - 3.34 (m,2H), 3.47 - 3.50 (m, 1H), 3.58 - 3.63 (m, 2H), 3.68 - 3.70 (m, 1H), 3.77 - 3.80 (m, 3H), 3.84 - 3.90 (m, 2H), 4.21 - 4.22 (m, 1H), 7.46 - 7.48 (m, 4H, arom., 7.96 - 7.98 (m, 4H, arom.); ^{13}C-NMR (150 MHz, CD_3OD):δ14.45 (CH_3), 23.75 (CH_2), 27.00 (CH_2), 30.49 (CH_2), 30.72 (CH_2), 30.78 (CH_2), 30.83 (CH_2), 30.87 (CH_2), 30.90 (CH_2), 33.08 (CH_2), 34.82 (CH_2), 41.33 (CH_2), 51.94 (CH), 68.03 (CH_2), 70.21 (CH), 70.55 (CH), 71.28 (CH), 71.30 (CH), 72.98 (CH), 75.49 (CH), 101.01 (CH, C-1), 129.95 (CH, arom.), 129.96 (CH, arom.), 130.84 (CH, arom.), 136.56 (C, arom.), 136.58 (C, arom.), 140.49 (C, arom.), 140.53 (C, arom.), 174.42 (C, CO), 175.32 (C, CO), 199.39 (C, CO), 199.52 (C, CO).

(*E*)-*N*-((2S,3S,4R)-3,4-dihydroxy-1-(((2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(((*E*)-3-(4-(trifluoromethyl)phenyl)acrylamido)methyl)tetrahydro-2H-pyran-2-yl)oxy)octadecan-2-yl)-3-(4-(trifluoromethyl)phenyl)acrylamide (**4**).

trans-4-(Trifluoromethyl) cinnamic acid (54 mg, 0.251 mmol) was used as the carboxylic acid for the conjugation. Column chromatography was performed using eluents of MeOH:CH_2Cl_2 = 1:10 → MeOH:$CHCl_2$ = 1:8 to provide the white crude product **4** in 27% yield (15 mg). After additional purification with HPLC using eluents of MeOH:CH_2Cl_2 = 1:7, a white solid was obtained in 22% yield (12 mg). The sample was further purified by using recrystallization from MeOH to provide the white solid in an overall yield of 13% (7 mg). t_R = 8.49 min. Anal. $C_{44}H_{60}F_6N_2O_9$, M (calcd.) = 874.4 (*m/z*); found:ESI + Q-TOF:$[M + H]^+$ = 875.4, $[M + Na]^+$ = 897.4; ^{1}H-NMR (500 MHz, CD_3OD):δ 0.88 (t, 3H, J = 7.0Hz, $H_{aliphatic}$), 1.20 - 1.42 (m, 24H, $H_{aliphatic}$), 1.45 - 1.65 (m, 2H, $H_{aliphatic}$), 3.35 - 3.40 (m, 1H), 3.44 - 3.49 (m, 1H), 3.52 - 3.56 (m, 1H), 3.61 - 3.77 (m, 3H), 3.79 - 3.82 (m, 1H),

3.85 (m, 1H), 3.92 - 3.98 (m, 2H), 4.34 - 4.37 (m, 1H), 4.89 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 6.72 (d, 1H, J = 16.0 Hz), 6.76 (d, 1H, J = 16.0 Hz), 7.45 (d, 1H, J = 16.0 Hz), 7.48 (d, 1H, J = 16.0 Hz), 7.63 - 7.66 (m, 8H, $H_{amide\text{-}aromatic}$); ^{13}C-NMR (125 MHz, CD_3OD):δ 14.44 (CH_3, $C_{aliphatic}$), 23.74 (CH_2), 26.79 (CH_2), 30.47 (CH_2), 30.71 (CH_2), 30.75 (CH_2), 30.78 (CH_2), 30.85 (CH_2), 33.07(CH_2), 33.20 (CH_2), 41.75 (CH_2), 52.56 (CH), 68.13 (CH_2), 70.50 (CH), 71.02 (CH), 71.26 (CH), 71.57 (CH), 72.83 (CH), 75.40 (CH), 100.93 (CH, C-1), 124.50 (CH), 124.74 (CH), 126.77 (CH), 129.35 (CH), 140.02 (CH), 140.08 (CH), 167.41 (C, CO_{amide}), 168.26 (C, CO_{amide}). ^{19}F-NMR (470 MHz, CD_3OD) δ −210.26, −210.28, −220.17, −221.67.

Conformers were speculated to assign for δ −220.17 and −221.67.

4. Acknowledgements

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