ANGEWANDTE CHEMIE
A Journal of the Gesellschaft Deutscher Chemiker

Supporting Information

for

Angew. Chem. Int. Ed. Z19315

69451 Weinheim, Germany

Facile Assembly of Cell Surface Oligosaccharide Mimics *via* Copolymerization of Carbohydrate Modules**

Kenji Sasaki, Yoshihiro Nishida,* Tomoaki Tsurumi, Hirotaka Uzawa, Hirosato Kondo, Kazukiyo Kobayashi*

[*] Dr. Y. Nishida, Prof. Dr. K. Kobayashi, K. Sasaki, T. Tsurumi
Department of Molecular Design and Engineering
Graduate School of Engineering, Nagoya University,
Chikusa-ku, Nagoya 464-8603 (Japan)
FAX: (+81) 52-789-2528
E-mail: nishida@mol.nagoya-u.ac.jp
kobayash@mol.nagoya-u.ac.jp

Dr. H. Uzawa,
National Institute of Advanced Industrial Science and Technology (AIST)

Dr. H. Kondo,
Research & Development Division, Nippon Organon K. K.

Experimentals

General information

All solvent was distilled and/or stored over 3 Å or 4 Å molecular sieves as appropriate. All other chemicals for the syntheses of target compounds were purchased from Kishida Chemical Co., Ltd. in Osaka, Wako Pure Chemical Industries Co., Ltd., and Tokyo Kasei Kogyo in Tokyo. Tin-layer chromatography (TLC) was performed on silica gel 60-F_{254} (Merck) detectable under UV lamp or 5% H_2SO_4, anisaldehyde in ethanol. Silica gel column chromatography on silica gel 60 (Merck 70~200mesh) or silica gel 60 RP-18 was accomplished with the solvent system (v/v) specified.

^{1}H NMR spectra were measured at 500 MHz with Varian INOVA-500, or at 300 MHz INOVA-300 at ambient temperature. Chemical shifts were reported in parts per milion. Coupling constants were reported in herz (Hz). Tetramethylsilane (TMS) and residual solvent peaks were used as internal references. Optical rotations were determined with a JASCO DIP-1000 digital polarimeter using a water-jacketed 100 mm cell at 25 °C. Size exclusion chromatography (SEC) was conducted with a JASCO 800 high-performance liquid chromatograph on Shodex B804 + B805 columns for copolymers using phosphate- buffered saline (pH =7.4) as the eluent, and a standard of molecular weight was used pullulan. Mass spectra analysis was measured on JEOL-JMS AX505HA MASS SPECTROMETER.

Allyl-2-*O*-TBDPS-3,4-*O*-isopropylidene-α-L-fucopyranoside (13).
tert-Butyldiphenylsilylchloride (2.1 mL, 8.21 mmol) was added to a DMF (20 mL) solution of Allyl 3,4-*O*-isopropylidene-α-L-fucopyranoside **12** (1.34 g 5.47 mmol). After stirring at room temperature for overnight, the mixture was diluted with $CHCl_3$, and the solution was washed with 1N HCl aq, brine, H_2O and dried over $MgSO_4$. After removal of solvent, the residue was purified by silica gel column chromatography (hexane : AcOEt = 2 : 1) to give **13** (2.32 g 82 %) as a colorless crystal. mp 55-57 °C; $[\alpha]_D^{24}$ = -93.3 (*c* = 0.5, in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32-7.76 (m, 10 H), 5.87-5.95 (m, 1 H), 5.28-5.32 (m, 1 H), 5.16-5.18 (m, 1 H), 4.33 (dd, 1 H, *J* = 7.5, 5.5 Hz, H-3), 4.28 (d, 1H, *J* = 3.5 Hz), 4.08 (dq, 1H, *J* = 2.5, 6.5 Hz, H-5), 4.03-4.05 (m, 1H), 4.01 (dd, 1H, *J* = 3.0, 5.5 Hz), 3.75 (dd, 1H, *J* = 3.5, 7.5 Hz), 3.71-3.75 (m, 1H), 1.32 (s, 1H), 1.26 (s, 1H), 1.24 (d, 1H, J= 6.5 Hz, H-6), 1.05 (s, 9H); FAB+: calcd for $C_{28}H_{37}O_5Si$ [M–H] 480.3, found 481.0

(*R*,*S*)-Glycidyl-2-*O*-TBDPS-3, 4-*O*-isopropylidene-α-L-fucopyranoside (14).
A CH_2Cl_2 (50 mL) solution of **13** (2.30 g 4.76 mmol) was cooled at 0 °C. mCPBA (4.11 g, 23.82 mmol) was added and the mixture stirred at room temperature for 17h. The excess mCPBA was quenched with saturated aqueous $Na_2S_2O_3$ and $NaHCO_3$-sol. The organic layer was washed with $NaHCO_3$-sol, brine H_2O and dried over $MgSO_4$. After removal of solvent, the residue was purified by silica gel column chromatography (hexane : AcOEt = 5 : 1) to give **14** (1.87 g, 89 %) as a colorless syrup. Mixture of diastereomers in a 76 : 24 ratio. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.75 (m, 10H), 4.33 (dd, 1H, *J* = 5.5, 7.5 Hz), 4.30 (dd, 1H, *J* = 5.5, 7.5 Hz), 4.26 (d, 1H, *J* = 3.5 Hz), 4.22 (d, 1H, *J* = 3.5 Hz), 4.12 (dq, 1H, *J* = 2.5, 6.5 Hz), 4.02 (dd, 1H, *J* = 2.5, 6.0 Hz), 3.75 (dd, 1H, *J* = 3.5, 7.3 Hz), 3.66 (dd, 1H, *J* = 3.5, 11.7 Hz), 3.43 (dd, 1H, J = 3.5, 11.7 Hz), 3.39 (dd. 1H, *J* = 6.0, 11.6 Hz), 3.29 (dd. 1H, *J* = 6.0, 11.6 Hz), 3.15 (m,1H), 3.12 (m, 1H), 2.80 (dd, 1H, *J* = 4.0, 5.0 Hz), 2.64 (dd, 1H, *J* = 2.5, 14.2 Hz), 2.55 (dd, 1H, *J* = 2.5, 14.2 Hz), 1.32 (s, 1H), 1.27 (s, 1H), 1.23 (d, 1H, *J* = 6.5 Hz), 1.09 (s, 9H); FAB+: calcd for $C_{28}H_{37}O_6Si$ [M–H] 497.2, found 497.0

1-*p*-Nitrophenyl-2-(*R*)-3-*O*-(2-*O*-TBDPS-3,4-*O*-isopropylidene-α-L-fucopyranosyl)glycerol (15).
Sodium *p*-nitrophenoxide (6.0 g, 37.3 mmol) was added to a DMF solution (30 mL) of **14** (1.86 g 3.73 mmol). After stirring at 80 °C for 13h under N_2 atmosphere, the mixture was diluted with $CHCl_3$, washed with 1N NaOH aq, brine, H_2O and dried over $MgSO_4$. After removal of solvent, the residue was purified by silica gel column chromatography (hexane : AcOEt = 5 : 1 to 2 : 1) to give a compound as a colorless oil. The residue was crystallized with EtOH/hexane to afford **15** as yellow crystal (1.18 g, 50 %). Mixture of diastereomers in a 90 : 10 ratio. mp 110-112 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20 (m, 2H), 7.20-7.75 (m, 10H), 6.92 (m, 2H), 4.34 (d, 1H, *J* = 3.5 Hz), 4.33 (d, 1H, *J* = 3.5 Hz), 4.30 (t, 1H, *J* = 6.5, 6.3 Hz), 4.29 (t, 1H, *J* = 6.5, 6.3 Hz), 4.17 (dq, 1H, *J* = 2.5, 6.5Hz), 4.10 (m, 1H), 4.00-4.08 (m, 3H), 3.87 (dd, 1H, *J* = 3.5, 6.5Hz), 3.85 (dd, 1H, *J* = 3.5, 6.5 Hz), 3.75 (dd, 1H, *J* = 4.0, 10.2 Hz), 3.69 (dd, 1H, *J* = 6.0, 10.5 Hz), 3.46 (dd, 1H, *J* = 4.5, 10,2 Hz), 3.40 (dd, 1H, *J* = 6.0, 10.5 Hz), 1.30 (s, 1H), 1.28 (s, 1H), 1.26 (d, 1H, *J* = 6.5 Hz), 1.25(d, 1H, *J* = 6.5), 1.06 (s, 9H); FAB+: calcd for $C_{34}H_{43}NO_9SiNa$ [M + Na] 660.3, found 660.1

1-*p*-Nitrophenyl-2-(*R*)-*O*-β-D-galactopyranosyl-3-*O*-(2-*O*-TBDPS-3,4-*O*-isopropylidene-β-L-fucopyranosyl)glycerol (17).

A CH_2Cl_2 solution of **15** (158 mg, 0.248 mmol), galactosyl imidate donor (244 mg, 0.496 mmol) and molecular sieves 4 Å was stirred at –40 °C under N_2 atmosphere for 1h. A solution of TMSOTf (30 μL, 0.149 mmol) was added to the mixture. After stirring at –40 °C to room temperature for 1h, the mixture was diluted with $CHCl_3$, filtered through a celitepad, washed with $NaHCO_3$-sol, brine H_2O and dried over $MgSO_4$. After removal solvent, the residue was purified by silica gel column chromatography (hexane : AcOEt = 2 : 1) to give crude oil of **16**. It is dissolved in MeOH (5 mL), and added catalytic amount of $NaOCH_3$. After stirring for 2h, the mixture was neutralized with Amberlist 15$^+$ (H^+ form), filtered and concentrated in vacuo. The residue was purified by silica column chromatography ($CHCl_3$: MeOH = 10 : 1) to afford **17** as a syrup (74 mg, 40 %). ^{1}H NMR (500 MHz, CD_3OD): δ8.20 (m, 2H), 7.70 (m, 4H), 7.40 (m, 10H), 7.11 (m, 2H), 4.48 (d, 1H *J* = 7.5 Hz), 4.42 (dd, 1H, *J* = 10.0, 5.0 Hz), 4.38 (d, 1H, *J* = 7.5 Hz), 4.33 (dd, 1H, *J* = 15.0, 4.0 Hz), 4.28(dd, 1H, *J* = 10.0, 5.0 Hz), 4.26 (d, 1H, *J* = 3.5 Hz), 4.24 (dd, 1H, *J* = 7.5, 5.5 Hz), 4.16 (dq, 1H, *J* = 2.5, 6.5 Hz), 4.03 (dd, 1H, *J* = 2.5, 5.5 Hz), 3.97 (dd, 1H, *J* = 2.5, 5.5 Hz), 3.88 (dd, 1H, *J* = 5.0, 5.5 Hz), 3.84 (dd, 1H, *J* = 1.0, 3.5 Hz), 3.73 (m, 2H), 3.65 (dd, 1H, *J* = 3.5, 9.0 Hz), 3.55-3.50 (m, 3H), 3.47 (dd, 1H, *J* = 3.0, 5,5 Hz), 1.24 (s, 1H), 1.26 (d, 1H, *J* = 6.5 Hz 1.12 (s, 1H) 1.06 (s, 9H)

1-*p*-Nitrophenyl-2-(*R*)-*O*-β-D-galactopyranosyl-3-*O*-(3, 4-*O*-isopropylidene-α-L-fucopyranosyl)glycerol (18).

A TBAF (600 μL, 0.6 mmol) in THF solution was added to a THF solution of **17** (100 mg, 0.125 mmol). After stirring for 24h, the mixture was concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$: MeOH = 10 : 1) to give **18** (63 mg, 90 %) as a syrup. ^{1}H NMR (500 MHz, CD_3OD) δ8.20 (m, 2H), 7.11 (m, 2H), 4.80 (d, 1H, *J* = 3.5 Hz), 4.48 (d, 1H *J* = 7.5 Hz), 4.36-4.27(m, 3H), 4.16 (dq, 1H, *J* = 2.5, 6.5 Hz), 4.10 (dd, 1H, *J* = 7.5, 5.5 Hz), 4.02 (dd, 1H, *J* = 2.5, 5.5 Hz), 4.00 (dd, 1H, *J* = 5.0, 5.5 Hz), 3.81 (dd, 1H, *J* = 1.0, 3.5 Hz), 3.73 (m, 2H), 3.62 (dd, 1H, *J* = 3.5, 9.0 Hz), 3.55-3.51(m, 3H), 3.47 (dd, 1H, *J* = 3.0, 5,5 Hz), 1.46 (s, 1H), 1.24 (d, 1H, *J* = 6.5 Hz), 1.12 (s, 1H)

1-*p*-Nitrophenyl-2-(*R*)-*O*-(3-*O*-sulfo-β-D-galactopyranosyl)-3-*O*-(3,4-*O*-isopropylidene-α-L-fucopyranosyl)glycerol triethylammmonium salt (19).

To a solution of **18** (56 mg, 0.1 mmol) in benzene (70 mL) was added dibutyltinoxide (27 mg, 0.11 mmol), and the mixture was refluxed. After 5 h, the mixture was concentrated and added *N*, *N*-dimethylformamide (2 mL), triethylamine (50 μL), and trimethylamine-sulfonic acid complex (15 mg, 0.11 mmol). After stirring at 40 °C for 2 h, the mixture was quenched by methanol (2 mL) and stirred at 40 °C for 2 h. The mixture was concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$: MeOH = 4 : 1) to afforded sulfated product **19** as a triethylammonium salt form (50 mg 76 %). ^{1}H NMR (500 MHz, CD_3OD) δ8.20 (m, 2H), 7.11 (m, 2H), 4.80 (d, 1H, *J* = 3.5 Hz), 4.61 (d, 1H *J* = 7.5 Hz), 4.35 (m, 3H), 4.22(m, 2H), 4.16 (dq, 1H, *J* = 2.5, 6.5 Hz), 4.10 (dd, 1H, *J* = 7.5, 5.5 Hz), 4.02 (dd, 1H,

J = 2.5, 5.5 Hz), 4.00 (dd, 1H, *J* = 5.0, 5.5 Hz), 3.75-3.68 (m, 4H), 3.62 (dd, 1H, *J* = 3.5, 9.0 Hz), 3.58 (m, 1H), 1.46 (s, 1H), 1.30 (s, 1H), 1.24 (d, 1H, *J* = 6.5 Hz)

1-*p*-Nitrophenyl-2-(*R*)-*O*-(3-*O*-sulfo-β-D-galactopyranosyl)-3-*O*-α-L-fucopyranosylglycerol triethylammonium salt (4a).

The compound of **19** (50 mg) was dissolved in H_2O : MeOH : TFA = 12 : 12 : 1 solution. After stirring for 1h, the mixture was lyophilized to give **4a** (49 mg, 95 %) as a white powder. ^{1}H NMR (500 MHz, CD_3OD) δ8.20 (m, 2H), 7.11 (m, 2H), 4.80 (d, 1H, *J*= 3.5 Hz), 4.64 (d, 1H *J*= 7.5 Hz), 4.40-4.24 (m, 5H), 4.00-3.94 (m, 2H), 3.70-3.58 (m, 8H), 1.17 (d, 1H, *J* = 6.5 Hz)

1-*p*-*N*-Acryloylamidophenyl-2-(*R*)-*O*-(3-*O*-sulfo-β-D-galactopyranosyl)-3-*O*-α-L-fucopyranosylglycerol sodium salt (4b).

A catalytic amount of $Pd(OH)_2$/C was added to the MeOH solution of **4a** (49 mg, 0.07 mmol). The mixture was hydrogenated at room temperature under H_2 atmosphere for 1h. The reaction mixture was filtered through a celitepad and concentrated in vacuo. The residue was added triethylamine (29 μL, 0.21 mmol) and MeOH (5 mL), and cooled at 0 °C. After 15min stirring, the solution was added Acryloylchloride (13 μL, 0.14 mmol) and warmed at room temperature. After stirring for 2h, the mixture was concentrated and purified by reverse phase silica gel column chromatography (H_2O : MeOH = 2 : 1). Treating the residue with Dowex 50 (Na^+ form) afforded **4b** (31 mg, 63%) as a white powder. Mixture of diastereomers in a 97.5 : 2.5 ratio. $[\alpha]_D^{24}$ = -78.0 (*c* = 0.03, in H_2O); ^{1}H NMR (500 MHz, D_2O) δ7.44 (m, 2H), 7.06 (m, 2H), 6.41 (dd, 1H, *J* = 10.5, 16.5 Hz), 6.32 (dd, 1H, *J* = 1.0, 16.5 Hz), 5.86 (dd, 1H, *J* = 1.0, 10.5 Hz), 4.92 (d, 1H, *J* = 3.5 Hz), 4.73 (d, 1H *J* = 7.5 Hz), 4.40 (br. m, 1H), 4.35-4.30 (m, 4H), 3.96 (dd, 1H, *J* = 6.0, 10.0 Hz), 3.85 (br. dq, 1H), 3.80-3.66 (m, 8H), 1.170 (d, 1H, *J* = 6.5 Hz) FAB+: calcd for $C_{24}H_{35}NNaO_{16}S$ [M+H] 648.1, found 648.0.

1-*p*-Nitrophenyl-2-(*R*)-*O*-β-D-galactopyranosyl-3-*O*-α-L-fucopyranosylglycerol (3a).

The compound of **16** (45 mg, 0.08 mmol) was dissolved in H_2O : MeOH : TFA = 12 : 12 : 1 solution. After stirring for 1h, the mixture was lyophilized to give **3a** (41 mg, 99 %) as a white powder. ^{1}H NMR (500MHz, CD_3OD) δ8.21 (m, 2H), 7.12 (m, 2H), 4.82 (d, 1H, *J* = 3.5 Hz), 4.52 (d, 1H *J* = 7.5 Hz), 4.83-4.27 (m, 3H), 3.98 (dd, 1H, J = 4.5, 11.0), 3.94 (m, 1H), 3.83-3.45 (m, 9H), 1.17 (d, 1H, *J* = 6.5 Hz)

1-*p*-*N*-Acryloylamidophenyl-2-(*R*)-*O*-β-D-galactopyranosyl-3-*O*-α-L-fucopyranosylglycerol (3b).

A catalystic amount of $Pd(OH)_2$/C was added to the MeOH solution of **3a** (41 mg, 0.08 mmol). The mixture was hydrogenated at room temperature under H_2 atmosphere for 1h. The reaction mixture was filtered through a celitepad and concentrated in vacuo. The residue was added triethylamine (28 μL, 0.22 mmol) and MeOH (5 mL), and cooled at 0 °C. After stirring for 15min, the solution was added Acryloylchloride (11 μL, 0.11 mmol) and warmed at room temperature. After stirring for 2 h, the mixture was concentrated and purified by reverse phase silica gel column chromatography (H_2O : MeOH = 5 : 1). Treating the residue with Dowex 50 (Na^+ form) afforded **3b** (28 mg,

65%) as a white powder. $[\alpha]_D^{24}$ = -63.0 (c = 0.05, in H_2O); ^{1}H NMR (500MHz, D_2O) δ7.45 (m, 2H), 7.07 (m, 2H), 6.44 (dd, 1H, J = 10.5, 16.5 Hz), 6.32 (dd, 1H, J = 1.0, 16.5 Hz), 5.86 (dd, 1H, J = 1.0, 10.5 Hz), 4.93 (d, 1H, J = 2.5 Hz), 4.64 (d, 1H J = 8.0 Hz), 4.39-4.30 (m, 3H), 3.98 (dd, 1H, J = 6.0, 10.5 Hz), 3.93 (d, 1H, J = 3.5 Hz), 3.85 (br. dd, 1H), 3.80-3.65 (m, 7H), 3.56 (dd, 1H, J = 7.5, 10.0 Hz), 1.15 (d, 1H, J = 6.5 Hz)

A typical procedure for polymerization

The typical procedure is as follows. A water solution of each of the sugar monomers, acrylamide and 2,2'-azobis(2-aminopropane)dihydrochloride (AAPD) in a sealed glass tube was cooled to –78 °C, and degassed under reduced pressure. The tube was sealed in vacuo and kept at 60 °C until the solution shows viscosity for 0.5-4 h. The solution was poured into methanol (20 mL) in centrifugation tube, and the mixture was centrifuged (3000 rpm). The precipitate dissolved in water was again poured into methanol. The precipitate was dissolved in water, dialyzed for 2 days in water (Mw 3500 cut off) and lyophilized to afford a white powder of the polymer.

Copolymer (6). $M_n = 3.1 \times 10^5$, M_w/M_n = 1.86 [SEC analysis in phosphate buffer saline (pH = 7.4), calibrated with pullulans]; ^{1}H NMR (500 MHz, D_2O, 60 °C) δ7.36 (brs, 2H, aromatic-H), 7.06 (brs, 2H; aromatic-H), 4.91 (brs, 1H; Fuc H-1), 4.61 (brd, 1H, J = 8.5 Hz; Gal H-1), 4.40-3.53 (brm, 15H), 2.40-2.17 (brm, -C*H*-CH_2-), 1.79-1.45 (brm, -CH-CH_2-), 1.11 (brs, 1H; Fuc H-6); (Le^X : Acrylamide = 11 : 89)

Copolymer (7). $M_n = 3.0 \times 10^5$, M_w/M_n = 1.87 [SEC analysis in phosphate buffer saline (pH = 7.4), calibrated with pullulans]; ^{1}H NMR (500 MHz, D_2O, 60 °C) δ7.56 (brs, 2H, aromatic-H), 7.25 (brs, 2H, aromatic-H), 5.11 (brs, 1H, Fuc H-1), 4.91 (brd, 1H, J = 7.0 Hz, Gal H-1), 4.50-3.84 (brm, 15H), 2.60-2.30 (brm, -C*H*-CH_2-), 2.00-1.75 (brm, -CH-CH_2-), 1.32 (brd, 1H, J = 6.5 Hz, Fuc H-6); (3'-sulfo-Le^X : Acrylamide = 8 : 92)

Copolymer (8). $M_n = 3.1 \times 10^5$, M_w/M_n = 1.74 [SEC analysis in phosphate buffer saline (pH = 7.4), calibrated with pullulans]; ^{1}H NMR (500 MHz, D_2O, 60 °C) δ7.60 (brs, 2H, aromatic-H), 7.27 (brs, 2H, aromatic-H), 5.32 (brs, 1H, GlcNAc H-1), 4.57 (br, 1H), 4.42 (br, 1H), 4.19 (br, 1H), 4.00 (br, 1H), 3.85 (brm, 1H), 3.78 (brm, 1H), 2.52-2.22 (brm, -C*H*-CH_2-), 2.10 (brs, 3H COCH_3), 2.00-1.75 (brm, -CH-CH_2-); (6-sulfo-GlcNAc : Acrylamide = 10 : 90)

Terpolymer (9). $M_n = 2.4 \times 10^5$, M_w/M_n = 1.77 [SEC analysis in phosphate buffer saline (pH = 7.4), calibrated with pullulans]; ^{1}H NMR (500MHz, D_2O, 60 °C) δ7.58 (brm, 4H, aromatic-H), 7.23 (brm, 4H, aromatic-H), 5.32 (brs, 1H, GlcNAc H-1), 5.11 (brs, 1H, Fuc H-1), 4.90 (brs, 1H, Gal H-1), 4.60-3.78 (brm, 22H), 2.60-2.30 (brm, C*H*-CH_2-), 2.20 (brs, 3H COCH_3), 2.12-1.75 (brm, CH-CH_2-), 1.29 (brs, 1H, Fuc H-6); (3'-sulfoLe^X : 6-sulfo-GlcNAc : Acrylamide = 7 : 6 : 87)

Inhibition assay of E-, L- and P-selectins/sLe^X binding (ELISA assay).

The assays were carried out by using sLe^X pentaceramide as a common ligand (the number of replicates= 2). The typical procedure is as follows. A solution of sLe^X

pentaceramide, in a 1:1 mixture of methanol and distilled water, was pipetted into microtiter plate wells (96 wells, Falcon PRO-BIND) at pmol/50 μL/well and was adsorbed by evaporating the solvent. The wells were washed twice with distilled water, blocked with 5% BSA (bovine serum albumin)-PBS (phosphate-buffered saline) for 2 h at room temperature, and washed three times with PBS.

Separately, a 1:1 volumetric mixture of a 1:500 dilution in 1 % BSA-PBS of biotin-anti-human IgG (Fc) (BioSource International Inc.) and a selectin-immunoglobulin fusion protein (E-, P- and L-selectin-Ig, respectively) was incubated at room temperature for 30 min to form a complex. The test compounds were dissolved in distilled water at 50 μg/mL and finally diluted to the final concentration at 1, 5, 10, 30, 40, and 50 μg/mL. Reactant solutions were prepared by incubating 30 μL of this solution at each concentration with 30 μL of the above complex solution for 30 min at room temperature. This reactant solution was then added to the above microtiter wells at 50 μL/well and incubated at 37 °C for 45 min. The wells were washed three times with PBS and distilled water, respectively, followed by addition of *p*-nitrophenylphosphate (1 mg/mL) and 0.01 % $MgCl_2$ in 1 M diethanolamine (pH 9.8) at 50 μL/well. The reactant was developed for 120 min at 23 °C, and absorbance at 405 nm was measured.