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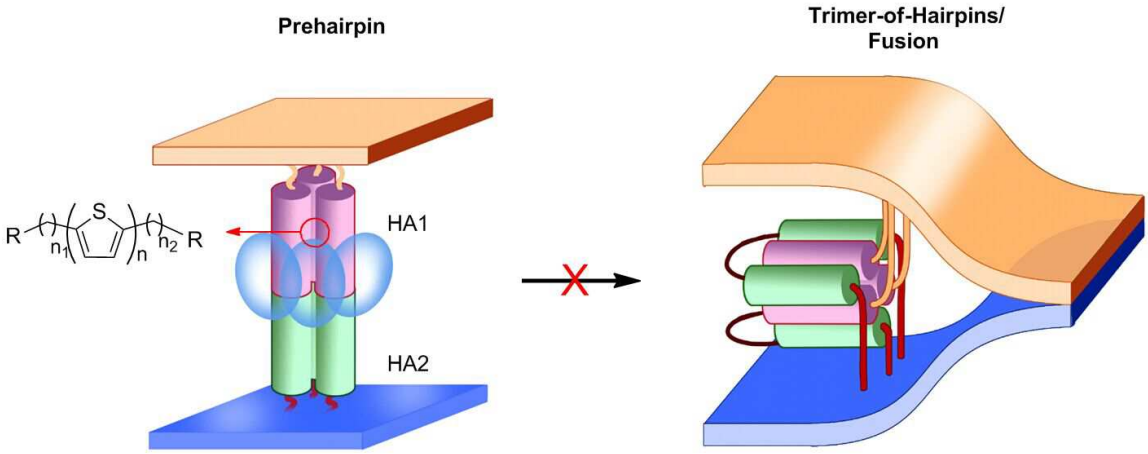

3sf against H5N1 influenza pseudovirus with an IC_{50} of 0.029 μM，against H1N1 influenza virus MDCK with an IC_{50} of 1.240 μM.

Oligothiophene compounds inhibit the membrane fusion between H5N1 avian influenza virus and the endosome of host cell

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Abstract:

Hemagglutinin (HA) which is essential for influenza viral infection and replication has become a target for the design of anti-influenza drugs. A novel series of oligothiophene compounds focused on the target were synthesized as specific inhibitors against the H5 subtype of influenza A viruses because oligothiophene has stronger π–π interactions with residues $F110_2$ and $M24_1$ of HA2 side chains. Oligothiophene compounds were designed and synthesized by a series of alkylation, azidation, amination and amidation reactions. The entry inhibitory activities of those compounds were tested at a cellular level against H5N1 influenza pseudovirus. Compound **3sf** was revealed as the most active inhibitor in this series with an IC_{50} of 0.029 μM. The activity of **3sf** is almost 1000 times that of the positive reference compound (CL-385319). A structure-activity analysis of these compounds demonstrated that the size of the oligothiophene compounds was very important for the inhibitory activity. Four compounds (**3sk, 3sf, 3sc** and **4sc**) of strong inhibitiory activity against H5N1 influenza pseudovirus were assessed against H1N1 influenza virus MDCK. They also showed strong inhibitiory activity with IC_{50s} of 3.292 μM, 1.240 μM, 1.119 μM and 0.768 μM, respectively.

Keywords: oligothiophene compounds, H5N1 influenza virus, hemagglutinin(HA), fusion inhibitor

1. Introduction

The H5N1 avian influenza A viruses, which spreads quickly by transferring viral particles into host cells is a serious threat to human health in the world.[1] Membrane fusion is an important step needed for the entry of the H5N1 avian influenza A viruses into host cells.[2] The fusion step is mediated by influenza virus hemagglutinin (HA), which is the viral envelope protein (ENV) organized as a trimer; each monomer consists of HA1 and HA2 subunits, linked by a single disulfide bond.[3] HA1 separates from HA2, which then turns inside out when the HA1:HA2 trimer encounters low pH in endosome after endocytosis. In detail, the hemagglutinin conformational changes are triggered following the attachment of the trimer to the cell surface, which is mediated by the interaction of HA1 with its receptor at the cell surface. The HA2 protein undergoes an irreversible conformation change from its metastable pre-fusion conformation to a low-pH hairpin structure. The N terminus of HA2 inserts into a pocket , which is created by a splaying apart from each other of the C termini of the HA2 coiled-coil helices.[4-6] The resulting extrusion of HA2 toward the endosomal membrane promotes the fusion of the viral and endosomal membranes.

Inhibition of these conformational changes could be an efficient means of blocking infection. Influenza A virus infection can be suppressed by multivalent sialylation of β-thio-glycoclusters.[7] Several small molecules have been found to be fusion inhibitors of the influenza A virus.[8-11] We previously found that CL-385319 could inhibit H5N1 influenza virus infection by blocking viral entry.[12] It was found that the recognition and binding of CL-385319 to HA proceeds by a process of ‘‘induced fit’’ and the binding

pocket is formed during their interaction. Those residues constituted the binding cavity: $V48_2$, $F110_2$, $M24_1$, $E105_2$, $R106_2$, $E103_2$, $T107_2$ and $K51_2$. Occupation of this pocket by CL-385319 could stabilize the neutral pH structure of hemagglutinin, thus inhibiting the conformational rearrangements required for membrane fusion.[12] A novel series of derivatives focused on the target pocket were synthesized as specific inhibitors against the H5 subtype of influenza A viruses.[10] We also found that molecule **1L** was the most active among these derivatives because the thiophene chromophore of molecule **1L** has stronger π–π interactions with the side chains of residues $F110_2$ and $M24_1$ compared with molecule CL-385319 according to a variety of theoretical calculations including docking, molecular dynamics simulations, free energy calculations and quantum calculations.[13,14] In this study, we concentrated on designing and synthesizing oligothiophene derivatives since thiophene could act strongly on some residues of the cavity. We also evaluated the activities of all synthesized compounds against H5N1 avian influenza A viruses.

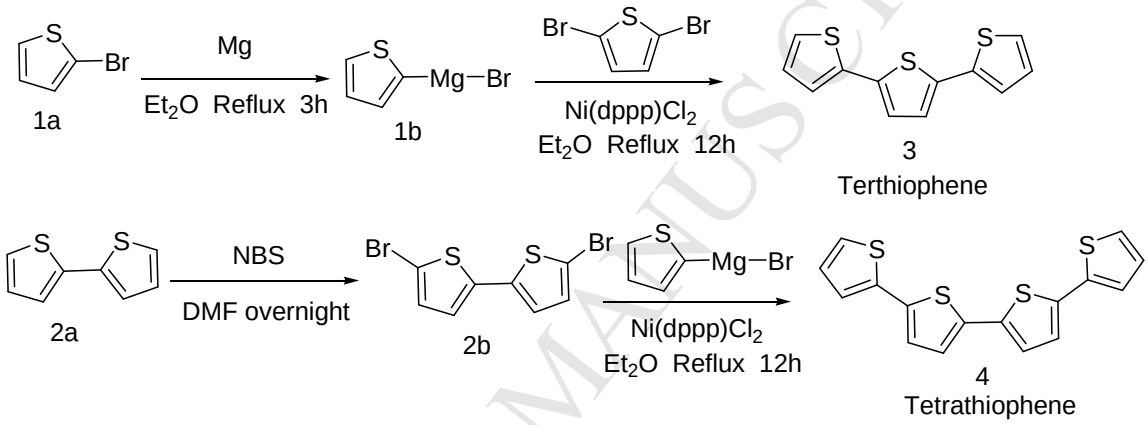
2. Chemistry

All the reactions were prepared under an atmosphere of dry N_2. Tetrahydronfuran (THF) and diethylether were dried over sodium/benzophenone, while the methanol was dried by distillation from CaF_2. To generate aminoalkyl thiophene derivatives, a general and scalable synthesis of the requisite oligothiophenes precursors were developed (Scheme 1).[15-17] 2,5-dibromo thiophene/5,5'-dibromo-2,2'-bithiophene were subjected to the Grignard reagent of thiophen-2-ylmagnesium bromide catalyzed by $Ni(dppp)Cl_2$ in the freshly distilled ether respectively to achieve terthiophene **3**/tetrathiophene **4** as

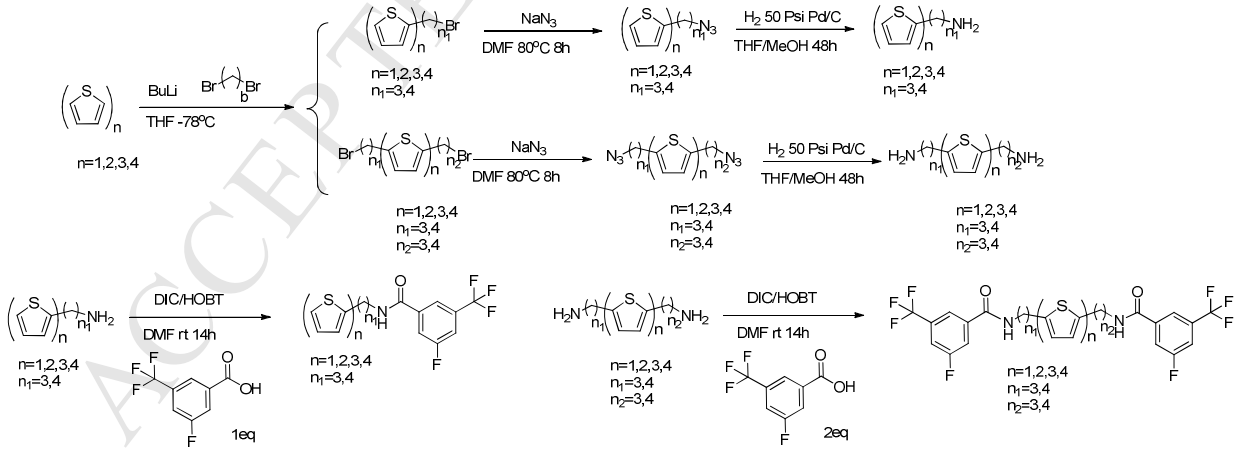
products. 5,5'-dibromo-2,2'-bithiophene was previously obtained through the bromination of 2,2'-bithiophene by NBS in DMF.[18] Subsequent alkylations of the oligothiophenes were carried out by the treatment with 4 equivalents of n-butyl lithium in hexane at -78 ℃ in dry THF, followed by the addition of 4 equivalents of corresponding dibromoalkanes to achieve the monoalkylation products (**3sd, 3sa, 4sa, 4sd)** and dialkylation products (**2sa, 3si, 3sg, 4sg**) at one pot.[19] Based on the above experimental results, the alkylation reactions with 1,4-dibromobutane yielded dialkyl-oligothiophenes as major products and monoalkyl-oligothiophenes as minor products with a ratio around 2:1. In contrast, alkylation of 1,3-dibromopropane provided a 1:2 ratio of di/mono products. However alkylation to monothiophene yielded only monoalkylation product. At the same time, we also tried the alkylation reactions with 1,2-dibromoethane; however this ethylation does not work.

The bromoalkylthiophenes and bisbromoalkylthiophenes were then treated with 2 equivalents and 4 equivalents of the NaN_3, respectively, in DMF at 90 ℃ to give azidoalkylthiophene (**3se, 3sb, 4sb, 4se**) and bisazidoalkylthiophene (**3sj, 4sh**) as products, followed by hydrogenation over 20 wt% Pd/C-H2 (50 Psi) in degased 1:1 MeOH/THF, affording aminoalkylthiophene and bisaminoalkylthiophene (**1sc, 2sb, 2sc, 2sd, 3sc, 3sf, 3sh, 3sk, 4sc, 4sf, 4si**). The amino thiophene derivatives were then coupled to 3-fluoro-5-(trifluoromethyl) benzoic acid by the treatment of 1 equivalent of N,N'-diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBT) in DMF providing F4-thiophene derivatives (**1sa, 1sd, 1se, 2se, 2sf, 3sm, 3sl**). While bisaminoalkylthiophenes were subjected to 2 equivalents of DIC/HOBT, and

3-fluoro-5-(trifluoromethyl)benzoic acid in DMF providing F4-thiophene-F4 derivatives (**1sf, 2sg, 2sh, 2si, 2sj, 3sn, 3so**) as products. For alkylation of monothiophene, the reactions with 1,4-dibromobutane only yielded mono alkyl products in the first step. A second alkylation of 2-(4-bromobutyl) thiophene yielded 2,5-bis(4-bromobutyl) -thiophene as final product **(1sb)**. The difference in structures of thiophene derivatives does not affect yields of following azido and hydrogenation reactions.



Scheme 1. Synthesis of oligothiophenes



Scheme 2. Synthesis of oligothiophenes derivatives

3. Results and discussion

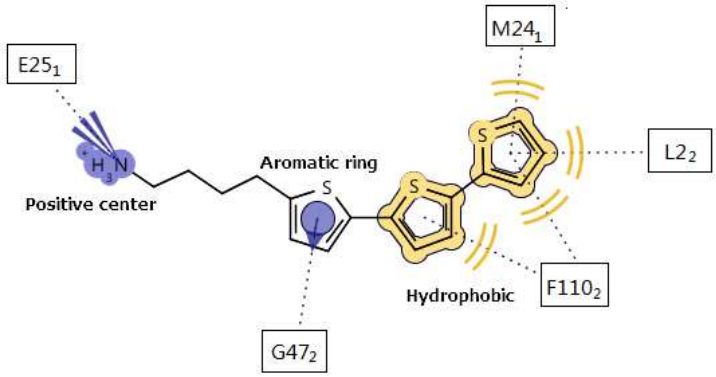
The compounds designed and synthesized are listed with the IC_{50} values for inhibition of H5N1 pseudovirus infection in Table 1. All compounds were evaluated for inhibitory

activity against the entry of H5N1 influenza virus.[10, 12] Several compounds exhibited stronger inhibitory activities against the infection of H5N1 virus compared with CL-385319. The most active compound was **3sf** with IC_{50} of 29 nM (Table 1).

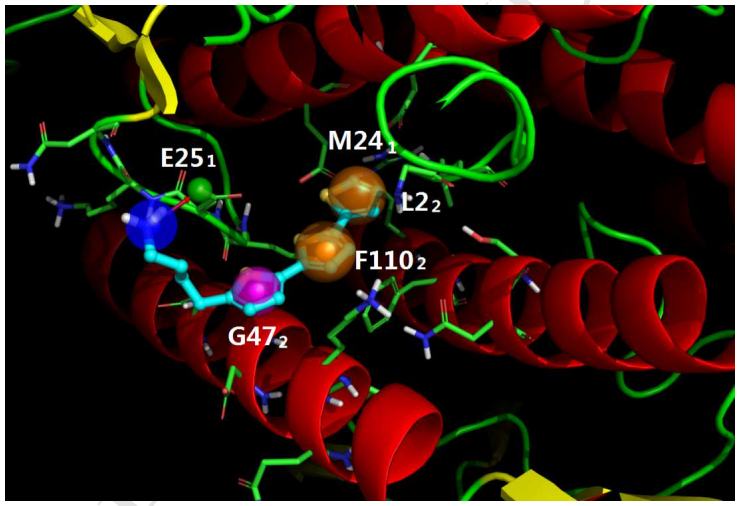
3.1 The activity of oligothiophene as virus entry inhibitor.

CL-385319 inhibits the infection[6,8] by H1-, H2-, H3- and H5-typed influenza A viruses by interfering with the fusogenic function of the viral hemagglutinin which consists of HA1 and HA2. CL-385319 is thought to distinguish[6,12] and bind to the cavity of H5N1 HA2 stem region, which contains $V48_2$, $F110_2$, $M24_1$, $E105_2$, $R106_2$, $E103_2$, $T107_2$ and $K51_2$. The recognition and binding of CL-385319 to HA proceeds by a process of ‘‘induced fit’’, whereby the binding pocket is formed during the course of this interaction. Occupation of this pocket by CL-385319 may stabilize the neutral pH structure of hemagglutinin, thus inhibiting the conformational rearrangements required for membrane fusion[6]. Thiophene has stronger π–π interactions with the side chains of residues $F110_2$ and $M24_1$ which are crucial for conformational changes in the HA2 protein[13] and for membrane fusion compared with piperidine of molecule CL-385319 by a variety of theoretical calculations, including docking, molecular dynamics simulations, free energy calculations, as well as quantum calculations. In this study, a lot of oligothiophene compounds are more active than CL-385319 (see Figure 2 and Table 1) and the highest activity of **3sf** is almost as 1000 times as CL-385319. Docking study was performed between 3sf and HA according to reference 6. 3sf also binds to the cavity of H5N1 HA2 stem region (see Figure 1). Two thiophenes involve in hydrophobic interaction with residues $M24_1$, $L2_2$, $F110_2$. Aromatic interaction takes place between one of thiophenes

and $G47_2$. Salt bridger forms between $E25_1$ and ammonium group of 3sf which is a stronger interaction. So 3sf is more active than CL-385319.



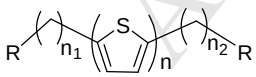
A



B

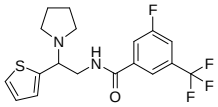
Figure 1 Mode of Interaction between 3sf and HA

Table 1. Inhibitory activities of compounds against H5N1 pseudovirus

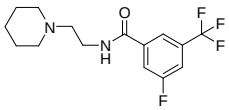


n = 1,2,3,4

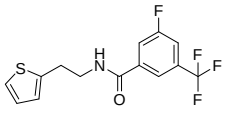
n_1=0, 3, 4; n_2=3,4



1 sa



CL-385319



1L

Compound	n	n_1	n_2	R	IC_{50}(μM)	CC_{50}(μM)
1L					**0.22** ± 0.11	**ND**
CL-385319					10.580±0.468	1373.002±99.185

1sa					6.414 ±0.095	239.378 ±14.086
1sb	1	4	4	bromo	NA	ND
1sc	1	4	4	amino	NA	ND
1sd	1	0	3	3-fluro-5-(trifluromethyl)-benzamide	7.236±0.355	40.16±6.24
1se	1	0	4	3-fluro-5-(trifluromethyl)-benzamide	4.733 ±0.474	43.541 ±9.677
1sf	1	4	4	3-fluro-5-(trifluromethyl)-benzamide	NA	ND
2sa	2	3	3	bromo	NA	ND
2sb	2	3	3	amino	NA	ND
2sc	2	0	4	amino	NA	ND
2sd	2	4	4	amino	0.0819±0.004	26.472±5.944
2se	2	0	3	3-fluro-5-(trifluromethyl)-benzamide	42.090±5.557	46.735 ±7.990
2sf	2	0	4	3-fluro-5-(trifluromethyl)-benzamide	19.763 ±2.609	27.354±3.788
2sg	2	3	3	3-fluro-5-(trifluromethyl)-benzamide	NA	ND
2sh	2	4	4	3-fluro-5-(trifluromethyl)-benzamide	NA	ND
2si	2	3	3	2-chloro-benzamide	NA	ND
2sj	2	3	3	3,5-dihydroxy-benzamide	NA	ND
3sa	3	0	3	bromo	12.538±1.869	35.322±5.433
3sb	3	0	3	azido	23.034±2.873	>200
3sc	3	0	3	amino	2.875 0.569	18.858 ±1.304
3sd	3	0	4	bromo	11.399±0.791	23.760 ±0.958
3se	3	0	4	azido	11.084±1.868	>200
3sf	3	0	4	amino	0.029±0.002	13.269±0.510
3sg	3	3	3	bromo	6.641±0.734	>200
3sh	3	3	3	amino	4.151±0.564	>200

3si	3	4	4	bromo	0.696±0.080	>200
3sj	3	4	4	azido	NA	ND
3sk	3	4	4	amino	0.037±0.002	12.977 ±1.700
3sl	3	0	3	3-fluro-5-(triflurомethyl)-benzamide	NA	ND
3sm	3	0	4	3-fluro-5-(triflurомethyl)-benzamide	NA	ND
3sn	3	3	3	3-fluro-5-(triflurомethyl)-benzamide	NA	ND
3so	3	4	4	3-fluro-5-(triflurомethyl)-benzamide	NA	ND
4sa	4	0	3	bromo	74.117±14.132	>200
4sb	4	0	3	azido	46.053 ±3.830	>100
4sc	4	0	3	amino	0.171±0.007	>200
4sd	4	0	4	bromo	NA	ND
4se	4	0	4	azido	NA	ND
4sf	4	0	4	amino	NA	ND
4sg	4	4	4	bromo	NA	ND
4sh	4	4	4	azido	NA	ND
4si	4	4	4	Amino	NA	ND

NA is no activity. ND is not determined .

3.2 The structure - activity relationship of oligothiophene compounds.

From Table 1, the antiviral activities of compounds 1sa, 1sd and 1se were a little higher than the positive control drug CL-385319, which suggested that the incorporation of 3-fluoro-5-(trifluoromethyl)benzamide segment and thiophene group to the basic structure was essential for the antiviral activities in the series of monothiophenes. Introduction of additional functional groups, such as 3-fluoro-5-(trifluoromethyl) benzamide segments did not result in enhanced activity.

Furthermore, the length of spacer between thiophene group and 3-fluoro-5-(trifluoromethyl)benzamide did not significantly affect the antiviral activities. It was found that the replacement of $-N_3$ with $-NH_2$ in R substituent increases the activity (**3sf** > **3se**; **4sc** > **4sb**) and the replacement of –Br with $-N_3$ in R substituent demonstrated the same results (**3se** > **3sd**; **4sb** > **4sa**). Similarly, the addition of an $-NH_2$ group was more effective than an azido function, which was more effetive than a bromo substitutuent for the series of terthiophene and tetrathiophene. The structures of **2sc** and **2sd** are very similar, in which **2sd** has another alkyl substituent in 5' position of the thiophene ring, while **2sc** doesn't have. Although **2sc** and **2sd** were differentiated only by the presence of 5' alkyl substituent in **2sd**, **2sd** was much more active than **2sc**, indicating that 2, 5'-disubstitution of the thiophene ring was the most important structural factor. For dithiophene and terthiophene based virus entry inhibitors with same functional groups ($-NH_2$, N_3, Br), compounds with four carbons alkyl chain exhibits higher activities than the conterparts with three carbons (**3sf** > **3sc**, **3sk** > **3sh**, **3si** > **3sg**, **3se**>**3sb**, **2sd** > **2sb**).

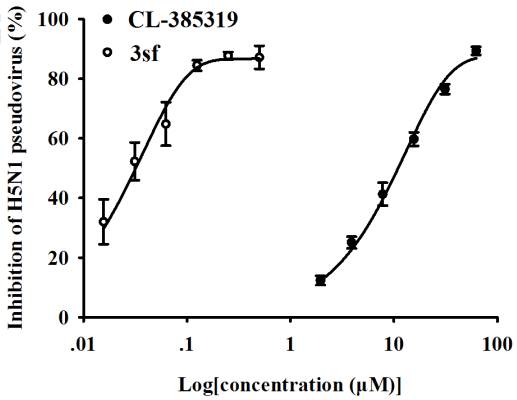


Figure 2. The inhibitory activity of **CL-385319** and **3sf** against A/Thailand/Kan353/2004 (H5N1) pseudovirus. The samples were tested in triplicate and the data were presented in mean ± SD. This experiment was repeated three times with similar results.

3.3 Inhibition activity against H1N1 influenza virus MDCK.

H5 subtype is the highly pathogenic virus. H1 and H5 are in the same clade.[20] H5N1 (strain A/Thailand/ Kan353/2004) and H1N1 (A/PuertoRico/8/1934) share 58.76% sequence identity (see supporting information). In order to assess inhibitiory activity against H5N1 influenza virus, we screened inhibition activity of some compounds (**3sk, 3sf, 3sc** and **4sc**) against H1N1 influenza virus MDCK (see table 2), which are powerfully active against H5N1 influenza pseudovirus (see table 1). They were all effective in inhibiting the infection of H1N1 influenza A virus (A/PuertoRico/8/1934). Compound **4sc** was the most active inhibitor in this series with an IC_{50} of 0.768 μM. It is interesting that compound **4sc** is more active than compound **3sc**. Thus compound **4sc** is safer than any other three compounds.

Table 2. Inhibition activity against H1N1 influenza virus MDCK of some compounds.

Compound	IC_{50}(μM)	CC_{50}(μM)
CL-385319	72.0±10.688	1373.002±99.185
3sk	3.292±0.594	12.977 ±1.700
3sf	1.240±0.156	13.269±0.510
3sc	1.119±0.381	18.858 ±1.304
4sc	0.768±0.245	>200

3. Conclusions

A novel series of oligothiophene compounds were synthesized as specific virus entry inhibitors against the H5 subtype of influenza A viruses to explore how the enhanced □ interactions with the side chains of residues $F110_2$ and $M24_1$ expected for thiophene

would impact activity. Compound 3sf was identified as the most active inhibitor in this series with an IC_{50} of 0.029 μM, which validated this design model. The structure-activity relationships analysis of these compounds showed that the size of the oligothiophene compounds was also very important for activity. This work suggested that compounds inhibited conformational changes of hemagglutinin (HA) could be an efficient way of blocking influenza virus infection. A cavity of H5N1 HA2 stem region which contained $V48_2$, $F110_2$, $M24_1$, $E105_2$, $R106_2$, $E103_2$, $T107_2$ and $K51_2$ could be a target to design new influenza virus entry inhibitors. Four compounds (**3sk, 3sf, 3sc and 4sc**) of strong inhibitiory activity against H5N1 influenza pseudovirus also strongly inhibite H1N1 influenza virus MDCK. In the future, we will study further on the mechanism of entry inhibition.

4. Experimental protocols

4.1 Chemical material and equipment.

Reagents for synthesis were obtained commmercially, unless otherwise noted. All reactions were operated under nitrogen atmosphere. Tetrahydrofuran (THF) was dried by distillation from sodium. Flash chromatography was performed on silica gel 60 (230-240 mesh, 60Å) with the indicated eluents. ^{1}H NMR was recorded at 400 or 500 MHz and ^{13}C NMR at 100 or 125 MHz on a Bruker DPX-400 or DPX-500 instrument. Hydrogenation was carried out with Dayton 5K906C hydrogenator. Mass spectra were recorded with Bruker MicroTof. Final products were dissolved in MeOH/MeCN purified using reverse-phase HPLC (C-18 column, H_2O/CH_3CN (10/90 to 100/0 over 25 minutes, 100/0

to 100/0 from 25 min to 40 min (0.1 % TFA)).

4.2 Cell lines, plasmids and materials.

Madin–Darby Canine Kidney (MDCK) cells and 293T cells were obtained from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) were obtained from Life technology; fetal calf serum (FCS) was supplied from Gemini Bio-Products; HA plasmid expressing HA of the H5 subtype strain A/Thailand/ Kan353/2004 (H5N1) strain was kindly provided by Frank Kirchhoff (University of Ulm, Germany). Cell culture lysis reagent and luciferase assay substrate were bought from Promega.

4.3 Chemistry procedures.

General procedure for synthesis of 5, 5'-dibromo-2, 2'-bithiophene. To a solution of 2, 2'-bithiophene (12 mmol, 2 g) in anhydrous DMF (30ml), NBS (24 mmol, 4.28 g) was added dropwisely (cooling with ice-water during addition of NBS solution) and stirred for 3h. The reaction mixture was then poured into 100 ml of ice water and the beige solid was separated by vacuum filtration. [1] The crude was purified with column chromatography (silica gel 100-200 mesh), using hexane as eluent to yield yellow solid as product (3.6 g, yield 90%).

General procedure for synthesis of ***2,2':5',2''-terthiophene*** *(3):* An established procedure was used for the synthesis of ***2,2':5',2''-terthiophene*** **3**. Mg turnings (0.20 g, 7.4 mmol) was added to a flame dried 100-mL two-necked flask containing 2-bromothiophene (1.1g, 6.4 mmol) in 10 mL anhydrous Ether at room temperature under N_2. The solution mixture was stirred vigorously and heated to reflux for 3h. After

cooling to room temperature the Grignard reagent of 2-bromothiophene (6.4 mmol) was added slowly to a solution of 2,5-dibromothiophene (0.74g, 3.1 mmol) catalyzed with Ni(dppp)Cl_2 (14mg, 0.026 mmol) in 20 mL anhydrous Ether and the solution was refluxing 14h. After cooling to temperature, the reaction was quenched with water and acidified with 6M HCl to pH 4-5. The solution was then extracted with $CHCl_3$ (5 × 20 mL). The combined organic solvent was washed with brine, dried over Na_2SO_4 and evaporated to dryness.[2] The crude was purified with column chromatography (silica gel 100-200 mesh), using hexane/DCM as eluent to yield bright yellow solid identified as tetrathiophene. Yield 0.77 g, 50%.

General procedure for synthesis of ***2,2':5',2'':5'',2'''-tetrathiophene (4):*** An established procedure was used for the synthesis of 2,2':5',2":5",2"'-Tetrathiophene **4**. Mg turnings (0.40 g, 14.7 mmol) was added to a flame dried 100-mL two-necked flask containing 2-bromothiophene (2.1g, 12.7 mmol) in 20 mL anhydrous Ether at room temperature under N_2. The solution mixture was stirred vigorously and heated to reflux for 3h. After cooling to room temperature the Grignard reagent of 2-bromothiophene (12.7 mmol) was added slowly to a solution of 5,5'-dibromo-2,2'-bithiophene (2.0g, 6.2 mmol) catalyzed with Ni(dppp)Cl_2 (28mg, 0.052 mmol) in 20 mL anhydrous Ether and the solution was refluxing 14h. After cooling to temperature, the reaction was quenched with water and acidified with 6M HCl to pH 4-5. The solution was then extracted with $CHCl_3$ (5 × 20 mL). The combined organic solvent was washed with brine, dried over Na_2SO_4 and evaporated to dryness.[2] The crude was purified with column chromatography (silica gel 100-200 mesh), using hexane/DCM as eluent to yield bright yellow solid

identified as tetrathiophene. Yield 1.98 g, 47%.

General procedure for synthesis of ***bromoalkylthiophene and bisbromoalkylthiophene*****:** To a solution containing 1 mmol of oligothiophene in freshly distilled THF (0.15 mol/L) under N_2, 4 equivalents of a 2.6 M solution of n-butyl lithium in hexane was added at -78 ℃ drop wisely. The solution was stirred at -78 ℃ for 1 h, and then 4 equivalents of dibromoalkane was added to the solution. The reaction was then allowed to warm to room temperature and stirred 12h. The mixture was quenched with 10 mL water and extracted with ether. The combined organic layer was washed with a saturated NaCl solution, dried over $MgS0_4$, and concentrated under reduced pressure. Column chromatography of the residue (hexane / DCM 10:1 to 5:1; Rf 0.3 and 0.5 with hexane / DCM 5:1) afforded pure bromo-alkylthiophene (**1sb, 3sd, 3sa, 4sa, 4sd, 2sa, 3si, 3sg, 4sg**).[14]

General procedure for synthesis of ***azido-alkylthiophene and bis-azido-alkylthiophene:*** Anhydrous DMF solution (2 mL) of 2 to 4 equivalents sodium azide and bromoalkylthiophene or bisbromoalkylthiophene (1 mmol) was stirred in round bottom flask under N_2 at 80 °C for 12h. Then water (10 mL) was added. The mixture was then extracted with dichloromethane (3 × 10 mL).[4] The organic layer was collected and then washed with a saturated NaCl solution, dried over $MgSO_4$, and concentrated under reduced pressure. Column chromatography of the residue (hexane / DCM 5:1; R_f 0.3-0.4 with hexane/DCM 5:1) afforded pure Azido-alkylthiophene (**3se, 3sb, 4sb, 4se**) and pure bis-Azido-alkylthiophene (**3sj, 4sh**).

General procedure for synthesis of ***aminoalkylthiophene or bisaminoalkylthiophene:***

To a solution of 10 mL N_2 degassed distilled THF/Methanol 1:1, 1 mmol of azido-alkylthiophene and 10% wt/wt palladium on activated carbon (10%) was added. The reaction was stirred under 50 psi of H_2 for 2 days. The mixture was then filtered, and the solution was evaporated under reduced pressure to give pure aminoalkylthiophene.

General procedure for synthesis of ***3-fluoro-5-(trifluoromethyl) benzoic acid -Thiophene*** *and* ***3-fluoro-5-(trifluoromethyl) benzoic acid -Thiophene-3-fluoro -5-(trifluoromethyl)*** *derivatives:* To a solution of aminoalkylthiophene or bisaminoalkylthiophene in DMF (10 mL), 1 equivalent of I'-diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBT), and 3-fluoro-5-(trifluoromethyl) benzoic acid (1mmol amino group for one equivalent) was added and stirred 12h. The solution was then evaporated under reduced pressure. The crude was purified with column chromatography (silica gel 100-200 mesh), using hexane/DCM as eluent to yield F4-Thiophene derivatives (**1sa, 1sd, 1se, 2se, 2sf, 3sm, 3sl**) and F4-Thiophene-F4 derivatives (**1sf, 2sh, 2si, 2sj, 2sg, 3sn, 3so**) as final products. Yield 60%~70%.

4.4 Measurement of the inhibitory activity against H5N1 pseudovirus.

MDCK cells and 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing glutamine, supplemented with 10% fetal calf serum (FCS). The H5N1 pseudoviruses were prepared by transfecting HA plasmid from the H5 subtype strain A/Thailand/ Kan353/2004(H5N1) strain and the NA plasmid from the N1 subtype strain A/Thailand/Kan353/2004. Briefly, 293T cells (70-80% confluent) were co-transfected with 1 μg HA plasmid, 1 μg NA plasmid and 3 μg HIV backbone plasmid (pNL4-3.luc.R_E_) into six-well plate with polyethylenimine (PEI)[5]. Forty-eight hours

after transfection, the culture supernatants were harvested and centrifuged at 2000 g for 10 min. Aliquots were stored at -80 °C. For measuring the inhibitory activities of test compounds, MDCK cells (10^4/well) were seeded in 96-well plates and grown overnight. Tested compounds at indicated concentrations were incubated with pseudotyped particles for 30 min at 37 °C. Subsequently, the virus-compound mixture was transferred to the cells and incubated for an additional 48 h. Cells were washed with phosphate buffer saline (PBS) and lysed with luciferase cell culture lysis reagent (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well flat bottom luminometer plates (Costar), followed by the addition of luciferase assay substrate (Promega). The luciferase activity was measured in a microplate luminometer (Genios Pro, Tecan, US). As a negative control, VSV-G pseudotyped particles were incubated with the tested compound instead of H5N1 pseudovirus.

4.5 Screening inhibition activity against H1N1 influenza virus MDCK.

cells were plated at a density of 2.5×104 cells per well in 96-well plates and incubated for 24 h at 37 °C in 5% CO_2. Influenza A/PuertoRico/8/1934 (H1N1) was pretreated at 37 °C for 1h with 7 concentrations of 2-fold serial dilutions of test compounds starting at 8-200 μM. Then cells were washed twice with PBS and infected with the mixture of virus-compound for 1h, thereafter refreshed and incubated with indicated concentration of test compounds in DMEM containing 1 μg/ml TPCK-treated trypsin, at 37 °C for 48 h. The progeny production in supernatant was measured by the modified neuraminidase activity (NA) assay. Briefly for NA activity assay, the supernatants were transferred to 96 black well plates and incubated with 20 μM

2-(4-Methylumbelliferyl-a-D-N-acetylneuraminic acid sodium salt (MUNANA, Sigma, cat. No M8639), dissolved in 33 mM 2-[N-morpholino]ethanesulfonic acid (pH 6.5) and 4 mM $CaCl_2$, at 37 °C for 1 h. The reaction was terminated by adding 0.14 M NaOH in 83% ethanol. The fluorescence intensity was measured at an excitation wavelength of 340 nm and an emission wavelength of 535 nm using an ELISA reader (Genios Pro, Tecan, US). The IC50 (the concentration of an agent causing 50% inhibition of progeny virus production) values were calculated as described previously [Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoint. Am. J. Hyg. 27, 493-497]. CL-385319 is used as a positive control.

4.6 Cytotoxicity assay

The cytotoxicity of compounds for MDCK cells was measured using the MTT assay. Briefly, 100 μl of MDCK cells (5×10^3/well) was seeded in a 96-well cell culture plate. On the second day, the medium was changed to a fresh one. Then 100 μl of test extract diluted in culture media was added. After incubation at 37 °C for 2 days, 10 μl of MTT solution (5 mg/ml) was added, followed by an incubation of 4 h. The absorbance at 570 nm was measured with an ELISA reader (Genios Pro, Tecan, US). The CC50 (the concentration of an agent causing 50% cytotoxicity) values were calculated using the Calcusyn software.

4.7 Characterization.

3-fluoro-5-(trifluoromethyl)-N-(4-(5-(thio-phene-2-yl)thio -phen-2-yl)butyl)benzamide (2sf). 345 mg of **2sf** as yellow solid, Yield 81%. Purity

96.67%. Mp 87-89 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.68–1.71 (m, 2H), 1.73–1.76 (m, 2H), 2.82–2.86 (m, 2H), 3.46–3.50 (m, 2H), 6.11 (s, 1H), 6.67–6.68 (d, 1H, *J* = 3.2 Hz), 6.96–6.97 (m, 2H), 7.06–7.07 (d, 1H, *J* = 2.4 Hz), 7.14–7.15 (d, 1H, *J* = 5.2 Hz), 7.42–7.44 (d, 1H, *J* = 8 Hz), 7.64–7.66 (d, 1H, *J* = 8.4 Hz), 7.75(s, 1H) , ^{13}C NMR (DMSO-D_6, 125 MHz) δ: 28.9, 29.0, 40.2, 115.6, 115.8, 117.9, 118.1, 119.4, 121.9, 123.2, 123.6, 124.1, 125.3, 127.8, 132.9, 133.2, 133.3, 135.4, 137.8, 138.1, 138.2, 144.1, 161.6, 163.6, 164.9; EI(+)-HRMS m/z , calcd for $C_{20}H_{17}F_4NOS_2$ (M + Na) 450.0579, found 450.0579.

2-(4-bromobutyl)-5-(thiophen-2-yl)thiophene. 164.5 mg of product as yellow liquid, yield 55%. Purity 95.56%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.81–1.84 (m, 2H), 1.91–1.95 (m, 2H), 2.80–2.84 (m, 2H), 3.40–3.43 (m, 2H), 6.67- 6.68 (d, 1H), 6.96–6.98 (m, 2H), 7.08–7.09 (d, 1H, *J* = 3.6 Hz), 7.14–7.16 (d, 1H, *J* = 6 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.5, 30.3, 32.3, 33.6, 123.5, 123.8, 124.2, 125.4, 128.0, 135.5, 138.1, 144.3; EI(+)-HRMS m/z , calcd for $C_{12}H_{13}BrS_2$ (M + Na) 322.9537, found 322.9534.

2-(4-azidobutyl)-5-(thiophen-2-yl)thiophene. 250 mg of product as yellow liquid, yield 95%. Purity 96.67%.. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.64–1.68 (m, 2H), 1.74–1.78 (m, 2H), 2.80–2.83 (m, 2H), 3.27–3.31 (m, 2H), 6.66–6.67 (d, 1H, *J* = 3.6 Hz), 6.96–7.01 (m, 2H), 7.07–7.08 (d, 1H, *J* = 3.2 Hz), 7.14–7.16 (d, 1H, *J* = 6 Hz), ^{13}C NMR (DMSO-D_6, 125 MHz) δ: 28.9, 29.3, 30.3, 51.9, 123.9, 124.1, 124.6, 125.8, 128.4, 135.9, 138.4, 144.7; EI(+)-HRMS m/z , calcd for $C_{12}H_{13}N_3S_2$ (M + Na) 286.0442, found 286.0443.

4-(5-(thiophen-2-yl)thiophen-2-yl)butan-1-amine (2sc). 225 mg of **2sc** as Yellow solid, yield 95%. Purity 98.90%. Mp 58-59 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz)

δ:1.50−1.56 (m, 4H), 1.69−1.73 (m, 2H), 2.70−2.73 (m, 2H), 2.78−2.81 (m, 2H), 6.66−6.67 (d, 1H, *J* = 3.6 Hz), 6.95−6.98 (m, 2H), 7.06−7.07 (d, 1H, *J* = 2.8 Hz), 7.13−7.15 (d, 1H, *J* =5. 6 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.9, 30.0, 33.1, 41.9, 123.1, 123.5, 123.8, 124.9, 127.7, 134.9, 137.9, 144.8; EI(+)-HRMS m/z , calcd for $C_{12}H_{15}NS_2$ (M + 1) 238.0718, found 238.0718.

3-fluoro-5-(trifluoromethyl)-N-(2-(pyrrolidin-1-yl)-2-(thiophen-2-yl)ethyl)benzamide (1sa). 367 mg of **1sa** as yellow solid, yield 95%. Purity 97.86%. Mp 117−118. ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.77−1.78 (s, 4H), 2.61−2.62 (m, 4H), 3.61−3.68 (m, 1H), 3.91−3.98 (m, 2H), 6.59 (s, 1H), 6.96−6.97 (m, 1H), 6.97−7.01 (m, 1H), 7.27−7.29 (m, 1H), 7.43−7.45 (d, 1H, *J* = 8 Hz), 7.59−7.61 (d, 1H, *J* = 8.2 Hz), 7.74 (s, 1H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 23.6, 44.7, 51.4, 62.5, 115.7, 115.9, 117.9, 118.0, 119.9, 125.4, 126.0, 126.6, 133.1, 138.3, 142.7, 161.7, 163.7, 164.9; EI(+)-HRMS m/z , calcd for $C_{18}H_{18}F_4N_2OS$ (M)387.1143, found 387.1148.

2,5-bis(4-bromobutyl)thiophene (1sb). 157 mg of **1sb** as white solid, yield 45%. Purity 98.87%. Mp 67−69 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.78−1.84 (m, 4H), 1.89−1.94 (m, 4H), 2.77− 2.81 (m, 4H), 3.41−3.44 (m, 4H), 6.58 (s, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.4, 30.3, 32.2, 33.6, 124.1, 142.6; EI(+)-HRMS m/z , calcd for $C_{12}H_{18}Br_2S$ (M+23)374.9388, found 374.9392.

N,N'-(thiophene-2,5-diylbis(butane-4,1-diyl))bis(3-fluoro-5-(trifluoromethyl)benzamide) (1sf). 515 mg of **1sf** as yellow solid, yield 85%. Purity 98.28%. Mp 87−89. ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.63−1.68 (m, 4H), 1.72−1.77 (m, 4H), 2.81− 2.84 (m, 4H), 3.44−3.48 (m, 4H), 6.31 (s, 2H), 6.56−6.58 (d, 2H, *J* = 7.2 Hz), 7.42−7.44 (d, 2H, *J* = 8.4

Hz), 7.63–7.65 (d, 2H, *J* = 8.4 Hz), 7.53 (s, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.9, 29.0, 29.8, 40.4, 115.6, 115.9, 117.9, 118.2, 119.6, 119.7, 121.9, 124.2, 133.0, 133.2, 138.3, 138.4, 142.7, 161.6, 163.6, 165.1; EI(+)-HRMS m/z , calcd for $C_{28}H_{26}F_8N_2O_2S$ (M+23)629.1497, found 629.1479.

3-fluoro-5-(trifluoromethyl)-N-(3-(thiophen-2-yl)propyl) benzamide (1sd). 264 mg of **1sd** as yellow liquid, yield 80%. Purity 96.21%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2,00–2.07 (m, 2H), 2.94–2.98 (m, 2H), 3.52– 3.57 (m, 2H), 6.27 (s, 1H), 6.92–6.94 (d, 1H, *J* = 8.0 Hz), 7.13–7.14 (d, 1H, *J* = 5.2 Hz), 7.42–7.44 (d, 1H, *J* = 7.6 Hz), 7.58–7.59 (d, 1H, *J* = 8.4 Hz), 7.66 (s, 1H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.0, 31.4, 40.3, 115.6, 115.7, 115.8, 115.9, 118.0, 118.2, 119.5, 119.5, 123.8, 124.8, 127.3, 138.1, 138.2, 144.3, 161.7, 163.7, 164.9; EI(+)-HRMS m/z , calcd for $C_{15}H_{13}F_4NOS$ (M+23)354.0538, found354.0546.

3-fluoro-5-(trifluoromethyl)-N-(4-(thiophen-2-yl)butyl) benzamide (1se). 289 mg of **1se** as yellow liquid, yield 84%. Purity 95.65%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.66–1.68 (m, 2H), 1.73–1.82 (m, 2H), 2.88– 2.92 (m, 2H), 3.47–3.52 (m, 2H), 6.10 (s, 1H), 6.79–6.80 (m, 1H), 6.90–6.93 (m, 1H), 7.11–7.13 (d, 1H, *J* = 6.4 Hz), 7.44–7.46 (d, 1H, *J* = 8.0 Hz), 7.65–7.67 (d, 1H, *J* = 8.4 Hz), 7.76 (s, 1H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.0, 29.2, 29.6, 115.7, 115.9, 118.0, 118.2, 119.5, 123.3, 124.6, 127.0, 133.0, 133.1, 133.3, 138.2, 138.3, 144.8, 161.7, 163.7, 164.9; EI(+)-HRMS m/z , calcd for $C_{16}H_{15}F_4NOS$ (M+23)368.0729, found 369.0733.

3-fluoro-5-(trifluoromethyl)-N-(4-(5-(thiophene-2-yl)-thiophen-2-yl)butyl)benzamid e (3sm). 407 mg of **3sm** as yellow solid, yield 80%. Purity 98.53%. Mp 115–117℃. ^{1}H

NMR ($CDCl_3$, 400 MHz) δ:1.69–1.73 (m, 2H), 1.73–1.78 (m, 2H), 2.84–2.87 (m, 2H), 3.49–3.50 (m, 2H), 6.09 (s, 1H), 6.68–6.69 (d, 1H, *J* = 3.6 Hz), 6.96–7.01 (m, 1H), 7.03–7.04 (d, 1H, *J* = 4 Hz), 7.13–7.14 (d, 1H, *J* = 4.4 Hz), 7.18–7.19 (m, 1H), 7.13–7.14 (d, 1H, *J* = 4.4 Hz), 7.43–7.45 (d, 1H, *J* = 8 Hz), 7.65–7.69 (d, 1H, *J* = 8.8 Hz), 7.76(s, 1H) , ^{13}C NMR (DMSO-D_6, 125 MHz) δ: 29.1, 29.2, 30.1, 40.4, 115.9, 118.3, 119.7, 123.7, 123.9, 124.6, 125.6, 128.2, 135.3, 136.1, 136.8, 137.5, 138.3, 144.6, 161.8, 163.8, 165.1; EI(+)-HRMS m/z , calcd for $C_{24}H_{19}F_4NOS_3$ (2M + Na) 1041.1022, found 1041.1053.

3-fluoro-5-(trifluoromethyl)-N-(3-(5-(thiophene-2-yl)thiophen-2-yl)propyl)benzamide (2se). 309 mg of **2se** as yellow solid, yield 75%. Purity 95.25%. Mp 50-52 ℃. ^1H NMR ($CDCl_3$, 400 MHz) δ:2.02–2.09 (m, 2H), 2.91–2.95 (m, 2H), 3.55–3.60 (m, 2H), 6.15 (s, 1H), 6.74–6.75 (d, 1H, *J* = 3.2 Hz), 6.98–7.00 (m, 2H), 7.07–7.08 (d, 1H, *J* = 3.6 Hz), 7.17–7.18 (d, 1H, *J* = 5.6 Hz), 7.42–7.44 (d, 1H, *J* = 8 Hz), 7.59–7.61 (d, 1H, *J* = 8.4 Hz), 7.74(s, 1H) , ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.1, 31.3, 40.2, 115.7, 115.8, 115.9, 117.9, 118.1, 119.6, 123.5, 123.7, 124.3, 125.6, 127.9, 135.8, 137.6, 138.0, 143.5, 161.6, 163.6, 165.0; EI(+)-HRMS m/z , calcd for $C_{19}H_{15}F_4NOS_2$ (M + Na) 436.0420, found 436.0423.

N,N'-([2,2'-bithiophene]-5,5'-diylbis(propane-3,1-diyl))bis-(3,5-dihydroxybenzamide) (2sj). 414 mg of **2sj** as yellow solid, yield 75%. Purity 99.31%. Mp 118–120 ℃. ^1H NMR ($CDCl_3$, 400 MHz) δ: 0.99–1.01 (d, 2H, *J* = 6.4 Hz), 1.82–1.85 (m, 4H), 2.78–2.82 (m, 4H), 3.25–3.26 (m, 4H), 6.34 (s, 2H), 6.66–6.67 (d, 4H, *J* = 1.6 Hz), 6.80–6.81 (d, 2H, *J* = 4.0 Hz), 7.00–7.01 (d, 2H, *J* = 3.2 Hz), 7.63–7.65 (d, 2H, *J* = 8.4 Hz), 8.27 (s,

2H), 9.40 (s, 4H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 23.3, 26.9, 30.9, 54.9, 104.9, 105.4, 122.9, 125.5, 134.5, 136.9, 143.5, 158.2, 166.5; EI(+)-HRMS m/z , calcd for $C_{28}H_{28}N_2O_6S_2$ (M+23)575.1285, found575.1281.

N,N'-([2,2'-bithiophene]-5,5'-diylbis(propane-3,1-diyl))bis -(2-chlorobenzamide) (2si). 361 mg of **2si** as yellow solid, yield 65%. Purity 95.38%. Mp 144–145 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.82–1.89 (d, 4H, *J* = 28.0 Hz), 2.84–2.88 (m, 4H), 3.26–3.32 (m, 4H), 6.81–6.82 (d, 2H, *J* = 3.2 Hz), 7.02–7.03 (d, 2H, *J* = 3.6 Hz), 7.36–7.45 (m, 6H), 7.49–7.51 (d, 2H, *J* = 7.6 Hz), 8.49 (s, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 26.8, 30.8, 38.2, 123.0, 125.6, 127.0, 128.7, 129.5, 129.7, 130.5, 134.5, 137.2, 143.4; EI(+)-HRMS m/z , calcd for $C_{28}H_{26}Cl_2N_2O_2S_2$ (M+23)579.0699, found579.0705.

5,5'-bis(3-bromopropyl)-2,2'-bithiophene (2sa). 170 mg of **2sa.** as yellow solid, yield 42%. Purity 97.38%. Mp 97–99 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.17–2.24 (m, 4H), 2.96– 2.99 (m, 4H), 3.43–3.47 (m, 4H), 6.71–6.72 (d, 2H, *J* = 3.6 Hz), 6.91–6.92 (d, 2H, *J* = 3.6 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.5, 32.7, 34.3, 123.6, 124.0, 126.1, 135.6, 136.3, 142.7; EI(+)-HRMS m/z , calcd for $C_{14}H_{16}Br_2S_2$ (M+23)428.8952, found 428.8952.

N,N'-([2,2'-bithiophene]-5,5'-diylbis(propane-3,1-diyl))bis (3-fluoro-5-(trifluoromethyl)benzamide) (2sg). 330 mg of **2sg** as yellow solid, yield 50%. Purity 97.94%. Mp 150–152 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.88–1.92 (m, 4H), 2.82– 2.86 (m, 4H), 3.31–3.38 (m, 4H), 6.81–6.82 (d, 2H, *J* = 3.2 Hz), 7.00–7.01 (d, 2H, *J* = 2.8 Hz), 7.88–7.90 (d, 2H, *J* = 9.6 Hz), 8.05 (s, 2H), 8.81–8.83 (m, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 26.9, 30.6, 38.9, 115.3, 115.5, 118.4, 118.5, 120.0, 122.0,

122.9, 124.2, 125.6, 130.9, 130.0, 131.1, 131.2, 134.5, 137.9, 138.1, 143.4, 160.9, 162.9, 163.4; EI(+)-HRMS m/z , calcd for $C_{30}H_{24}F_8N_2O_2S_2$ (M+23)683.1045, found683.1043.

N,N'-([2,2'-bithiophene]-5,5'-diylbis(butane-4,1-diyl))bis-(3-fluoro-5-(trifluoromethyl)benzamide) (2sh). 340 mg of **2sh** as yellow solid, yield 50%. Purity 98.28%. Mp 152–154 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.57–1.61 (m, 4H), 1.65–1.69 (m, 4H), 2.78– 2.82 (m, 4H), 3.29–3.34 (m, 4H), 6.76–6.77 (d, 2H, *J* = 3.6 Hz), 6.97–6.98 (d, 2H, *J* = 3.6 Hz), 7.87–7.89 (d, 2H, *J* = 8.4 Hz), 8.05 (s, 2H), 8.78–8.80 (m, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.2, 28.4, 28.9, 39.0, 115.3, 115.5, 118.3, 120.0, 122.0, 122.8, 124.2, 125.4, 130.9, 131.2, 134.4, 138.0, 138.1, 143.8, 160.9, 162.9, 163.3; EI(+)-HRMS m/z , calcd for $C_{32}H_{28}F_8N_2O_2S_2$ (M+23)711.1357, found711.1356.

2-(5-(3-bromopropyl)thiophen-2-yl)-5-(thiophen-2-yl)thio-phen (3sa). 351 mg of **3sa** as yellow solid, yield 90%. Purity 95.68%. Mp 95–96 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:2.17–2.24 (m, 2H), 2.96–2.99 (m, 2H), 3.43–3.46 (m, 2H), 6.72–6.74 (d, 1H, *J* = 7.2 Hz), 6.95–7.00 (m, 3H), 7.14–7.15 (d, 1H, *J* = 5.2 Hz), 7.19–7.20 (d, 2H, *J* = 8 Hz) , ^{13}C NMR (DMSO-D_6, 125 MHz) δ: 28.5, 32.7, 34.3, 116.8, 123.6, 123.7, 123.8, 123.9, 124.0, 124.5, 124.6, 126.1, 128.1, 135.5, 136.0, 136.3, 136.6, 137.4, 142.7; EI(+)-HRMS m/z , calcd for $C_{15}H_{13}BrS_3$ (M + Na) 390.9255, found 390.9254.

2-(5-(3-azidopropyl)thiophen-2-yl)-5-(thiophen-2-yl) thiophene (3sb). 321 mg of **3sb** as yellow solid, yield 95%. Purity 95.68%. Mp 121–122 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.95–1.99 (m, 2H), 2.89–2.93 (m, 2H), 3.35–3.39 (m, 2H), 6.72–6.73 (d, 1H, *J* = 3.2 Hz), 6.97–7.02 (m, 3H), 7.06–7.07 (d, 1H, *J* = 3.6 Hz), 7.16–7.17 (d, 1H, *J* = 2.8 Hz), 7.21–7.22 (d, 1H, *J* = 4.4 Hz) , ^{13}C NMR (DMSO-D_6, 125 MHz) δ: 27.3, 30.8, 50.5,

123.7,123.8, 124.0, 124.5, 124.6, 125.9, 128.1, 135.4, 136.1, 136.3, 136.6, 137.4, 143.2; EI(+)-HRMS m/z , calcd for $C_{15}H_{13}N_3$ (M + Na) 354.0164, found 354.0157.

3-fluoro-5-(trifluoromethyl)-N-(3-(5-(5-(thiophen-2-yl)thio-phen-2-yl)thiophen-2-yl) propyl)benzamide (3sl). 198 mg of **3sl** as white solid, yield 40%. Purity 97.02%. Mp 144–146 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.02–2.09 (m, 2H), 2.92–2.96 (m, 2H), 3.55–3.60 (m, 2H), 6.13 (s, 1H), 6.75–6.76 (d, 2H, *J* = 3.2 Hz), 6.96–6.98 (m, 1H), 7.01–7.02 (d, 1H, *J* = 4.0 Hz), 7.15–7.16 (d, 1H, *J* = 3.2 Hz), 7.20–7.21 (d, 1H, *J* = 4.8 Hz), 7.42–7.44 (d, 1H, *J* = 8.0 Hz), 7.59–7.62 (d, 1H, *J* = 8.8 Hz), 7.74 (s, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.1, 31.3, 40.1, 117.9, 118.1, 119.6, 123.6, 123.8, 124.0, 124.4, 124.6, 125.7, 128.1, 135.5, 136.1, 136.4, 137.3, 138.1, 143.7, 163.7, 164.9; EI(+)-HRMS m/z , calcd for $C_{23}H_{17}F_4NOS_3$ (M+23)518.0292, found518.0300.

N,N'-([2,2':5',2''-terthiophene]-5,5''-diylbis(butane-3,1-diyl))bis(3-fluoro-5-(trifluoro methyl)benzamide) (3so). 539 mg of **3so** as yellow solid, yield 70%. Purity 98.48%. Mp 162–164 ℃. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.70–1.72 (m, 4H), 1.74–1.77 (m, 4H), 2.83– 2.85 (m, 4H), 3.48–3.50 (m, 4H), 6.67–6.68 (d, 2H, *J* = 3.6 Hz), 6.94 (s, 4H), 7.43–7.45 (d, 2H, *J* = 7.6Hz), 7.64–7.67 (d, 2H, *J* = 8.4 Hz), 7.75 (s, 2H), ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 28.2, 28.4, 28.9, 39.2, 115.5, 118.3, 118.5, 120.0, 122.0, 123.7, 124.0, 125.7, 133.6, 135.1, 138.0, 138.1, 144.6, 160.9, 162.9, 163.3; EI(+)-HRMS m/z , calcd for $C_{36}H_{30}F_8N_2O_2S_3$ (M+23)793.1228, found798.1234.

2,5-bis(5-(3-bromopropyl)thiophen-2-yl)thiophene (3sg). 413 mg of **3sg** as yellow solid, yield 85%. Purity 95.37%. Mp 112–114 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:2.17–2.22 (m, 4H), 2.96–2.99 (m, 4H), 3.43–3.47 (m, 4H), 6.71–6.72 (d, 2H, *J* = 3.6

Hz), 6.91–6.92 (d, 4H, *J* = 3.6 Hz) , ^{13}C NMR (DMSO-D_6, 125 MHz) δ: 28.5, 32.7, 34.3, 123.6, 123.9, 126.1, 135.6, 136.3, 142.7; EI(+)-HRMS m/z , calcd for $C_{18}H_{18}Br_2S_3$ (M + Na) 510.8829, found 510.8834.

N,

N'-([2,2':5',2''-terthiophene]-5,5''-diylbis(propane-3,1-diyl))bis(3-fluoro-5-(trifluoro methyl)benzamide) (3sn). 549 mg of **3sn** as yellow solid, yield 74%. Purity 96.94%. Mp 140–142 ℃. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.87–1.94 (m, 4H), 2.84–2.88 (m, 4H), 3.34–3.38 (m, 4H), 6.85–6.86 (d, 2H, *J* = 3.2 Hz), 7.11–7.13 (m, 4H), 7.87–7.89 (d, 2H, *J* = 8.4 Hz), 7.96–7.99 (d, 2H, *J* = 9.2 Hz) , 8.06 (s, 2H), 8.83 (s, 2H), ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 26.9, 30.6, 39.2, 115.3, 115.5, 118.4, 118.5, 120.0, 122.0, 123.8, 124.1, 125.8, 130.8, 130.9, 131.2, 131.2, 133.7, 135.1, 138.0, 138.1, 144.2, 160.9, 162.9, 163.4; EI(+)-HRMS m/z , calcd for $C_{34}H_{26}F_8N_2O_2S_3$ (M+23)765.0917, found765.0920.

2-(4-bromobutyl)-5-(5-(5-(4-bromobutyl)thiophen-2-yl)thio-phen-2-yl)thiophene (3si). 262 mg of **3si** as yellow solid, yield 50%. Purity 96.61%. Mp 103–105 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.81–1.86 (m, 4H), 1.92–1.99 (m, 4H), 2.82– 2.86 (m, 4H), 3.42–3.45 (m, 4H), 6.69–6.70 (d, 2H, *J* = 3.6 Hz), 6.96–6.97 (d, 2H, *J* = 3.6 Hz), 6.98 (s, 2H), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.5, 30.2, 32.1, 33.5, 123.5, 123.9, 125.4, 135.2, 136.3, 144.2; EI(+)-HRMS m/z , calcd for $C_{20}H_{22}Br_2S_3$(M+23)538.9169, found538.9142.

2-(4-azidobutyl)-5-(5-(5-(4-azidobutyl)thiophen-2-yl) thioph-en-2-yl)thiophene (3sj). 398 mg of **3sj** as yellow solid, yield 90%. Purity 97.71%. Mp 78-79 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.65–1.72 (m, 4H), 1.76–1.82 (m, 4H), 2.82– 2.86 (m, 4H),

3.30–3.33 (m, 4H), 6.69–6.70 (d, 2H, *J* = 3.6 Hz), 6.96–6.97 (d, 4H, *J* = 3.6 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.4, 28.8, 29.8, 51.4, 123.5, 123.8, 125.4, 135.2, 136.3, 144.3; EI(+)-HRMS m/z , calcd for $C_{20}H_{22}N_6S_3$ (M+23)465.0956, found465.0960

4-5-(5-(5-(4-aminobutyl)thiophen-2-yl)thiophen-2-yl)thio-phene-2-yl)butan-1-amine (3sk). 385 mg of **3sk** as yellow solid, yield 99%. Purity 98.80%. Mp 118-119 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.42–1.47 (m, 4H), 1.64–1.69 (m, 4H), 2.54– 2.60 (m, 4H), 2.79–2.83 (m, 4H), 6.84–6.85 (d, 2H, *J* = 3.2 Hz), 7.14–7.17 (d, 4H, *J* = 10.8 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.1, 28.9, 32.3, 40.9, 123.4, 123.7, 125.1, 133.2, 135.0, 144.8; EI(+)-HRMS m/z , calcd for $C_{20}H_{26}N_2S_3$ (M+1)391.1322, found391.1330

2-(4-bromobutyl)-5-(5-(thiophen-2-yl)thiophen-2-yl) thiophene (3sd). 112 mg of **3sd** as yellow solid, yield 40%. Purity 95.23%. Mp 60-62 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.83–1.89 (m, 2H), 1.92–1.97 (m, 2H), 2.79–2.86 (m, 2H), 3.42–3.45 (m, 2H), 6.69–6.70 (d, 1H, *J* = 7.8 Hz), 6.96–7.02 (d, 4H, *J* = 22.4 Hz), 7.15–7.16 (d, 1H, *J* = 2.8 Hz), 7.20–7.21 (d, 1H, *J* = 5.2 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.5, 30.2, 32.1, 33.5, 123.7, 123.8, 123.9, 124.5, 125.5, 128.1, 135.1, 135.9, 136.8, 137.5, 144.4; EI(+)-HRMS m/z , calcd for $C_{16}H_{15}BrS_3$(M+1)382.9584, found382.9592.

2-(4-azidobutyl)-5-(5-(thiophen-2-yl)thiophen-2-yl)thioph-ene (3sb). 327 mg of **3sb** as yellow solid, yield 95%. Purity 99.02%. Mp 63-65 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.66–1.72 (m, 2H), 1.75–1.82 (m, 2H), 2.83–2.86 (m, 2H), 3.30–3.33 (m, 2H), 6.69–6.71 (d, 1H, *J* = 3.2 Hz), 6.98–7.03 (d, 3H, *J* = 17.6 Hz), 7.05–7.07 (d, 1H, *J* = 3.6 Hz), 7.16–7.17 (d, 1H, *J* = 3.2 Hz), 7.20–7.22 (d, 1H, *J* = 5.2 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.4, 28.8, 29.9, 51.4, 123.6, 123.8, 123.9, 124.5, 124.6, 125.4, 128.1, 135.1,

135.9, 136.8, 137.4, 144.4; EI(+)-HRMS m/z , calcd for $C_{16}H_{15}N_3S_3$(M+1)346.0500, found346.0496.

4-(5-(5-(thiophen-2-yl)thiophen-2-yl)thiophene-2-yl) butan-1-amine (3sf). 315 mg of **3sf** as yellow solid, yield 99%. Purity 98.54%. Mp 95-97 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.44–1.48 (m, 2H), 1.64–1.70 (m, 2H), 2.60–2.63 (m, 2H), 2.80–2.84 (m, 2H), 6.85–6.86 (d, 1H, *J* = 3.6 Hz), 7.13–7.15 (m, 1H), 7.17–7.18 (d, 1H, *J* = 3.6 Hz), 7.20–7.21 (d, 1H, *J* = 3.6 Hz), 7.27–7.28 (d, 1H, *J* = 3.6 Hz), 7.35–7.36 (d, 1H, *J* = 2.8 Hz), 7.55–7.56 (d, 1H, *J* = 4.4 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.4, 29.2, 31.8, 40.9, 123.9, 124.1, 125.5, 125.7, 128.4, 133.4, 134.7, 135.7, 136.0, 145.0; EI(+)-HRMS m/z , calcd for $C_{16}H_{17}NS_3$(M+1)320.0584, found320.0595.

2-(4-bromotubyl)-5-(5-(5-(5-(4-bromobutyl)thiophen-2-yl)thiophen-2-yl)thiophene-2-yl)thiophene (4sg). 208 mg of **4sg** as yellow solid, yield 35%. Purity 95.24%. Mp 153-155 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.82–1.87 (m, 4H), 1.94–1.99 (m, 4H), 2.83– 2.86 (m, 4H), 3.43–3.46 (m, 4H), 6.70–6.71 (d, 2H, *J* = 3.2 Hz), 6.98–7.00 (m, 4H), 7.03–7.04 (d, 2H, *J* = 3.6 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.5, 30.2, 32.1, 33.5, 123.7, 123.9, 124.3, 125.5, 135.1, 135.7, 136.8, 144.5; EI(+)-HRMS m/z , calcd for $C_{24}H_{24}Br_2S_4$ (M+23) 620.9035, found 620.9019.

2-(4-azidotubyl)-5-(5-(5-(5-(4-azidobutyl)thiophen-2-yl)-thio-phen-2-yl)thiophene-2-yl)thiophene (4sh). 498 mg of **4sh** as yellow solid, yield 95%. Purity 96.77%. Mp 153-155 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.66–1.72 (m, 4H), 1.78–1.82 (m, 4H), 2.83– 2.86 (m, 4H), 3.30–3.34 (m, 4H), 6.70–6.71 (d, 2H, *J* = 3.2 Hz), 6.98–7.00 (m, 4H), 7.03–7.04 (d, 2H, *J* = 3.6 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.4, 28.8, 29.8, 51.4,

123.6, 123.9, 124.3, 125.4, 135.1, 135.7, 136.7, 144.5; EI(+)-HRMS m/z , calcd for $C_{24}H_{24}N_6S_4$ (M+23) 547.0839, found 547.0837.

4-5-(5-(5-(5-(4-aminobutyl)thiophen-2-yl)thiophen-2-yl)-thiophen-2-yl)thiophen-2-yl)butan-1-amine (4si). 467 mg of **4si** as yellow solid, yield 99%. Purity 98.80%. Mp 175-178 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.43–1.53 (m, 4H), 1.65–1.71 (m, 4H), 2.61– 2.63 (m, 4H), 2.79–2.82 (m, 4H), 6.82–6.83 (d, 2H, *J* = 2.8 Hz), 7.12–7.13 (d, 2H, *J* = 3.2 Hz), 7.15–7.16 (d, 2H, *J* = 3.2 Hz), 7.22–7.23 (d, 2H, *J* = 3.2 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.1, 28.9, 32.3, 40.9, 123.7, 123.8, 124.5, 125.2, 133.1, 134.1, 135.7, 138.7, 145.1; EI(+)-HRMS m/z , calcd for $C_{24}H_{28}N_2S_4$ (M+1) 473.1208, found 473.1191.

2-(4-bromotubyl)-5-(5-(5-(5-(thiophen-2-yl)thiophen-2-yl)-thiophen-2-yl)thiophene (4sd). 185 mg of **4sd** as yellow solid, yield 40%. Purity 95.15%. Mp 150-152 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.83–1.87 (m, 2H), 1.92–1.97 (m, 2H), 2.79–2.86 (m, 2H), 3.43–3.46 (m, 2H), 6.68–6.71 (d, 1H, *J* = 11.6 Hz), 6.98–7.02 (m, 3H), 7.05–7.09 (m, 3H), 7.17–7.18 (d, 1H, *J* = 3.6 Hz), 7.21–7.23 (d, 1H, *J* = 6.0 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 29.5, 30.2, 32.1, 33.5, 123.6, 123.7, 123.9, 124.3, 124.6, 124.7, 125.5, 128.1, 135.1, 135.6, 136.2, 136.4, 136.9 137.3;EI(+)-HRMS m/z , calcd for $C_{20}H_{17}BrS_4$ (M+1) 464.9471, found 464.9469.

2-(4-azidotubyl)-5-(5-(5-(thiophen-2-yl)thiophen-2-yl) thiophen-2-yl)thiophene (4se). 405 mg of **4se** as yellow solid, yield 95%. Purity 95.02%. Mp 147-149 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.67–1.72 (m, 2H), 1.77–1.82 (m, 2H), 2.79–2.86 (m, 2H), 3.30–3.34 (m, 2H), 6.70–6.71 (d, 1H, *J* = 2.8 Hz), 6.99–7.07 (m, 6H), 7.17–7.18 (d, 1H,

J = 2.8 Hz), 7.22–7.23 (d, 1H, *J* = 4.4 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.4, 28.8, 29.9, 51.4, 123.6, 123.7, 123.9, 124.0, 124.2, 124.3, 124.4, 124.6, 124.7, 125.1, 125.5, 128.1, 135.1, 136.2, 136.4, 136.9, 137.3, 144.5; EI(+)-HRMS m/z , calcd for $C_{20}H_{17}N_3S_4$ (M+Na) 450.0197, found 450.0197.

4-5-(5-(5-(thiophen-2-yl)thiophen-2-yl)thiophen-2-yl)thio-phen-2-yl)butal-1-amine (4sf). 400 mg of **4sf** as yellow solid, yield 99%. Purity 95.26%. Mp 150-152 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.46–1.61 (m, 2H), 1.62–1.69 (m, 2H), 2.79– 2.84 (m, 2H), 3.36–3.39(m, 2H), 6.82–6.84 (d, 1H, *J* = 7.6 Hz), 7.11–7.19 (m, 3H), 7.35–7.44 (m, 3H), 7.53–7.54 (d, 1H, *J* = 3.2 Hz), 7.73–7.78 (d, 1H, *J* = 18.4 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 27.3, 27.7, 28.5, 50.1, 123.8, 123.9, 124.6, 124.7, 125.3, 125.4, 128.0, 133.2, 134.0, 134.6, 135.3, 135.6, 135.8, 144.4; EI(+)-HRMS m/z , calcd for $C_{20}H_{19}NS_4$ (M+1) 402.0473, found 402.0474.

2-(3-bromotubyl)-5-(5-(5-(thiophen-2-yl)thiophen-2-yl)-thio-phen-2-yl)thiophene (4sa). 180 mg of **4sa** as yellow solid, yield 40%. Purity 98.79%. Mp 155-157 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.19–2.54 (m, 2H), 2.96–3.01 (m, 2H), 3.45–3.48 (m, 2H), 6.75–6.76 (d, 1H, *J* = 3.6 Hz), 6.99–7.03 (m, 2H), 7.03–7.04 (m, 1H), 7.05–7.09 (m, 2H), 7.17–7.18 (d, 1H, *J* = 2.8 Hz), 7.22–7.23 (d, 1H, *J* = 4.4 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 28.5, 32.7, 34.2, 123.8, 123.9, 124.1, 124.4, 124.5, 124.6, 124.7, 126.1, 128.1, 135.5, 135.7, 136.2, 136.5, 136.8, 137.3, 142.9; EI(+)-HRMS m/z , calcd for $C_{19}H_{15}BrS_4$ (M+23) 472.9132, found 472.9225.

2-(3-azidotubyl)-5-(5-(5-(thiophen-2-yl)thiophen-2-yl)thio-phen-2-yl)thiophene (4sb). 392 mg of **4sb** as yellow solid, yield 95%. Purity 95.85%. Mp 170-172 ℃. ^{1}H NMR

($CDCl_3$, 400 MHz) δ: 1.91–1.98 (m, 2H), 2.88–2.91 (m, 2H), 3.33–3.37 (m, 2H), 6.70–6.71 (d, 1H, *J* = 3.6 Hz), 6.97–7.07 (m, 2H), 7.15–7.16 (d, 1H, *J* = 2.8 Hz), 7.20–7.21 (d, 1H, *J* = 5.2 Hz), ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 27.26, 30.8, 50.5, 123.7, 123.9, 124.1, 124.3, 124.4, 124.6, 124.7, 125.9, 128.1, 135.4, 136.1, 136.4, 136.7, 137.3, 143.3; EI(+)-HRMS m/z , calcd for $C_{19}H_{15}N_3S_4$ (M+23) 436.0043, found 436.0041.

3-5-(5-(5-(thiophen-2-yl)thiophen-2-yl)thiophen-2-yl) thiophene-2-yl)propan-1-amine (4sc). 388 mg of **4sc** as yellow solid, yield 99%. Purity 98.45%. Mp 160-162 ℃. ^{1}H NMR (DMSO-D_6, 70 ℃, 400 MHz) δ: 1.64 (s, 2H), 2.63 (s, 2H), 3.11 (s, 2H), 6.83 (s, 1H), 7.11–7.18 (m, 3H), 7.27 (s, 3H), 7.34 (s, 1H), 7.53 (s, 1H), ^{13}C NMR (DMSO-D_6, 70 ℃, 125 MHz) δ:26,4, 33,7, 40.1, 123.7, 123.8, 123.9, 124.5, 124.6, 124.7, 125.2, 125.3, 127.9, 133.1, 133.9, 134.6, 135.3, 135.7, 135.8, 144.8; EI(+)-HRMS m/z , calcd for $C_{19}H_{17}NS_4$ (M+1) 388.0310, found 388.0316.

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Highlights

- Oligothiophene compounds are novel skeletons that inhibit influenza virus.
- Compound 3sf is the most active inhibitor with an IC_{50} of 0.029 μM.
- Docking study shows that compound 3sf binds to the cavity of H5N1 HA2 stem region.