Supporting information

A Novel STING Agonist for Cancer Immunotherapy and SARS-CoV-2 Vaccine Adjuvant

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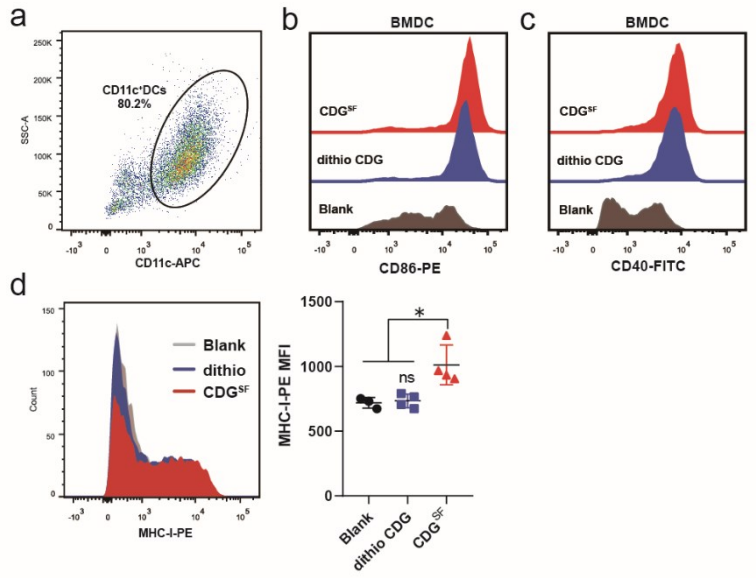


Fig. S1 (a) Representative flow cytometry dot plot of cultured bone marrow-derived dendritic cells (BMDCs) population. Representative flow cytometry histogram of CD86 (b) and CD40 (c) expression in BMDCs. (d) Representative flow cytometry histogram and quantification of MHC-I expressions on BMDCs stimulated by 10 μM of CDG^{SF} and dithio CDG without liposome transfection. Results are shown as mean ± SD of three or four separate experiments, ns: no significant difference, *p<0.05 by Student's *t*-test.

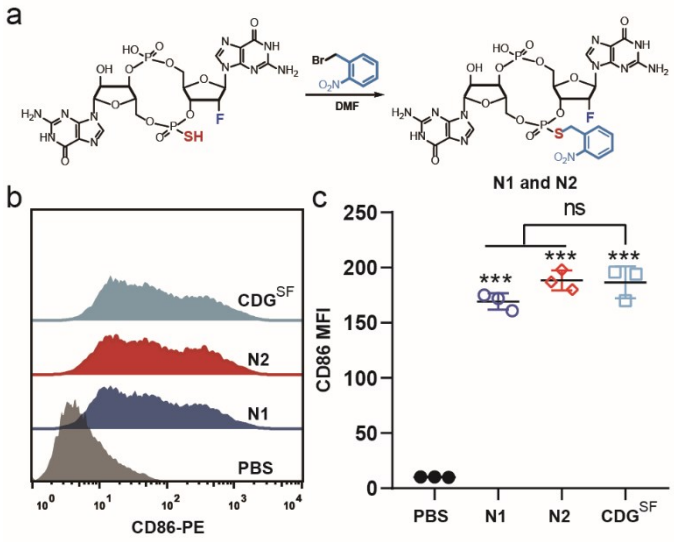


Fig. S2 (a) Chemical synthesis of 2-nitrobenzyl-caged CDG^{SF} **N1** and **N2** through mixing CDG^{SF} and 2-Nitrobenzyl bromide in DMF. Representative flow cytometry histogram (b) and quantification (c) of CD86 expressions on J774A.1 cells stimulated by **N1**, **N2** and CDG^{SF} with liposome transfection (10 μM). PBS was used as the negative control. Results are shown as mean ± SD of three separate experiments, ns: no significant difference, ***p<0.001 by Student's *t*-test.

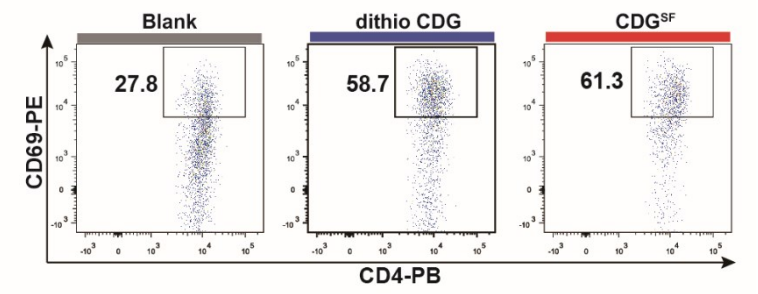


Fig. S3 Representative flow cytometry dot plot of tumor infiltrating $CD69^{+}CD4^{+}$ T cells.

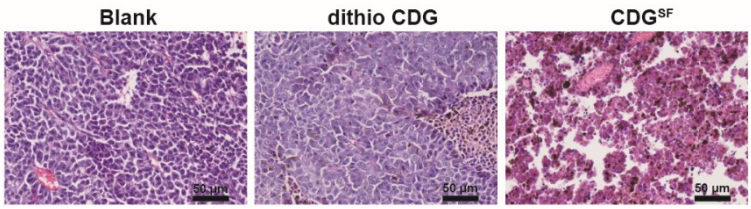


Fig. S4 Representative images of tumor tissues H&E (scale bar=50 μm).

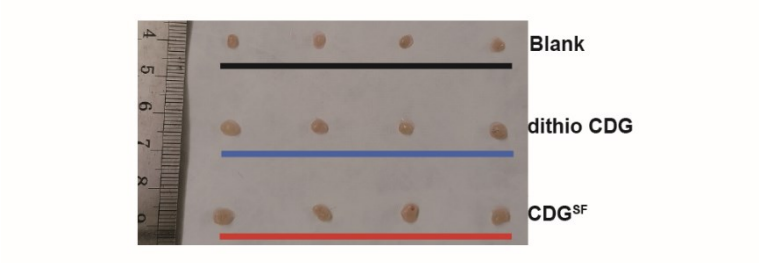


Fig. S5 Image of TDLNs (inguinal lymph nodes) from mice with established B16.F10 melanoma.

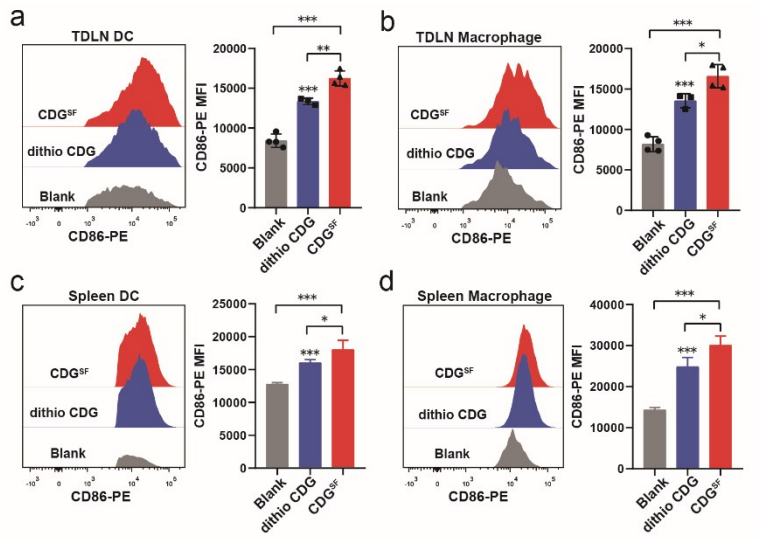


Fig. S6 Representative flow cytometry histogram (left) and quantification (right) of CD86 expressions on DCs (a) and macrophages (b) of inguinal TDLNs from mice with established B16.F10 melanoma. Representative flow cytometry histogram (left) and quantification (right) of CD86 expressions on DCs (c) and macrophages (d) of spleens from mice with established B16.F10

melanoma. Results are shown as mean ± SD, ns: no significant difference, *p<0.05, ***p<0.001 by Student's *t*-test.

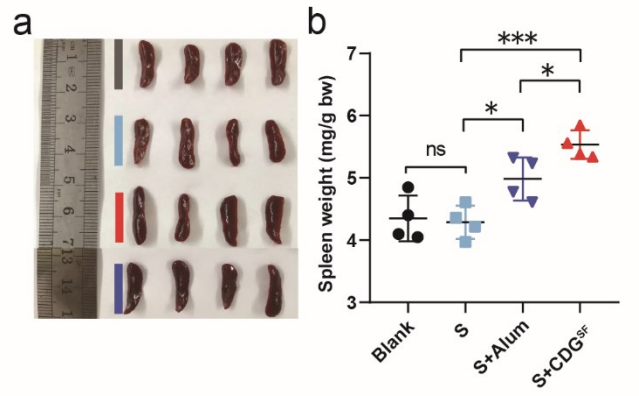


Fig. S7 Spleen images (a) and weight data (b) from vaccine immunized mice.

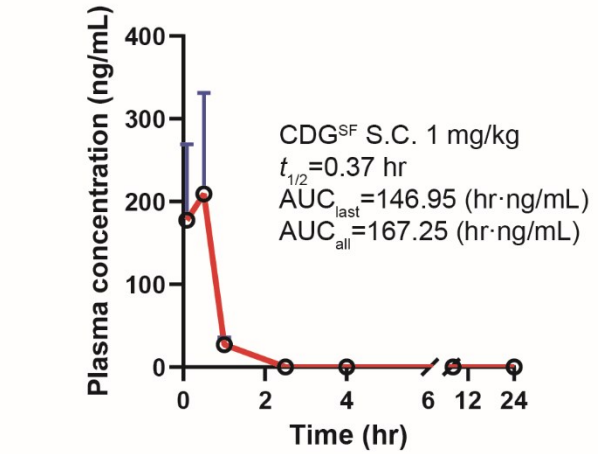


Fig. S8 Concentration-time profile of CDG^{SF} in the mouse plasma through a subcutaneous injection (1 mg/kg, mean ± SD, n = 3).

General information.

All reagents used in experiments were obtained from commercial companies and used without further purification. All solvents were reagent/HPLC grade. Anhydrous solvents were commercially available or dehydrated and distilled. The purifications of cyclic dinucleotides (CDNs) and conjugated compounds were carried on Shimadzu LC-6AD reversed-phase HPLC (YMC C18 column, 5 μm, 20×250mm, Japan) at a flow rate of 10 mL/min. The analyses of compounds above were performed on Shimadzu LC-2010A HPLC (YMC analytic C18 column, Japan, 5μm, 4.6×150 mm) at a flow rate of 0.8 mL/min. Thermo Scientific UltiMate 3000 (ESI-MS) and Shimadzu LCMS-IT/TOF (ESI-HRMS) were used to identify compounds above. NMR spectrum were recorded on Jeol-ECA-400 spectrometer (400 MHz).

Synthesis of CDG^{SF}

The CDG^{SF} was synthesized based on the one-flask synthesis strategy by Jones et al with some adjustments.[1]

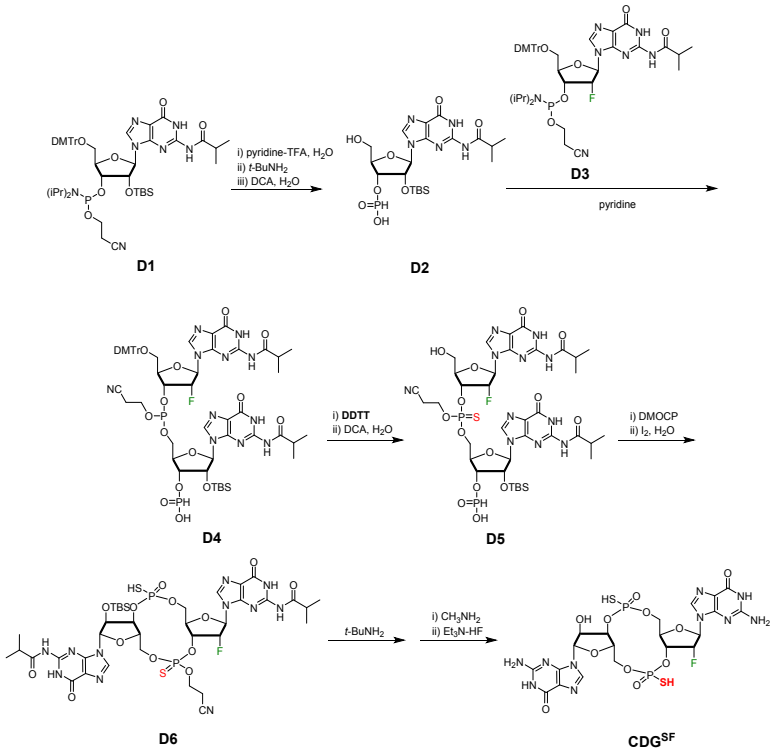
Compound **D4**

0.557 g (0.65 mmol) of N-isopropyl-2'-F-guanosine phosphoramidite monomer (**D3**) was added into a 10 mL round bottom flask. 4 mL anhydrous acetonitrile was added to dissolve the solid which was then dried by evaporation on a rotary evaporator. This process was repeated three times and 2 mL acetonitrile was left at the last time. The dried **D3** was injected into **D2** solution with a syringe and the mixture was stirred for 2 min to form **D4**.

Compound **D5**

0.113 g 3-((dimethylaminomethylidene)-amino)-3H-1,2,4-dithiazole-5-thione (DDTT, 0.55 mmol, 1.1 equiv) was added into **D4** solution to oxidize the trivalent phosphorus. The mixture was stirred for 30 min and concentrated to an oily state by evaporation. 8 mL DCM was added to dissolve the oil. 0.09 mL water and 8 mL of 6% DCA in DCM (1.5 mmol) were added and stirred for 10 min. Samples were taken during the reaction for ESI-MS detection to determine the removal of the DMTr protecting group. ESI-MS (positive mode): $C_{37}H_{55}FN_{11}O_{14}P_2SSi^+$ $[M+H]^+$ calculated 1018.2; found 1018.2. After the reaction was completed, 5 mL pyridine was added and concentrated to 2 mL volume. And then 15 mL pyridine was added and concentrated to 10 mL. Other steps were the same as the one-flask synthesis strategy by Jones et al.[1]

Finally, the compound CDG^{SF} (with two phosphorothioate stereoisomers) was obtained as triethylammonium salt with a crude yield of 40%. The crystals were then analyzed with analysis HPLC (solution A: 0.01M triethylamine acetate pH=7.0, solution B: acetonitrile, gradient is 2% to 40% of solution B in solution A in 30 min on the C18 column, 0.8 mL/min). Rt=13.6 min. To record NMR spectra, triethylammonium group was further exchanged with ammonium through using 0.05M NH_4HCO_3 as solution A. ^{1}H NMR (400M, D_2O) δ 8.26-7.71 (m, 2H), 6.38-5.87 (m, 2H), 5.86-5.45 (m, 2H), 4.58-4.32 (m, 4H), 4.07 (d, J = 11.1 Hz, 2H). ^{31}P NMR (400M, D_2O, no internal standard) δ 55.35, 54.96, -1.05. ^{19}F NMR (400M, D_2O, no internal standard) δ -122.42, -130.51. ESI-HRMS (negative mode): $C_{20}H_{22}FN_{10}O_{12}P_2S^-$ $[M-H]^-$ calculated 707.0604; found 707.0606.



Scheme S1 Synthesis of CDG^{SF}.[1]

Synthesis of N1/N2

8 mg (8.8 μmol) CDG^{SF} was dissolved in 2 mL anhydrous DMF. To the solution was added 3.8 mg (17.6 μmol, 2 equiv) o-nitrobenzyl bromide and the mixture was reacted for 1 h. The mixture was then purified with semi-preparative HPLC (solution A: 0.01M triethylamine acetate, solution B: acetonitrile, pH=7.0, gradient is 2% to 40% of solution B in solution A in 30 min on the C18 column, 10 mL/min) to obtain 1 mg of compound **N1** and1 mg of compound **N2**. **N1** Rt=18.6 min, **N2** Rt=22.4 min. ESI-HRMS (negative mode): $C_{27}H_{27}FN_{11}O_{14}P_2S^-$ $[M-H]^-$ calculated 842.0913; found **N1** 842.0918, **N2** 842.0917.

Evaluation of CDG^{SF} plasma pharmacokinetics

Six week-old C57BL/6 mice (n = 3 for each time point) were subcutaneously injected with CDG^{SF} at the dose of 1 mg/kg. Blood samples were collected at 0.083, 0.5, 1, 2.5, 4, 8 and 24 h into the tubes containing the 10X anticoagulant EDTA. 2K. Through the centrifugation (8000 rpm for 8 min), plasma was separated. Then, internal standard (Nimodipine, Fig. S9) and CH_3CN/CH_3OH (1/1) were in turn added into the plasma. After the vortex for 1 min, the mixture was centrifuged at 13000 rpm for 8 min. The supernatant was concentrated for 2 h and 10% CH_3OH/H_2O was added to dissolve the compounds. After the ultrasound and centrifugation, the solution was collected for LC-MS/MS analysis (AB Sciex QTRAP® 4500 LC/MS/MS system). WinNonlin software was adopted to calculate pharmacokinetic parameters. We thanks to Pharmaceutical Technology Center of Tsinghua University for the help in this experiment.

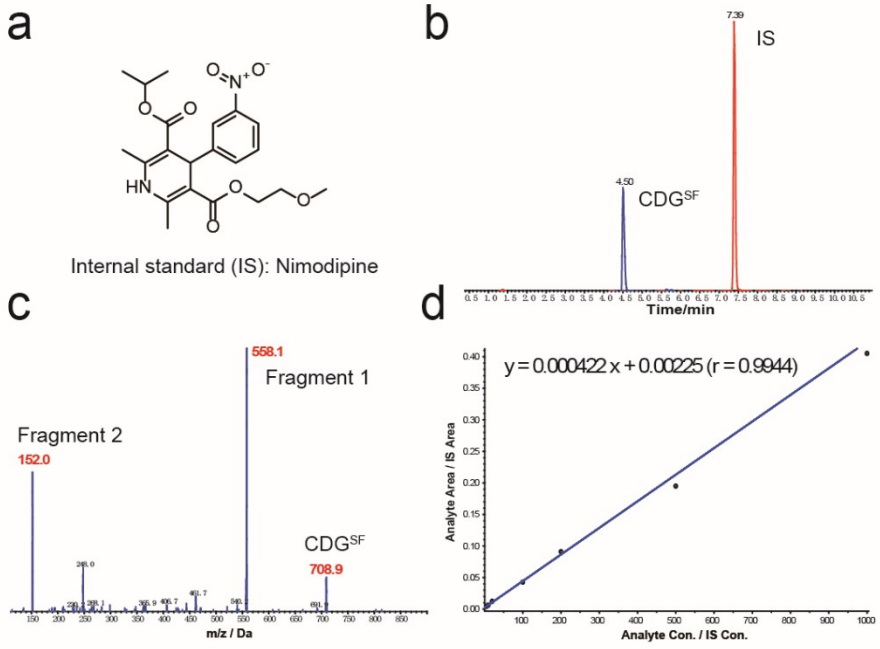


Fig. S9 Experiment details of CDG^{SF} pharmacokinetics evaluation. (a) Chemical structure of internal standard (IS) Nimodipine. (b) and (c) LC-MS/MS spectra of CDG^{SF} (CDG^{SF} Rt=4.5 min, IS Rt=7.39 min). (c) Standard curve for the quantification.

Evaluation of macrophage activation in vitro using J774A.1 cell line

J774A.1 cells (mouse monocyte macrophage cell line) were cultured in DMEM (dulbecco's

modified eagle medium) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37℃, 5% CO_2. After harvest, the cells were planted on 24-well culture plates with a density of 5×10^5 cells/well and cultured overnight. Then, 10 μM of the compounds were added respectively and incubated for 18 h. For the transfection group, Lipofectamine® 3000 was used as a transfection reagent and conducted according to manufacturer's protocol. Samples were mixed with DMEM as an A solution (150μL). Lipofectamine® 3000 (4.5 μL) was mixed with DMEM as a B solution (150μL). 5 min later, solution A was added to the solution B. Waiting for 15 min, the mixture was added dropwise to 24-well plate at a final concentration of 10 μM and incubated for 18h. Then, cells were harvested and stained with mouse anti-CD86-phycoerythrin antibodies (BD Pharmingen, dilution 1/200) on ice for 1 h. After washing, the cells were analyzed on BD Calibur flow cytometry.

Evaluation of dendritic cell (DC) activation in vitro using bone marrow-derived dendritic cells (BMDCs)

Bone marrow was isolated from the hind legs of female C57BL/6 mice and cultured in RPMI-1640 media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37℃, 5% CO_2. Granulocyte-macrophage colony stimulating factor (GM-CSF) was added (10 ng/mL). Media was replenished on day 3 or 4 and half of media was changed with fresh RPMI-1640 media containing 10 ng/ml GM-CSF at day 7-8. The cells were harvested at day 9 and planted on 24-well culture plates with a density of 5×10^5 cells/well. 10 μM of the compounds were added respectively and incubated for 24 h. Then, cells were harvested and stained with mouse anti-CD11c, anti-MHC-I, anti-CD40, and anti-CD86 antibodies (Biolegend, dilution 1/200) on ice for 1h. After washing, the cells were analyzed on BD LSRFortessa flow cytometry. Mice used in the experiments were raised in Animal Facility of Center of Biomedical Analysis in Tsinghua University and treated in compliance with the animal ethics guidelines. The animal protocol (approval number: 16-LYM2) was approved by Institutional Animal Care and Use Committee (IACUC) of Tsinghua University. Animal Facility of Center of Biomedical Analysis in Tsinghua University has been authenticated by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Tumor therapy experiment

Six week-old mice (n = 8 for each group) were subcutaneously injected with 1.5×10^5 B16.F10 melanoma cells (100 μL PBS) into the right flank of mice. The tumor volume was measured every other day. The calculating equation of tumor volume was V = L×W×W×0.5. Mice were subcutaneously injected with 20 μg CDG^{SF} in Hepes buffer for four times as shown in Figure 3a. Then, mice were euthanized when the tumor diameter reached 15 mm.

Flow cytometric analysis of tumor-infiltrating lymphocytes in B16.F10 TME

Six week-old mice (n = 8 for each group) were subcutaneously injected with 1.5×10^5 B16.F10 melanoma cells (100 μL PBS) into the right flank of mice. Mice were subcutaneously injected with 20 μg CDG^{SF}, 20 μg dithio CDG and HEPES buffer as the blank group, respectively, for two times at the interval of three days. 24 h after the second injection, mice were euthanized to harvest the tumor tissues, inguinal draining lymph nodes and spleens. Tumor tissues were dissociated and filtered with a 70 μm cell strainer to get single cells in PBS. After centrifugation, 5 mL 40% Percoll in PBS was added into the glass tube to get lymphocytes through centrifugation. The lymphocytes were stained with APC-αCD3 (17A2), PB-αCD4 (RM4-4), FITC-αCD8α (53-6.7) and PE-αCD69

(H1.2F3) for FACS analysis on BD LSRFortessa flow cytometry.

Evaluation of macrophages and DCs activation in inguinal TDLN and spleen

The spleens and inguinal TDLNs were grinded and filtered through a 40 μm cell strainer. And red blood cells (RBCs) were lysed using lysis buffer for RBC. The left cells were first blocked with anti-CD16/32 antibody (BD Pharmingen, dilution 1/200) and then labeled with mouse antibodies for CD11c (anti-CD11c-FITC, BD Pharmingen, dilution 1/200), F4/80 (anti-F4/80-APC, BD Pharmingen, dilution 1/200) and CD86 (anti-CD86- phycoerythrin, BD Pharmingen, dilution 1/200) at 4℃ for 1h. After washing, the cells were analyzed on BD LSRFortessa flow cytometry.

SARS-CoV-2 vaccines immunization

6-8 week old Babl/c mice (4-5 mice per group, female) were separately subcutaneously vaccinated with SARS-CoV-2 S protein 5 μg/mouse, CDG^{SF} 20 μg/mouse, Alhydrogel® adjuvant 2% 100 μg/mouse. SARS-CoV-2 Spike protein (S1+S2 ECD, gene: YP_009724390.1) was purchased from Sino Biological Inc. Alhydrogel® adjuvant 2% was purchased from InvivoGen. Immunizations were conducted for three times biweekly. Antisera and spleens were collected one week after the last administration.

IFN-γ enzyme-linked immunospot assay (IFN-γ ELISPOT)

IFN-γ ELISPOT Kit was purchased from Dakewe Biotech Co., Ltd. Spleens from immunized mice were grinded and filtered through a 40 μm cell strainer. And red blood cells (RBCs) were lysed using lysis buffer for RBC. Splenocytes were counted and added to 96-well kit at 1000,000 amount/ 100 μL per well. Then SARS-CoV-2 S protein was added to the well (final concentration: 50 μg/mL) and cells were stimulated for 36 h. The spot forming procedure was performed according to the Kit instruction.

SARS-CoV-2-specific antibody titers

1 μg/mL SARS-CoV-2 S protein in coating buffer (0.1M $NaHCO_3$ solution, pH=9.6) was added to high-binding 96-well ELISA plate (Costar 3590, 100 μL/well). After incubating overnight at 4℃ and washing with PBST solution (0.05% Tween in PBS buffer), the wells were blocked by 0.25% gelatin PBS solution for 3 h at room temperature (RT). After washing with PBS and PBST, the diluted antisera (1:200) was added to each well (100 μL per well) and incubated for 1.5h at 37℃. After washing again, diluted rabbit anti-mouse IgG-Peroxidase antibodies (1/2000 dilution, Sigma) were added to each well (100 μL per well) and incubated for 1 h at 37℃. After washing and spin-drying, 3,3',5,5'-Tetramethylbenzidine (TMB) was added to plate (200 μL per well) and incubated for 4 min and then stopped by 2 M H_2SO_4 (50 μL per well). Optical absorption was measured at wavelength of 450 nm (Synergy 4, Biotek) and antibody titer was defined as highest dilution yielding an optical absorption of 0.1 or greater over that of negative control antisera.

SARS-CoV-2-specific antibody isotypes

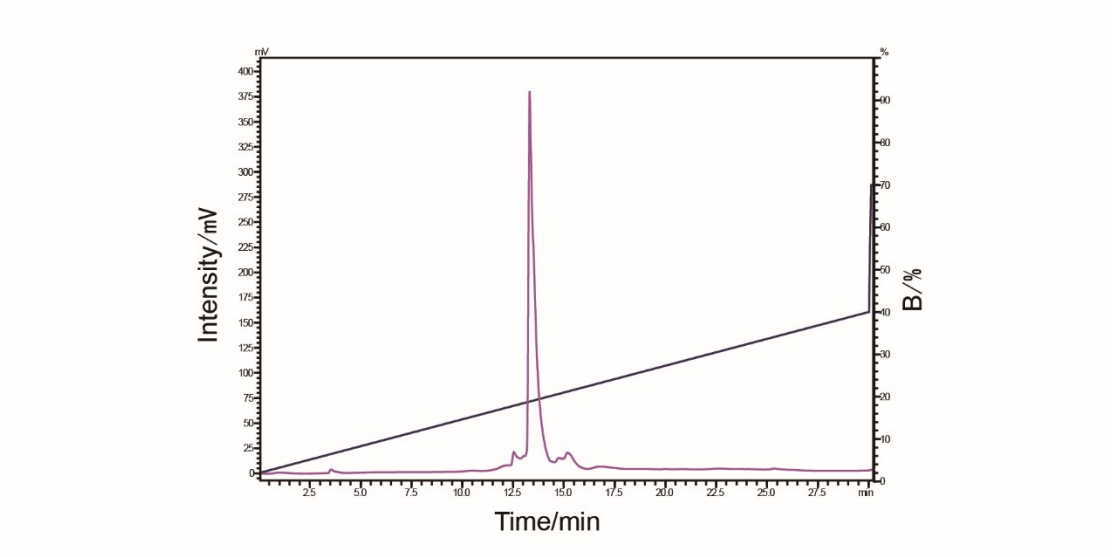
96-well ELISA plate was coated with SARS-CoV-2 S protein according to the procedure described above. The antisera were diluted to 1:12800 and added to each well, incubating for 1.5 h at 37℃. After washing with PBS and PBST, isotype antibodies IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (anti-mouse antibodies from goat, Sigma) were diluted to 1:1000 and added to each well (100 μL per well). After incubation for 1.5 h at 37℃ and washing, 200 μL TMB substrate described above

was added to plate, incubated for 4 min and stopped by 2 M H_2SO_4 (50 μL per well). Optical absorption was also measured at wavelength of 450 nm (Synergy 4, Biotek).

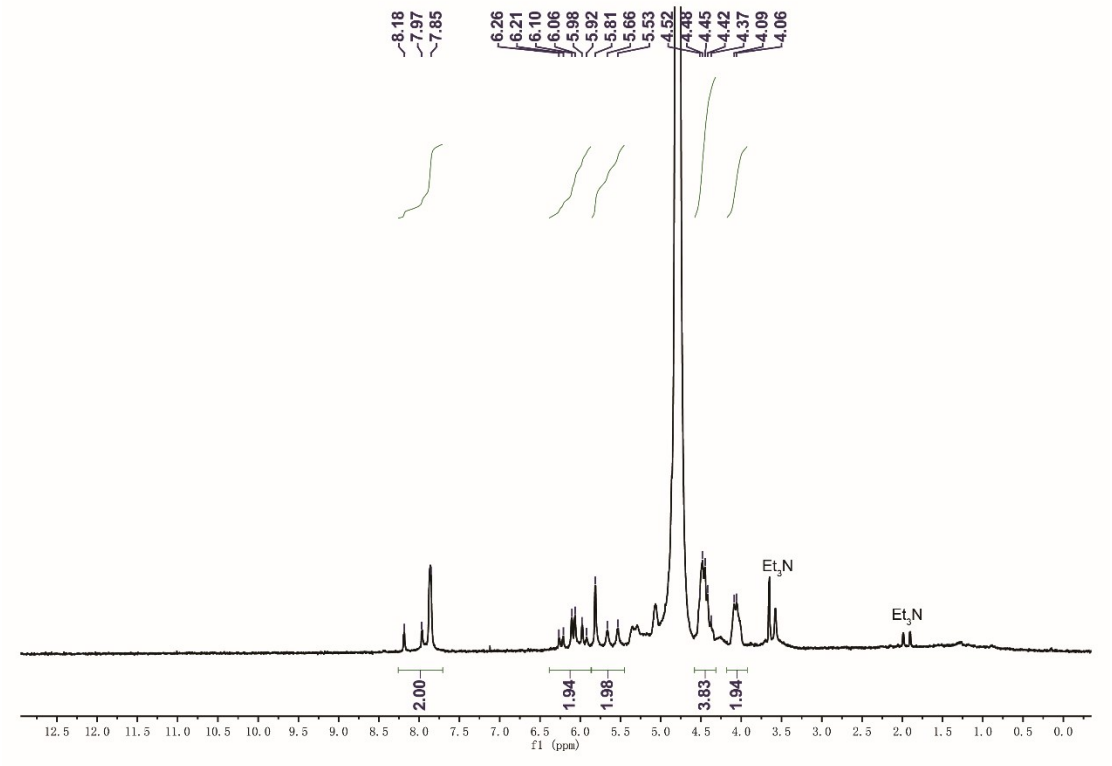
Analytic data of compounds

CDG^{SF}. Analytic gradient is 2% to 40% of solution B (acetonitrile) in solution A (0.01M triethylamine acetate, pH=7.0) in 30 min on the analytic C18 column (λ=260nm). Retention time is 13.6 min.

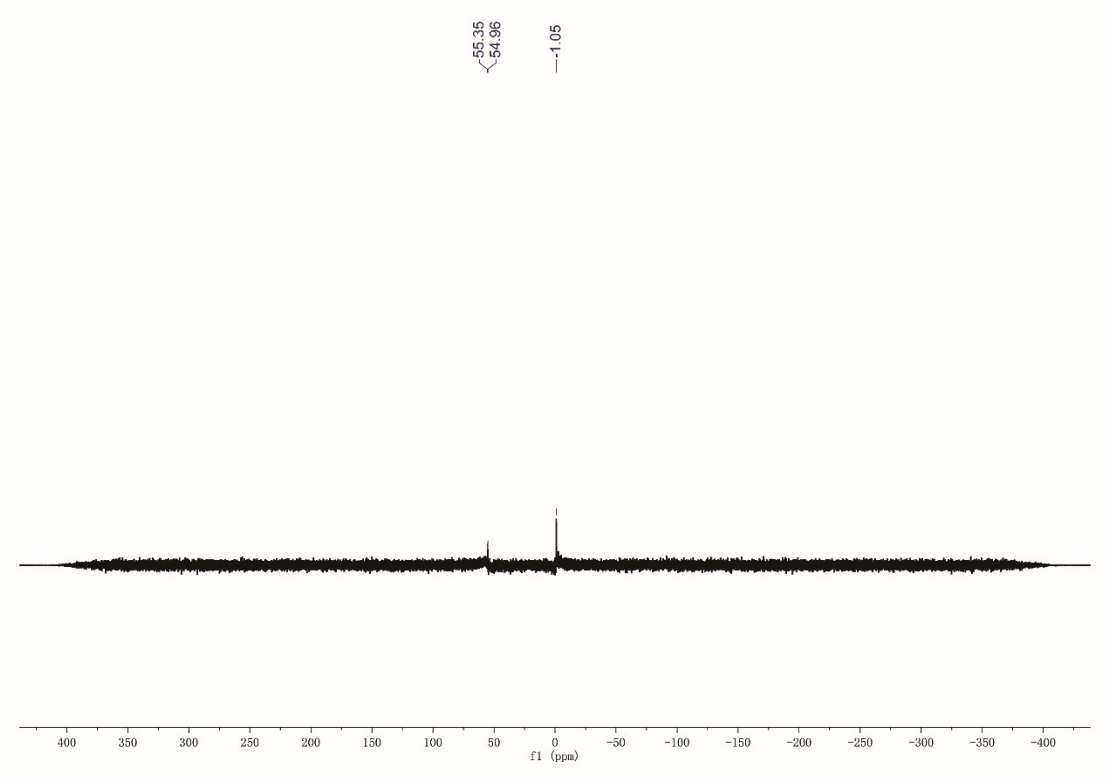
Analytic HPLC trace of **CDG^{SF}**:



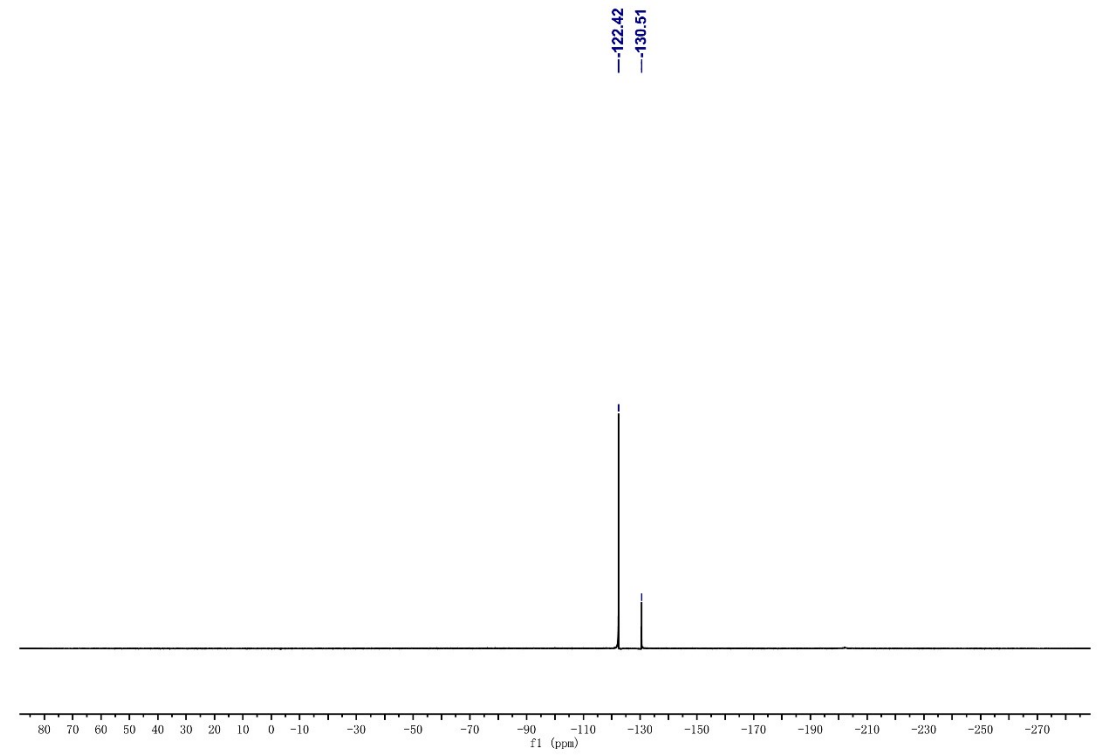
^{1}H NMR (400M, D_2O) of **CDG^{SF}**:



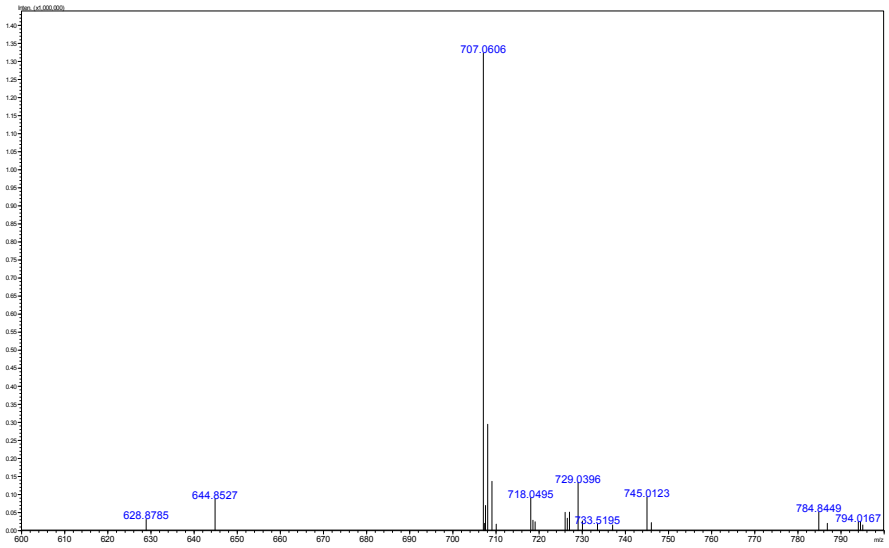
^{31}P NMR (400M, D_2O, no internal standard) of **CDG^{SF}**:



^{19}F NMR (400M, D_2O, no internal standard) of **CDG^{SF}**:

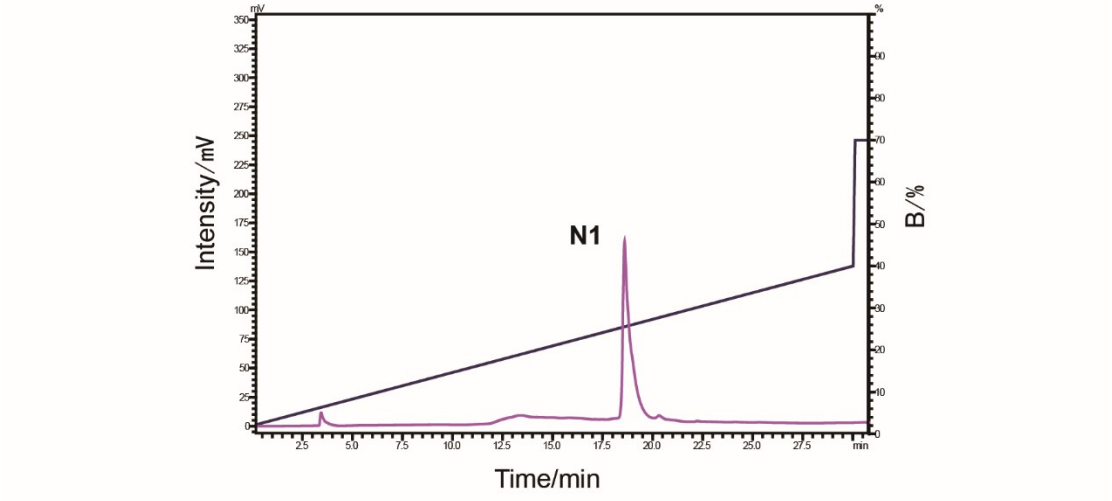


ESI-HRMS (negative mode) of **CDG^{SF}**:



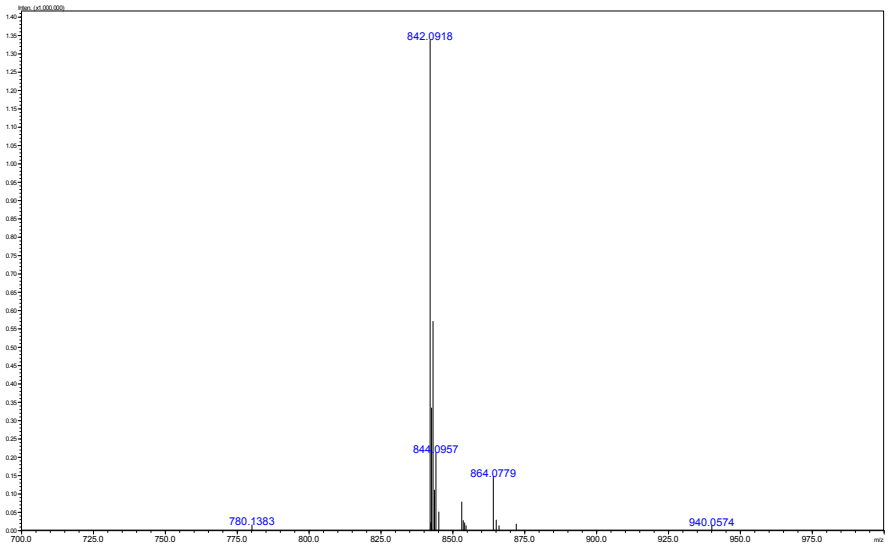
N1 and N2. Analytic gradient is 2% to 40% of solution B (acetonitrile) in solution A (0.01M triethylamine acetate, pH=7.0) in 30 min on the analytic C18 column (λ=260nm). Retention time is 18.6 min (**N1**) and 22.4 min (**N2**). ESI-HRMS (negative mode): $C_{27}H_{27}FN_{11}O_{14}P_2S^-$ $[M-H]^-$ calculated 842.0913; found **N1** 842.0918, **N2** 842.0917.

Analytic HPLC trace of **N1 and N2**:

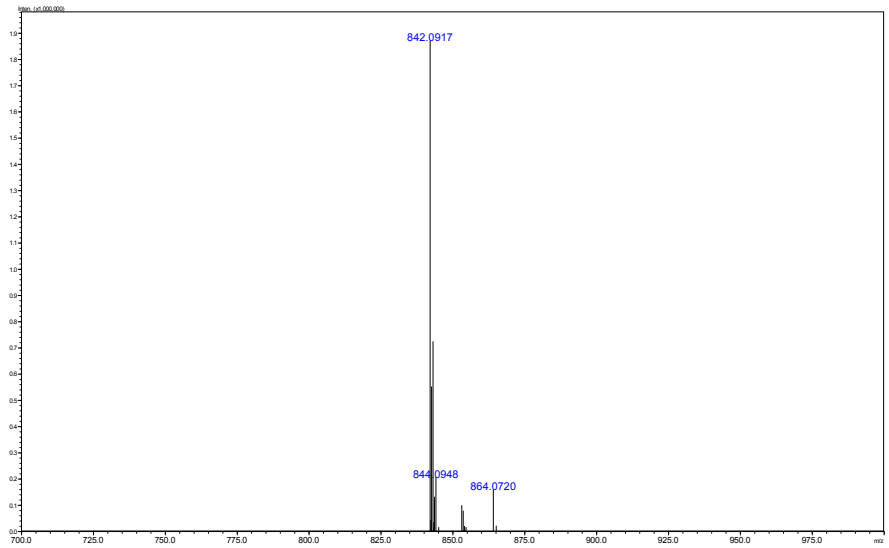


ESI-HRMS (negative mode):

N1



N2



Reference

1. (a)B. L. Gaffney, E. Veliath, J. Zhao and R. A. Jones, *Org. Lett.*, 2010, **12**, 3269; (b)B. L. Gaffney and R. A. Jones, *Curr. Protoc. Nucleic Acid Chem.*, 2012, **Chapter 14**, Unit 14 8 1.