Supporting Information

Pyridobenzothiazolones exert potent anti-dengue activity by hampering multiple functions of NS5 polymerase

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1. Chemistry

1.1. Synthesis of pyridobenzothiazolones 2-9

Compounds **2-9** were synthesized following the procedure outlined in Scheme 1. 2-Methyl-1,3-benzothiazol-5-ol was reacted with cycloheptanol employing Mitsunobu reaction to afford compound **12** which was reacted with diethylcarbonate by using NaH as base to obtain acetate derivative **13**. This latter was further functionalized by reaction with DMF-DMA to give acrylate intermediate **14** that was combined in neat conditions with phenylacetic anhydride to afford PBTZ ester **15** in good yield. Esters **15** and the C-8-cycloheyloxy analogue **16**, prepared as previously reported,[1] were hydrolyzed under basic conditions into carboxylates **17** and **18**[1] which were amidated with methyl ester amino acid hydrochlorides, in presence of TBTU, to afford derivatives **19-22**. Final mild ester cleavage, using LiOH in dioxane provided target acids **2-5** in fairly good yield. Employing the previous amidation conditions, the key intermediate **18**[1] was coupled with benzylamines to give target compounds **6** and **7** in moderate yield, while compound **8** was synthesized by refluxing ester **16**[1] in ethanolamine. Decarboxylation of acid **18**[1] in a mixture of Dowtherm A/ethylene glycol under MW heating afforded the C-4 unsubstituted PBTZ **9**.

1.2. Synthesis of pyridones 10 and 11

The synthesis of pyridones **10** and **11** is depicted in Scheme 2. Chan-Lam oxidative *N*-arylation with (3-methoxyphenyl)boronic acid of pyridone **23**[2] led to derivative **24** that was subsequently coupled with phenylboronic acid by Suzuki-Miyaura reaction under MW irradiation to give **25** in high yield. Intermediate **25** was de-methylated with BBr_3 at 0 °C affording phenol **26,** which was alkylated with cyclohexanol under Mitsunobu conditions to give ether intermediate **27**, in moderate yield. Moreover, *N*-methylpyridone intermediate **28**,[3] prepared according literature, was arylated with phenylboronic acid to afford compound **29** in good yield. Then, similarly to the procedure described in Scheme 1 for the preparation of compounds **2-5**, esters **27** and **29** were hydrolyzed into acid intermediates **30** and **31**, which were submitted to amidation with Tyr methyl ester hydrochloride. The obtained intermediates **32** and **33** were finally hydrolyzed into target carboxylic acid derivatives **10** and **11**.

2. Experimental procedures: general chemistry

Unless otherwise indicated, all starting materials were commercially available. Reagents and solvents were purchased from common commercial suppliers and were used as received. HPLC-grade solvents used for HPLC analysis were purchased by Sigma-Aldrich and all the employed mobile phases were degassed with 20 min sonication before use. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a Büchi rotary evaporator under reduced pressure. All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel 60F254 (Merck) and visualized by using UV or iodine. Flash column chromatography separations were

carried out on Merck silica gel 60 (mesh 230-400) or using a semi-automatic Biotage SNAP Cartridge KP-Sil - 50μM irregular silica and Biotage SNAP Cartridge KP-C18-HS - 50μM irregular silica eluting with the indicated solvents. Melting points were determined in capillary tubes (BüchiElectrotermal model 9100) and are uncorrected. Yields were of purified products and were not optimized. ^{1}H NMR spectra were recorded at 200 or 400 MHz (Bruker Avance DRX-200 or 400, respectively) while ^{13}C NMR spectra were recorded at 100 MHz (Bruker Avance DRX-400). Chemical shifts are given in ppm (δ) relative to TMS and calibrated using residual undeuterated solvent as internal reference. Spectra were acquired at 298 K and coupling constants (J) are reported in Hz. Data processing was performed with standard Bruker software XwinNMR and the spectral data are consistent with the assigned structures. Microwave assisted reactions were conducted with a microwave oven type Biotage Initiator Sixty (400W) and the reaction conditions are reported for each compound. All compounds were ≥95% pure as determined by HPLC analysis that were carried out employing an HPLC Waters LC Module I Plus and the purity was determined as the area of each peak. The peaks were revealed at 254 nm and at λ max of each compound using a X-Terra MS C18 column reversed-phase (3.5 μm spherical hybrid, 4.6 mm x 150 mm, 3.5 μm particle size) with 12 min at 1.0 mL/min isocratic of: 70% CH_3CN in 30% H_2O with 0.1% formic acid for compounds **2-5**; 70% CH_3CN in 30% H_2O for compounds **6** and **8**; 90% CH_3CN in 10% H_2O for compounds **7** and **9**; 60% CH_3CN in 40% H_2O with 0.1% formic acid for compound **10**; 30% CH_3CN in 70% H_2O with 0.1% formic acid for compound **11**. Injection volume was of 20 μL and column temperature of 30 °C, peaks retention time (r_t) are given in minutes. High resolution mass detection was based on electrospray ionization (ESI) in positive polarity using Agilent 1290 Infinity System equipped with a MS detector Agilent 6540A Accurate Mass Q-TOF.

2.1. Synthesis of pyridobenzothiazoles 2-9

General Procedure for Amidation Reaction. (Method A). To a mixture of the appropriate acid **18**, (1.0 mmol) and the appropriate aa methyl ester hydrochloride or the desired amine (1.3 mmol) in dry DMSO (15mL), TBTU coupling reagent (1.3 mmol), and DIPEA (4.6 mmol) were added. After stirring at room temperature for 1.45-24 h, the reaction mixture was poured into ice/water and acidified with 2N HCl (pH=5 - 6) to give a precipitate which was filtered under vacuum to give the relative solid which was purified, as described below for each compound, in order to obtain the desired pyridobenzothiazolone derivatives.

Methyl N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-L-serinate (20). Following the general procedure and starting from intermediate **18**[1] and using L-Ser methyl ester hydrochloride, compound **20** was obtained after purification by semi-automated column chromatography eluting with $CHCl_3$/Acetone 98:2 and subsequently tritured with EtOH giving a yellow solid in 47% yield (reaction time: 2 h): mp 158-159 °C; TLC ($CHCl_3$/MeOH 95:5v/v) R_f = 0.6. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.86-8.84 (m, 2H, Ser-NH and H-9), 8.52 (s, 1H, H-3), 7.89 (d, J = 8.8 Hz, 1H, H-6), 7.76 (d, J = 7.4 Hz, 2H, Ar-H), 7.46-7.42 (m, 2H, Ar-H), 7.36-7.32 (m, 1H, Ar-H), 7.18 (dd, J = 2.2 and 8.8 Hz, 1H, H-7), 5.08 (t, J = 6.0 Hz, 1H, OH), 4.60-4.55 (m, 1H, Ser CH), 4.39-4.37 (m, 1H, Cy-OCH), 3.76 (t, J = 5.8 Hz, 2H, Ser-CH_2), 3.62 (s, 3H, CH_3), 1.90 (m, 2H, Cy-CH_2), 1.68-1.56 (m, 2H, Cy-CH_2), 1.55-1.18 (m, 6H, Cy-CH_2).

Methyl N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-beta-alaninate (21). Following the general procedure and starting from

intermediate **18**[1] and using β-Ala methyl ester hydrochloride, compound **21** was obtained after purification by semi-automated column chromatography eluting with CH_2Cl_2 100% to give a yellow solid in 27% yield: mp 218-219 °C; TLC ($CHCl_3$/MeOH 99:1 v/v) R_f=0.5. ^{1}H NMR (400 MHz, DMSO-d_6): δ8.84 (d, *J* = 2.2 Hz, 1H, H-9), 8.72 (t, *J* = 5.2 Hz, 1H, β-Ala NH), 8.35 (s, 1H, H-3), 7.88 (d, *J* = 8.7 Hz, 1H, H-6), 7.74 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.45-7.41 (m, 2H, Ar-H), 7.35-7.31 (m, 1H, Ar-H), 7.17 (dd, *J* = 2.2 and 8.6 Hz, 1H, H-7), 4.38-4.34 (m, 1H, Cy-OCH), 3.82 (s, 3H, CH_3), 3.51-3.46 (m, 2H, β-Ala NHC$H_2$$CH_2CO_2CH_3$), 2.58 (t, *J* = 6.9 Hz, 2H, β-Ala $NHCH_2CH_2CO_2CH_3$), 1.91-1.90 (m, 2H, Cy-CH_2), 1.69-1.66 (m, 2H, Cy-CH_2), 1.54-1.17 (m, 6H, Cy-CH_2).

Methyl N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-3-hydroxy-L-phenylalaninate (22). Following the general procedure and starting from intermediate **18**[1] and using L-*m*-OH Phe methyl ester hydrochloride, compound **22** was obtained after purification by flash column chromatography CH_2Cl_2/acetone 98:2 giving a yellow solid in 49% yield (reaction time: 2 h): TLC ($CHCl_3$/MeOH 98:2 v/v)R_f = 0.4. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.27 (s, 1H, Phe OH), 8.98 (d, *J* = 8.0 Hz, 1H, Phe NH), 8.83 (s, 1H, H-9), 8.45 (s,1H, H-3), 7.86 (d, *J* = 8.6 Hz, 1H, H-6), 7.75 (d, *J* = 8.0 Hz , 2H, Ar-H), 7.45 (t, *J* = 7.6 Hz , 2H, Ar-H), 7.37-7.31 (m, 1H, Ar-H), 7.17 (m, 1H, H-7), 7.01 (t, *J* = 8.8 Hz, 1H, Ar-H), 6.67-6.64 (m, 2H, Ar-H), 6.54 (d, *J* = 8.2 Hz, 1H, Ar-H), 4.66-4.61 (m, 1H, Phe CH), 4.36 (m, 1H, Cy-OCH), 3.60 (s, 3H, CH_3), 3.07-2.94 (m, 2H, Phe CH_2), 1.90 (m, 2H, Cy-CH_2), 1.67 (m, 2H, Cy-CH_2), 1.46-1.18 (m, 6H, Cy-CH_2).

8-(Cyclohexyloxy)-N-(4-hydroxybenzyl)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazole-4-carboxamide (6). Following the general procedure and starting from intermediate **18**[1] and using 4-Hydroxybenzylamine, compound **6** was obtained after purification by flash column chromatography CH_2Cl_2/acetone 95:5 giving a yellow solid in 20% yield (reaction time: 24h): mp 244-245 °C; TLC (CH_2Cl_2/MeOH 95:5v/v) R_f = 0.5. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.27 (s, 1H, Bn OH), 9.08 (t, *J* = 5.6 Hz, 1H, Bn NH), 8.84 (d, *J* = 2.3 Hz, 1H, H-9), 8.43 (s, 1H, H-3), 7.87 (d, *J* = 8.7 Hz, 1H, H-6) , 7.76 (d, *J* = 7.4 Hz, 2H, Ar-H), 7.41 (t, *J* = 7.4 Hz, 2H, Ar-H), 7.32 (m, 1H, Ar-H), 7.17 (dd, *J* = 2.3 and 8.7Hz, 1H, H-7), 7.12 (d, *J* = 8.3 Hz, 2H, Bn Ar-H), 6.67 (d, *J* = 8.4 Hz, 2H, Bn Ar-H), 4.36-4.35 (m, 3H, Bn $CH_2$and Cy-OCH), 1.90 (m, 2H, Cy-CH_2), 1.67 (m, 2H, Cy-CH_2), 1.49-1.13 (m, 6H, Cy-CH_2). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 164.07, 161.28, 156.47, 156.17, 151.47, 138.81, 136.64, 133.75, 129.97, 129.25, 129.20, 128.35, 127.75, 122.98, 122.35, 120.93, 116.46, 115.31, 107.38, 105.73, 75.61, 42.51, 31.45, 25.41, 23.37. UV max λ = 231 nm; HPLC: r_t 5,630 minutes. HRMS (ESI) calculated for $C_{31}H_{28}N_2O_4S$ $[M + H]^+$ 525.1848, found 525.1843.

N-benzyl-8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazole-4-carboxamide (7). Following the general procedure and starting from intermediate **18**[1] and using benzylamine, compound **7** was obtained after purification by crystallization by 6:1 EtOH/DMF giving a yellow solid in 25% yield (reaction time: 24h): mp 285 °C; TLC ($CHCl_3$/MeOH 99:1 v/v) R_f = 0.5. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.24-9.21 (m, 1H, BnNH) 8.89 (d, *J* = 2.2 Hz, 1H, H-9), 8.50 (s, 1H, H-3), 7.91 (d, *J* = 8.7 Hz, 1H, H-6) 7.83-7.81 (m, 2H, Ar-H), 7.51-7.45 (m, 2H, Ar-H), 7.39-7.24 (m, 5H, Ar-H), 7.27-7.23 (m, 1H Ar-H), 7.21 (dd, *J* = 2.2 and 8.7 Hz, 1H, H-7), 4.54-4.53 (m, 2H, BnCH_2), 4.43-4.37 (m, 1H, Cy-OCH), 2.09-1.95 (m, 2H, Cy-CH_2),

1.74-1.64 (m, 2H, Cy-CH_2), 1.54-1.11 (m, 6H, Cy-CH_2).^{13}C-NMR (100 MHz, DMSO-d_6): δ 164.33, 161.27, 156.23, 151.61, 139.83, 138.90, 136.74, 133.80, 129.27, 128.70, 128.32, 127.79, 127.69, 127.24, 122.99, 122.30, 121.00, 116.43, 107.49, 105.69, 75.62, 43.10, 31.51, 25.46, 23.41. UV max λ = 237 nm; HPLC: r_t 5,379 minutes. HRMS (ESI) calculated for $C_{31}H_{28}N_2O_3S$ $[M + H]^+$ 509.1899, found 509.1883

8-(Cyclohexyloxy)-N-(2-hydroxyethyl)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazole-4-carboxamide (8). A mixture of starting pyridobenzothiazolone ethyl ester **16**[1] (0.10g, 0.22 mmol) and excess of 2-ethanolamine (1 mL) was heated to reflux in neat conditions for 36 h. After cooling, the residue was poured into ice/water and acidified with 2N HCl (pH = 5) to give a precipitate which was filtered under vacuum and purified via crystallization by 1:1 cyclohexane/EtOAc, giving the desired compound **8** (30 mg, 29%) as a light yellow solid: mp 151-152 °C; TLC($CHCl_3$/MeOH 95:5 v/v) R_f= 0.3. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.89 (d, J = 2.3 Hz, 1H, H-9), 8.70 (t, J = 5.4 Hz, 1H, NH), 8.46 (s, 1H, H-3), 7.92 (d, J = 8.7 Hz, 1H, H-6), 7.81 (d, J = 7.4 Hz, 2H, Ar-H), 7.49-7.45 (m, 2H, Ar-H), 7.39-7.36 (m, 1H, Ar-H), 7.21 (dd, J = 2.4 and 8.7 Hz, 1H, H-7), 4.79 (t, J = 5.6 Hz, 1H, OH), 4.43-4.39 (m, 1H, Cy-OCH), 3.55-3.51 (q, J = 6.0 Hz, 2H, $NHCH_2CH_2OH$), 3.37 (q, J = 5.8 Hz, 2H, $NHCH_2CH_2OH$), 2.08-1.94 (m, 2H, Cy-CH_2), 1.73-1.71 (m, 2H, Cy-CH_2), 1.54-1.21 (m, 6H, Cy-CH_2). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 164.52, 161.34, 156.17, 151.30, 138.72, 136.58, 133.89, 129.27, 128.46, 127.88, 122.93, 122.42, 120.84, 116.56, 107.32, 105.85, 75.73, 59.95, 42.47, 31.45, 25.41, 23.38. UV max λ = 234 nm; HPLC: r_t 5,305 minutes. HRMS (ESI) calculated for $C_{26}H_{26}N_2O_4S$ $[M + H]^+$ 463.1691, found 463.1675.

General Procedure for the Hydrolysis of Methyl Esters (Method B). A solution of the appropriate methyl ester (**20-22**) (1mmol) and aq 1N LiOH (5mmol) in 1,4-dioxane (5mL) was stirred for 40 min to 1.30 h at room temperature. Then, the reaction mixture was poured into ice/water, acidified with 2N HCl (pH =4) to give a precipitate which was filtered under vacuum and purified as reported below for each compound.

N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-L-serine (3). Starting from **20** the target acid **3** was obtained as light yellow solid (reaction time: 1 h, purification method: crystallization by EtOH, yield 47%): mp 252-254 °C; $[\alpha]_D$ = -0.15 (0.5% p/v, DMSO). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.73 (bs, 1H, COOH), 8.98 (s, 1H, H-9), 8.74 (d, J = 7.4 Hz, 1H, Ser-NH), 8.52 (s, 1H, H-3), 7.89 (d, J = 8.7 Hz, 1H, H-6), 7.77 (d, J = 7.4 Hz, 2H, Ar-H), 7.46-7.42 (m, 2H, Ar-H), 7.36-7.32 (m, 1H, Ar-H), 7.19-7.16 (m, 1H, H-7), 5.00 (bs, 1H, OH), 4.52-4.50 (m, 1H, Ser CH), 4.37 (m, 1H, Cy-OCH), 3.77 (m, 2H, Ser-CH_2), 1.90 (m, 2H, Cy-CH_2), 1.67 (m, 2H, Cy-CH_2), 1.46-1.26 (m, 6H, Cy-CH_2). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 172.35, 164.59, 161.30, 156.25, 151.84, 138.94, 136.73, 134.19, 129.35, 128.35, 127.72, 123.10, 122.35, 120.90, 116.47, 107.48, 105.45, 75.61, 61.45, 56.12, 31.50, 25.45, 23.41. UV max λ = 238 nm; HPLC: r_t 4,450 minutes. HRMS (ESI) calculated for $C_{27}H_{26}N_2O_6S$ $[M + H]^+$ 507.1590, found 507.1580

N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-*β*-alanine (4). Starting from **21** the target acid **4** was obtained as light yellow solid (reaction time: 1 h, purification method: crystallization by 1:1 cyclohexane/EtOAc, yield 44%): mp 197-198 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.27 (bs, 1H, COOH), 8.83 (s, 1H, H-9), 8.75 (bs, 1H, β-Ala

NH), 8.38 (s, 1H, H-3), 7.87 (d, *J* = 8.5 Hz, 1H, H-6), 7.75 (d, *J* = 7.4 Hz, 2H, Ar-H), 7.44-7.42 (m, 2H, Ar-H), 7.35-7.31 (m, 1H, Ar-H), 7.17-7.15 (m, 1H, H-7), 4.35 (m, 1H, Cy-OCH), 3.48-3.43 (m, 2H, β-Ala NHCH_2CH$_2$CO$_2$H), 2.51 (t, *J* = 7.0 Hz, 2H, β-Ala NHCH$_2$CH_2CO$_2$H), 1.90 (m, 2H, Cy-CH$_2$), 1.67 (m, 2H, Cy-CH$_2$), 1.46-1.23 (m, 6H, Cy-CH$_2$). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 173.93, 164.31, 161.25, 156.18, 151.36, 138.86, 136.73, 133.81, 129.27, 128.33, 127.69, 123.00, 122.27, 120.99, 116.41, 107.45, 105.76, 75.60, 36.33, 34.70, 31.49, 25.45, 23.40. UV max λ = 234.5 nm; HPLC: r$_t$ 6,530 minutes. HRMS (ESI) calculated for $C_{27}H_{26}N_2O_5S$ [M + H]$^+$ 491.1640, found 491.1637.

***N*-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-3-hydroxy-L-phenylalanine (5).** Starting from **22** the target acid **5** was obtained as yellow solid (reaction time: 1.30 h, purification method: semi-automatic chromatography column: $CHCl_3$/MeOH 93:7, yield 29%): mp 202-203 °C; [α]$_D$ = -0.08 (0.5% p/v, DMSO). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.26 (bs, 1H, Phe-OH), 8.81 (s, 2H, H-9 and Phe-NH), 8.48 (bs,1H, H-3), 7.83 (d, *J* = 8.6 Hz , 1H, H-6), 7.78-7.76 (m, 2H, Ar-H), 7.44 (t, *J* = 7.1 Hz , 2H, Ar-H), 7.34 (m, 1H, Ar-H), 7.06 (d, *J* = 8.5 Hz , 1H, H-7), 6.95 (t, *J* = 7.9 Hz , 1H, PheAr-H), 6.67 (s, 2H, PheAr-H), 6.48 (d, *J* = 7.7 Hz, 1H, PheAr-H), 4.55-4.47 (m, 1H, Phe-CH), 4.35 (m, 1H, Cy-OCH), 3.12-3.11 (m, 1H, PheCH_aCH$_b$), 2.94-2.84 (m, 1H, PheCH$_a$CH_b), 1.90 (m, 2H, Cy-CH$_2$), 1.67 (m, 2H, Cy-CH$_2$), 1.46-1.18 (m, 6H, Cy-CH$_2$). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 174.31, 164.05, 161.28, 157.13, 156.23, 151.38, 140.46, 138.71, 136.53, 133.79, 129.39, 129.27, 128.41, 127.79, 122.89, 122.47, 120.77, 120.24, 116.56, 116.24, 113.46, 107.45, 105.69, 75.80, 55.43, 37.27, 31.44, 25.37, 23.31. UV max λ = 226 nm; HPLC: r$_t$ 5,948 minutes. HRMS (ESI) calculated for $C_{33}H_{30}N_2O_6S$ [M + H]$^+$ 583.1903, found 583.1895.

8-(Cyclohexyloxy)-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-1-one (9). To a stirred mixture of Dowtherm A (1 mL), Ethylene glycol (0.5 mL)and the acid **18**[1] (0.1g, 0.24mmol) the reaction was carried out by MW irradiation T = 250°C, P = 10 bar, FHT on, cooling off, Absorption High, time = 10 min. After cooling, the mixture was treated with cyclohexane and the precipitated dark brown solid was filtered under vacuum. The filtrate was then concentrated under reduced pressure and purified by flash column chromatography eluting with CH_2Cl_2/cyclohexane 50:50 to CH_2Cl_2 100% to give the derivative **9** (51 mg, 56% yield) as a yellow solid: TLC ($CHCl_3$ 100%): R_f = 0.5; mp 125 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.91 (d, J = 2.5 Hz, 1H, H-9), 7.88 (d, J = 8.8 Hz, 1H, H-6), 7.78 (d, J = 7.8 Hz, 1H, H-3), 7.75-7.73 (m, 2H, Ar-H), 7.44-7.41 (m, 2H, Ar-H), 7.34-7.31 (m, 1H, Ar-H), 7.19 (dd, J = 2.5 and 8.8, 1H, H-7), 7.07 (d, J = 7.8 Hz, 1H, H-4), 4.43-4.38 (m, 1H, Cy-OCH), 2.06-1.95 (m, 2H, Cy-CH_2), 1.72-1.51 (m, 2H, Cy-CH_2), 1.50-1.24 (m, 6H, Cy-CH_2). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 161.37, 156.23, 147.01, 139.91, 137.29, 137.20, 129.06, 128.41, 127.41, 123.38, 123.05, 117.75, 116.21, 108.09, 101.11, 75.63, 31.55, 25.51, 23.47. UV max λ = 227 nm; HPLC: r_t 5,371 minutes. HRMS (ESI) calculated for $C_{23}H_{21}NO_2S$ $[M + H]^+$ 376.1371, found 376.1356.

5-(Cycloheptyloxy)-2-methyl-1,3-benzothiazole (12). To a stirred solution of starting 2-methyl-1,3-benzothiazol-5-ol (2.0 g, 12.1 mmol), cycloheptanol (4.36 mL, 36.3 mmol),and PPh_3 (4.76 g, 18.15 mmol) in dry THF (40mL), DIAD (3.57 mL, 18.15 mmol) was added at 0°C. After sonication for 24 h at r.t., the solvent was removed under vacuum,the residue was poured into ice/water, and extracted three times with EtOAc; the collected organic layers were consecutively washed with 10% NaOH (to remove the unreacted starting benzothiazole), brine, dried and

evaporated under vacuum. The crude slurry residue was treated with cyclohexane and the insoluble material was removed. The filtrate was evaporated to dryness and the crude oil was purified by flash column chromatography eluting with cyclohexane/ EtOAc 90:10 to give compound **12** (2.2g,70%) as yellow oil: TLC (cyclohexane/ EtOAc 60:40v/v): Rf = 0,40.^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (d, J = 8.7 Hz, 1H, H-7), 7.37 (d, J = 2.2 Hz, 1H, H-4), 6.92 (dd, J= 2.2 and 8.6 Hz, 1H, H-6), 4.44-4.40 (m, 1H, Cycloheptyl OCH), 2.78 (s, 3H, CH_3), 2.07-2.02 (m, 2H, Cycloheptyl CH_2), 1.81-1.71 (m, 5H, Cycloheptyl CH_2), 1.60-1.57 (m, 3H, Cycloheptyl CH_2), 1.46-1.35 (m, 2H, Cycloheptyl CH_2).

Ethyl [5-(Cycloheptyloxy)-1,3-benzothiazol-2-yl]acetate (13). To a suspension of 60% NaH in mineraloil (3.2 g, 80.3 mmol) in dry THF (63 mL), a solution of 2-methylbenzothiazole derivative **12** (2.1 g, 8.03 mmol) in dry THF(63 mL) was added dropwise under N_2 atmosphere and stirred for 20 min. At r.t. Then, diethylcarbonate (1.93 mL, 16.06 mmol) was added and the reaction mixture was stirred at reflux for 12h. After quenching with EtOAc and water at 0°C, the reaction mixture was concentrated under vacuum and poured into ice/water. The aqueous phase was neutralized (pH=7) with 2N HCl, extracted four times with EtOAc, and the combined organic layers were washed with brine, dried, and evaporated under vacuum to give compound **13** as a brown oil in 95% yield: TLC (cyclohexane/EtOAc 80:20v/v): Rf = 0,35. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, J = 8.7 Hz, 1H, H-7), 7.42 (d, J = 2.2 Hz, 1H, H-4), 6.97 (dd, J = 1.5 and 8.7 Hz, 1H, H-6), 4.46-4.40 (m, 1H, Cycloheptyl OCH), 4.22 (q, J = 7.2 Hz, 2H, O$CH_2$$CH_3$), 4.11 (s, 2H, CH_2CO), 2.06-2.02 (m, 2H, Cycloheptyl CH_2), 1.80-1.70 (m, 5H, Cycloheptyl CH_2), 1.58-1.57 (m, 3H, Cycloheptyl CH_2), 1.47-1.46 (m, 2H, Cycloheptyl CH_2), 1.29-1.22 (m, 3H, OCH_2CH_3).

Ethyl (2E)-2-[5-(cycloheptyloxy)-1,3-benzothiazol-2-yl]-3-(dimethylamino) acrylate (14). To a solution of the benzothiazole acetate **13** (0.2 g, 0.6 mmol) in dry DMF (2.5 mL), DMF/DMA (0.24 mL, 1.79 mmol) was added and the reaction was carried out under MW irradiation Temperature = 80 °C, FHT on, Cooling off, Absorption high, time = 10 minutes. After cooling, the reaction mixture was filtered to remove an insoluble residue and the filtrate was extracted four times with EtOAc, washed with brine, dried, and evaporated under vacuum to give **14** in 80% yield as yellow oil, used without further purification for the next reaction step: TLC ($CHCl_3$/MeOH 99:1 v/v): Rf = 0.2.^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80 (s, 1H, C*H*NCH_3CH_3), 7.63 (d, *J* = 8.5 Hz, 1H, H-7), 7.40 (m, 1H, H-4), 6.91 (d, *J* = 7.4 Hz, 1H, H-6), 4.44-4.39 (m, 1H, Cycloheptyl OCH), 4.19 (q, *J* = 6.8 Hz, 2H, OCH_2CH_3), 2.92 (s, 6H, $CHNCH_3CH_3$), 2.15-2.03 (m, 2H, Cycloheptyl CH_2), 1.82-1.71 (m, 6H, Cycloheptyl CH_2), 1.47-1.46 (m, 4H, Cycloheptyl CH_2), 1.24 (t, *J* = 7.0 Hz, 3H, OCH_2CH_3).

Ethyl 8-(Cycloheptyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazole-4-carboxylate (15). A mixture of starting benzothiazole acrylate **14** (1.0 g, 2.57 mmol) and phenilacetic anhydride (1.31 g, 5.14 mmol) was heated at 110 °C for 4 h. After cooling, the residue was triturated with Et_2O and compound **15** was obtained as yellow solid without any further purification in 58% yield: mp 167-168 °C;TLC ($CHCl_3$ 100%): Rf = 0.65.^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (s, 1H, H-9), 8.03 (s, 1H, H-3), 7.93 (d, *J* = 8.8 Hz, 1H, H-6), 7.68-7.66 (m, 2H, Ar-H), 7.46-7.40 (m, 2H, Ar-H), 7.36-7.32 (m, 1H, Ar-H), 7.14 (d, *J* = 8.8 Hz, 1H, H-7), 4.51-4.49 (m, 1H, Cycloheptyl OCH), 4.31 (q, *J* = 7.0 Hz, 2H, OCH_2CH_3), 2.02-1.92 (m, 2H,

Cycloheptyl CH_2), 1.73-1.61 (m, 5H, Cycloheptyl CH_2), 1.51-1.43 (m, 5H, Cycloheptyl CH_2), 1.29 (t, J = 7.0 Hz, 3H, OCH_2CH_3).

8-(Cycloheptyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazole-4-carboxylic acid (17). A mixture of starting ethyl ester **15** (0.67 g, 1.45 mmol) in aqueous 10% NaOH/MeOH (1:4) (65mL) was heated at 75 °C for 4h. After cooling at r. t. the mixture was concentrated to one-third volume, poured into ice/water, and acidified with 2N HCl (pH = 4). The obtained precipitated was collected by filtration to give the acid **17** as yellow powder, in 94% yield, used in the next step reaction without further purification: mp > 290 °C; TLC (CH_2Cl_2/MeOH 95:5 v/v) R_f=0.3. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.87 (s, 1H, H-9), 8.16 (s, 1H, H-3), 7.76 (d, J = 8.3 Hz, 1H, H-6), 7.71-7.69 (m, 2H, Ar-H), 7.41-7.37 (m, 2H, Ar-H), 7.29-7.26 (m, 1H, Ar-H), 7.06 (d, J = 8.6, 1H, H-7), 4.55-4.50(m, 1H, Cycloheptyl-OCH), 2.04-1.94 (m, 2H, Cycloheptyl-CH_2), 1.74-1.44 (m, 10H, Cycloheptyl-CH_2).

Methyl N-{[8-(Cycloheptyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-L-tyrosinate (19). Following the general procedure (Method A), starting from intermediate **17** and using L-Tyr methyl ester hydrochloride, compound **19** was obtained after purification by flash column chromatography and eluting with $CHCl_3$/MeOH 98:2, as a yellow solid in 44%yield (reaction time: 2 h): mp 139-140 °C; TLC ($CHCl_3$/MeOH 95:5 v/v) R_f= 0.6. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.24 (s, 1H, Tyr OH), 8.99 (d, J = 7.7 Hz, 1H, Tyr NH), 8.86 (d, J = 2.2 Hz, 1H, H-9), 8.50 (s, 1H, H-3), 7.90 (d, J = 8.7 Hz, 1H, H-6), 7.81-7.79 (m, 2H, Ar-H), 7.53-7.49 (m, 2H, Ar-H), 7.42-7.39 (m, 1H, Ar-H), 7.18 (dd, J = 2.2 and 8.7 Hz, 1H, H-7) 7.10-7.08 (m, 2H, Tyr Ar-H), 6.67-6.64 (m, 2H, Tyr Ar-H), 4.67-4.55 (m, 2H, Cycloheptyl-OCH

and Tyr-CH), 3.59 (s, 3H, Tyr-CO_2CH_3), 3.09-2.96 (m, 2H, Tyr-CH_2), 2.02-1.97 (m, 2H, Cycloheptyl-CH_2), 1.8-1.57 (m, 4H, Cycloheptyl-CH_2), 1.56-1.34 (m, 6H, Cycloheptyl-CH_2).

N-{[8-(Cycloheptyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-L-tyrosine (2). Following the general procedure (Method B) and starting from intermediate **19** the target acid **2** was obtained as yellow solid (reaction time: 40 minutes, purification method: crystallization by 3:4 Cyclohexane/EtOAc followed by flash column chromatography eluting with $CHCl_3$/MeOH 90:10, yield 18%): mp 191-193 °C; $[\alpha]_D$ = -0.07 (0.5% p/v, DMSO). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.71 (bs, 1H, CO_2H), 9.16 (s, 1H, Tyr-OH), 8.85-8.80 (m, 2H, Tyr-NH, and H-9), 8.47 (s, 1H, H-3), 7.86 (d, J = 8.8 Hz, 1H, H-6), 7.77-7.75 (m, 2H, Ar-H), 7.50-7.45 (m, 2H, Ar-H), 7.38-7.34 (m, 1H, Ar-H), 7.13 (dd, J = 1.6 and 8.8 Hz, 1H, H-7), 7.05-7.00 (m, 2H, Tyr Ar-H), 6.61-6.58 (m, 2H, Tyr Ar-H), 4.58-4.50 (m, 2H, Cycloheptyl-OCH and Tyr-CH), 3.05 (dd, J = 4.7 and 14.0 Hz, 1H, Tyr CH_aH$_b$), 2.91 (dd, J = 10.6 and 14.0 Hz, 1H, Tyr CH$_a$$H_b$), 2.00-1.95 (m, 2H, Cycloheptyl-CH_2), 1.75-1.40 (m, 10H, Cycloheptyl-CH_2). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 173.69, 164.45, 161.28, 156.35, 156.22, 151.84, 138.94, 136.72, 133.87, 130.34, 129,75, 129.29, 128.41, 127.76, 123.08, 122.34, 120.69, 116.53, 115.40, 107.28, 105.31, 78.14, 55.03, 36.04, 33.41, 28.26, 22.76. UV max λ = 229.5 nm; HPLC: r_t 5,985 minutes.

2.2. Synthesis of pyridones 10 and 11.

Methyl 5-bromo-1-(3-methoxyphenyl)-6-oxo-1,6-dihydropyridine-3-carboxylate

(24). To a mixture of starting material **23** (0.2 g, 0.86 mmol) in dry CH_2Cl_2, (3-methoxyphenyl)boronic acid (0.16 g, 1.03 mmol), $Cu(OAc)_2 \cdot H_2O$ (0.26 g, 1.3 mmol), pyridine (0.14 mL, 1.72 mmol) and 4 Å MS were added and the reaction was stirred for 23h at room temperature. Then, the mixture was filtered to remove MS, water and 2N HCl were added to the filtrate and the mixture was extracted with CH_2Cl_2, washed with brine, dried and concentrated under vacuum to provide a crude white residue which was purified by flash column chromatography eluting with $CHCl_3$ 100% followed by $CHCl_3$/acetone 99:1, to give the desired derivative **24** (218 mg, 75%) as pale brown solid: mp 108-109 °C; TLC (CH_2Cl_2/MeOH, 99:1 v/v): R_f = 0.55. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.31 (d, J = 2.4 Hz, 1H, H-2), 8.20 (d, J = 2.3 Hz, 1H, H-4), 7.41-7.37 (m, 1H, Ar-H), 7.01-6.98 (m, 1H, Ar-H), 6.92-6.88 (m, 2H, Ar-H), 3.85 (s, 3H, OCH_3), 3.81 (s, 3H, CH_3).

Methyl 1-(3-methoxyphenyl)-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylate (25). To a stirred mixture of **24** (0.1 g, 0.30 mmol), $PhB(OH)_2$ (0.043 g, 0.35 mmol), $Pd(PPh_3)_4$ (0.017 g, 0.015 mmol), DME (1.6 mL), and 2M K_2CO_3 solution (0.3 mL, 0.59 mmol) were added successively. The resulting mixture was put in the MW at T = 100 °C, P = 8 bar, cooling on, FHT on, Absorption normal, time = 15 minutes. The reaction was cooled to room temperature and filtered through Celite, washed with $CHCl_3$. Water was added to the filtrate, and extracted with $CHCl_3$ and the organic layers were washed with brine, dried and the solvent evaporated to dryness giving a dark brown slurry which was purified by semi-automatic column chromatography CH_2Cl_2 (100%), giving the title compound **25** in 74% yield as white solid: mp 114-115 °C; TLC (Cyclohexane/EtOAc, 80:20 v/v): R_f = 0.5. ^{1}H NMR (400 MHz, $CDCl_3$): δ

8.27 (d, J = 2.6 Hz, 1H, H-2), 8.13 (d, J = 2.5 Hz, 1H, H-4), 7.77-7.75 (m, 2H, Ar-H), 7.46-7.33 (m, 4H, Ar-H), 7.04-6.97 (m, 3H, Ar-H), 3.93 (s, 3H, OCH_3), 3.85 (s, 3H, CH_3).

Methyl 1-(3-hydroxyphenyl)-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylate (26). Under N_2 atmosphere, to a stirred solution of **25** (0.10g, 0.30 mmol) in dry CH_2Cl_2 (3 mL) at 0 °C, BBr_3 1M in CH_2Cl_2 (1.19 mL, 1.19 mmol) was added dropwise. The reaction mixture was stirred for 15 minutes and quenched at 0 °C with few drops of MeOH. The solvent was evaporated under reduced pressure and the crude mixture was poured into ice/water and acidified with 2N HCl (pH = 5). The obtained precipitate was collected by filtration under vacuum giving a yellow powder which was purified by flash column chromatography eluting with $CHCl_3$/acetone (98:2), to give the desired derivative **26** as yellow solid in 30% yield: mp 177-178 °C; TLC (Cyclohexane/EtOAc, 80:20 v/v): R_f = 0.4. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, J = 2.4 Hz, 1H, H-2), 8.13 (d, J = 2.5 Hz, 1H, H-4), 7.71 (d, J = 7.5 Hz, 2H, Ar-H), 7.57 (s, 1H, OH), 7.44-7.29 (m, 3H, Ar-H), 7.28-7.25 (m, 1H, Ar-H), 6.79- 6.77 (m, 1H, Ar-H), 6.77-6.72 (m, 2H, Ar-H), 3.85 (s, 3H, CH_3).

Methyl 1-[3-(cyclohexyloxy) phenyl]-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylate (27). Following the same procedure used for compound **12**, starting from intermediate **26**, compound **27** was obtained after 30 h of sonication a tr.t., the solvent was removed under vacuum, the residue was poured into ice/water, and extracted three times with EtOAc; the collected organic layers were consecutively washed with 10% NaOH (to remove the unreacted starting material), brine, dried and evaporate under vacuum. The crude slurry residue was treated with cyclohexane and the insoluble material was removed. The filtrate was evaporate to dryness

and the crude oil was purified by flash column chromatography eluting with 100% $CHCl_3$ obtaining **27** as white solid (240 mg, 68% yield): mp 94-95 °C; TLC (Cyclohexane/EtOAc, 80:20 v/v): R_f = 0.4. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, J = 2.4 Hz, 1H, H-2), 8.08 (d, J = 2.4 Hz, 1H, H-4), 7.72 (d, J = 7.2 Hz, 2H, Ar-H), 7.40-7.31 (m, 4H, Ar-H), 6.97-6.91 (m, 3H, Ar-H), 4.28-4.22 (m, 1H, Cy-OCH), 3.86 (s, 3H CH_3), 2.04-1.94 (m, 2H, Cy-CH_2), 1.85-1.75 (m, 2H, Cy-CH_2), 1.56-1.47 (m, 2H, Cy-CH_2), 1.40-1.19 (m, 4H, Cy-CH_2).

Methyl 1-methyl-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylate (29). To a stirred mixture of compound **28** (0.20 g, 0.81 mmol) in DME (1.62 mL), $PhB(OH)_2$ (0.12 g, 0.97 mmol), $Pd(PPh_3)_4$ (0.046 g, 0.05 mmol), and 2M K_2CO_3 solution (2 mmol) were added successively. The resulting mixture was degassed and replaced with N_2 atmosphere and stirred at 80°C in a pre-heated oil bath for 4 h. The reaction was cooled to room temperature, filtered through celite and washed with $CHCl_3$. The filtrate was extracted with water and $CHCl_3$ then, the organic layers were washed with brine, dried and the solvent was evaporated to dryness giving a dark brown slurry which was purified by flash column chromatography eluting CH_2Cl_2/acetone (97:3) followed by CH_2Cl_2/acetone (95:5). Derivative **29** was obtained in 81% yield as white solid: TLC (Cyclohexane/EtOAc, 50:50 v/v): mp 113-114 °C; R_f = 0.5. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.56 (d, J = 2.4 Hz, 1H, H-2), 7.86 (d, J = 2.4 Hz, 1H, H-4), 7.64-7.62 (m, 2H, Ar-H), 7.40-7.30 (m, 3H, Ar-H), 3.83 (s, 3H, CH_3), 3.56 (s, 3H, NCH_3).

1-[3-(Cyclohexyloxy)phenyl]-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylic acid (30). To a solution 0.1M of compound **27**, (0.10g, 0.30mmol) in MeOH/THF 1:1 (3.0 mL), 2N NaOH (0.89 mL, 1.79 mmol) was added and reaction mixture was stirred for 2 h. The solution was

concentrated to a one-third of the volume, poured into ice/water and acidified with 2N HCl (pH = 4). The obtained precipitate was collected by filtration under vacuum to give the acid **30** as white powder used in the next step of reaction without any further purification, in 95% yield: mp 162-163 °C; TLC ($CHCl_3$/MeOH, 90:10 v/v): R_f= 0.25. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.33 (d, J = 2.0 Hz, 1H, H-2), 8.11 (d, J = 2.1 Hz, 1H, H-4), 7.74 (d, J = 7.7 Hz, 2H, Ar-H), 7.44-7.34 (m, 4H, Ar-H), 7.01-6.95 (m, 3H, Ar-H), 4.44-4.26 (m, 1H, Cy-OCH), 1.99-1.96 (m, 2H, C-CH_2), 1.78 (m, 2H, Cy-CH_2), 1.57-1.50 (m, 2H, Cy-CH_2), 1.43-1.22 (m, 4H, Cy-CH_2).

1-Methyl-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylic acid (31). To a solution (0.1 M) of compound **29**, (0.10g, 0.41mmol) in MeOH/THF 1:1 (4.1 mL), 2N NaOH (1.23 mL, 2.47mmol) was added and the reaction mixture was stirred for 2 h. The solution was concentrated to a one-third of the volume, poured into ice/water and acidified with 2N HCl (pH = 4). The obtained precipitate was collected by filtration under vacuum to give acid **31** as white powder used in the next step of reaction without any further purification in 82% yield: mp 255-256 °C; TLC ($CHCl_3$/MeOH, 90:10 v/v): R_f= 0.25. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.88 (bs, 1H, CO_2H), 8.50 (d, J = 2.4 Hz, 1H, H-2), 7.86 (d, J = 2.5 Hz, 1H, H-4), 7.64-7.62 (m, 2H, Ar-H), 7.39-7.29 (m, 3H, Ar-H), 3.51 (s, 3H, NCH_3).

(Methyl N-({1-[3-(cyclohexyloxy)phenyl]-6-oxo-5-phenyl-1,6-dihydropyridin-3-yl}carbonyl)-L-tyrosinate (32). Following the general procedure (Method A), starting from acid **30,** compound **32** was obtained after purification by flash column chromatography eluting with $CHCl_3$/Acetone (98:2) as a light yellow solid in 50% yield: mp 189-190 °C; TLC (CH_2Cl_2/MeOH, 95:5 v/v): R_f= 0.5. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (d, J = 2.6 Hz, 1H, H-

2), 7.74 (d, J = 2.6 Hz, 1H, H-4), 7.68 (d, J = 8.3 Hz, 2H, Tyr Ar-H), 7.41-7.31 (m, 4H, Ar-H), 6.98-6.86 (m, 5H,Tyr Ar-H andAr-H), 6.64 (d, J = 8.5 Hz, 2H, Tyr Ar-H), 6.41 (d, J = 7.7 Hz, 1H, Tyr-NH), 5.76 (bs, 1H, Tyr-OH), 5.02-4.97 (m, 1H, Tyr-CH), 4.30-4.24 (m, 1H, Cy-OCH), 3.78 (s, 3H, CH_3), 3.18 (dd, J = 14.06 and 5.8, 1H, Tyr CH_aH$_b$), 3.08 (dd, J = 14.1 and 5.7, 1H, Tyr CH$_a$$H_b$), 1.98-1.96 (m, 2H, Cy-$CH_2$), 1.78-1.77 (m, 2H, Cy-$CH_2$), 1.56-1.42 (m, 2H, Cy-$CH_2$), 1.28-1.21 (m, 4H, Cy-$CH_2$).

Methyl N-[(1-methyl-6-oxo-5-phenyl-1,6-dihydropyridin-3-yl)carbonyl]-L-tyrosinate (33). Following the general procedure (Method A) and starting from acid **31,** compound **33** was obtained after purification by flash column chromatography eluting with $CHCl_3$/Acetone (80:20) as a light yellow solid in 75% yield: mp 122-123 °C; TLC (CH_2Cl_2/MeOH, 95:5 v/v): R_f = 0.5. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.18 (s, 1H, Tyr-OH), 8.63 (d, J = 7.7 Hz, 1H, Tyr-NH), 8.32 (d, J = 2.5 Hz, 1H, H-2), 8.01 (d, J = 2.6 Hz, 1H, H-4), 7,67 (d, J = 9.7 Hz, 2H, Ar-H), 7.41-7.37 (m, 2H, Ar-H), 7.33-7.29 (m, 1H, Ar-H), 7.03 (d, J = 8.5 Hz, 2H, Tyr Ar-H), 6.60 (d, J = 8.5 Hz, 2H, Tyr Ar-H), 4.55-4.49 (m, 1H, Tyr-CH), 3.57 (s, 3H, NCH_3), 3.51 (s, 3H, Tyr-CH_3), 3.02-2.85 (m, 2H, Tyr-CH_2).

N-({1-[3-(Cyclohexyloxy)phenyl]-6-oxo-5-phenyl-1,6-dihydropyridin-3-yl}carbonyl)-L-tyrosine (10). Following the general procedure (Method B) and starting from **32**, target acid **10** was obtained after purification by flash column chromatography eluting with $CHCl_3$/MeOH (85:15) as white solid, yield: 30%; mp 201-202 °C; $[\alpha]_D$ = -0.18 (0.5% p/v, MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.13 (bs, 1H, Tyr OH), 8.37 (bs, 1H, Tyr NH), 8.17 (bs, 1H, H-2), 8.06 (bs, 1H, H-4), 7.65 (d, J = 7.5 Hz, 2H, Tyr Ar-H), 7.44-7.25 (m, 4H, Ar-H), 7.10-6.94 (m, 5H,

Tyr Ar-H and Ar-H), 6.53 (d, *J* = 8.1 Hz, 2H, Ar-H), 4.50-4.35 (m, 2H, Tyr-CH and Cy-OCH), 3.06-3.03 (3, 1H, Tyr-CH_aH$_b$), 2.82-2.70 (m, 1H, Tyr-CH$_a$$H_b$), 2.07-2.02 (m, 2H, Cy-CH$_2$), 1.75-1.64 (m, 2H, Cy-CH$_2$), 1.46-1.11 (m, 6H, Cy-CH$_2$).^{13}C-NMR (100 MHz, DMSO-d$_6$): δ 163.11, 160.59, 158.01, 155.79, 142.46, 140.07, 136.72, 136.65, 130.38, 129.66, 128.11, 119.07, 116.34, 114.60, 113.44, 74.85, 56.53, 36.97, 31.55, 29.41, 25.44, 23.49. UV max λ = 226 nm; HPLC: r$_t$ 4,676 minutes. HRMS (ESI) calculated for $C_{33}H_{32}N_2O_6$ $[M + H]^+$ 553.2338, found 553.2339.

***N*-[(1-Methyl-6-oxo-5-phenyl-1,6-dihydropyridin-3-yl)carbonyl]-L-tyrosine (11).** Following the general procedure (Method B) and starting from intermediate **33**, the target acid **11** was obtained after purification by semi-automatic reversed-phase column chromatography eluting with H$_2$O/CH$_3$CN (80:20) as white solid, yield: 27%; mp 163-164 °C; $[\alpha]_D$ = -0.35 (0.5% p/v, DMSO). ^{1}H NMR (400 MHz, DMSO-*d$_6$*): *δ* 12.72 (bs, 1H, COOH), δ 9.16 (bs, 1H, Tyr-OH), δ 8.71 (d, *J* = 7.7 Hz, 1H, Tyr-NH), 8.32 (d, *J* = 2.5 Hz, 1H, H-2), 8.02 (d, *J* = 2.5 Hz, 1H, H-4), 7.68 (d, *J* = 7.3 Hz, 2H, Ar-H),7.45-7.41 (m, 2H, Ar-H), 7.37 (m, 1H, Ar-H), 7.05 (d, *J* = 8.5 Hz, 2H, Tyr Ar-H), 6.64 (d, *J* = 8.4 Hz, 2H, Tyr Ar-H), 4.56-4.53(m, 1H, Tyr-CH), 3.59 (s, 3H, NCH$_3$), 3.00-2.97 (m, 1H Tyr-CH_aH$_b$), 2.90-2.84 (m, 1H, Tyr-CH$_a$$H_b$). ^{13}C-NMR (100 MHz, DMSO-d$_6$): *δ* 173.72, 163.89, 161.16, 156.20, 142.00, 136.81, 135.68, 130.33, 129.75, 128.79, 128.34, 128.10, 128.02, 115.35, 112.12, 54.80, 38.45, 36.12. UV max λ = 229 nm; HPLC: r$_t$ 4.862 minutes. HRMS (ESI) calculated for $C_{22}H_{20}N_2O_5$ $[M + H]^+$ 393.1450, found 393.1440.

3. Experimental procedures for the *in silico* studies

3.1 Sequence Alignments

Multiple sequence alignments (performed with BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) of 20 strains belonging to DENV1-4 highlighted that these residues were well conserved with identity of 100% (Table S1). High identity was observed for cavity B residues in WNV and ZIKV, with the exception of amino acids Lys330 and Thr858 which are replaced by Tyr and Glu in YFV and TBEV, respectively (Table S1). Thr329 (DENV2 numeration) was replaced by a Ser in WNV, ZIKV and TBEV, while was well conserved in all DENV serotypes.

Table S1: Cavity B identity among DENV1-4 strains, TBEV, YFV and ZIKV. Non conserved residues are highlighted in bold red.

Organism	Taxonomy	UniProt ID	UniProt Name	Residues (Dengue 2 numeration)[a]								
				327	328	329	330	858	859	862	863	866
Dengue virus type 1	DEN1W	P17763	POLG_DEN1W	L	L	T	K	T	W	N	I	A
Dengue virus type 1	DEN1B	P27909	POLG_DEN1B	L	L	T	K	T	W	N	I	A
Dengue virus type 1	DEN1S	P33478	POLG_DEN1S	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN26	P29990	POLG_DEN26	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN27	P29991	POLG_DEN27	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN28	P14337	POLG_DEN28	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN2J	P07564	POLG_DEN2J	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN2N	P14340	POLG_DEN2N	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN2P	P12823	POLG_DEN2P	L	L	T	K	T	W	N	I	A
Dengue virus type 2	DEN2Q	Q9WDA6	POLG_DEN2Q	L	L	T	K	T	W	N	I	A
Dengue virus type 3	DEN3C	Q99D35	POLG_DEN3C	L	L	T	K	T	W	N	I	A
Dengue virus type 3	DEN3I	Q5UB51	POLG_DEN3I	L	L	T	K	T	W	N	I	A
Dengue virus type 3	DEN3M	Q6YMS3	POLG_DEN3M	L	L	T	K	T	W	N	I	A
Dengue virus type 3	DEN3P	P27915	POLG_DEN3P	L	L	T	K	T	W	N	I	A
Dengue virus type 3	DEN3S	Q6YMS4	POLG_DEN3S	L	L	T	K	T	W	N	I	A
Dengue virus type 4	DEN4D	P09866	POLG_DEN4D	L	L	T	K	T	W	N	I	A
Dengue virus type 4	DEN4H	Q2YHF2	POLG_DEN4H	L	L	T	K	T	W	N	I	A
Dengue virus type 4	DEN4P	Q58HT7	POLG_DEN4P	L	L	T	K	T	W	N	I	A
Dengue virus type 4	DEN4S	Q5UCB8	POLG_DEN4S	L	L	T	K	T	W	N	I	A
Dengue virus type 4	DEN4T	Q2YHF0	POLG_DEN4T	L	L	T	K	T	W	N	I	A
Tick-borne encephalitis virus	TBEVS	P07720	POLG_TBEVS	L	L	**S**	K	**E**	W	N	I	A
West Nile virus	WNV	P06935	POLG_WNV	L	L	**S**	K	T	W	N	I	A
Yellow fewer virus	YEFVT	Q9YRV3	POLG_YEFVT	L	L	T	**Y**	T	W	N	I	**V**
Zika virus	ZIKVF	A0A024B7W1	POLG_ZIKVF	L	L	**S**	K	T	W	N	I	**T**

[a] The alignment was performed with BLAST. Only the reviewed sequences found using the keyword “NS5 Dengue” were considered.

3.2. Docking studies

Docking studies were performed using the software AutoDock 4.2.[4] The crystal structure of Dengue virus NS5 RNA dependent RNA polymerase domain (PDB code 2J7U)[5] was retrieved from the RCSB Protein Data Bank[6] and used as target structure for our modeling studies cause of its high resolution (1.85 Å) and the absence of missing residues close to the explored pocket. The cavity B is defined by residues Leu326, Leu327, Lys329, Thr858, Trp859, Asn862, Ile863, and Ala866.

Before the docking run, the complex was submitted to Schrodinger's Protein Preparation Wizard:[7] water molecules were deleted, hydrogen atoms were added, bond orders and charges were then assigned, and the orientation of hydroxyl groups on Ser, Thr, and Tyr, the side chains of Asn and Gln residues, and the protonation state of His residues were optimized.

Compounds **1**, **2**, **4** and **5** were built using the Schrodinger Maestro Interface and then submitted to LigPrep preparation protocol.[8]

Ligands and receptor structures were converted to AD4 format files using AutoDockTools, and then, the Gesteiger–Marsili partial charges were assigned. The dimensions of the grid were 60 X 60 X 60 with grid points separated by a 0.375 Å. The grid was centered on Lys329. The Lamarckian genetic algorithm local search method was used, and for each compound, the docking simulation was composed of 100 runs. Clustering of docked conformations was performed on the basis of their root mean square deviation (rmsd) with a tolerance of 2.0 Å, and the results were ranked based on the estimated free energy of binding (i.e. LBE) (Table S1). Figures 1A was generated using the Ligand Interaction Diagram tool of Maestro GUI, whereas Figures 1B, S1 and S2 were prepared using the software PYMOL.[9] The cavity B residues

involved in ligand contacts were calculated using the script poseviewer_interactions.py provided as part of Schrodinger Suite.

Table S2. Predicted LBE, NiC and residues involved in the ligand-protein interaction for the best docked pose of each compound.

Compound	LBE (Kcal/mol)[a]	NiC[b]	Interacting Residues[c]
5	-9.56	45	322;325;**326**;327;328;**329**;332;**859**;862;**863**;866;869
1	-9.24	40	322;325;**326**;327;328;**329**;**859**;862;**863**;865;866;869
2	-9.05	28	322;325;**326**;327;328;**329**;332;**859**;862;**863**;865;866;869
4	-7.78	22	322;325;**326**;**329**;858;**859**;862;**863**;866;869
[a]Ligand binding energy; [b] numbers in cluster in the first-ranked pose; [c] DENV3 RdRp cavity B residues interacting with the docked PBTZs; critical residues for RdRp activity and NS3-NS5 interaction are highlighted in magenta and cyan, respectively.			

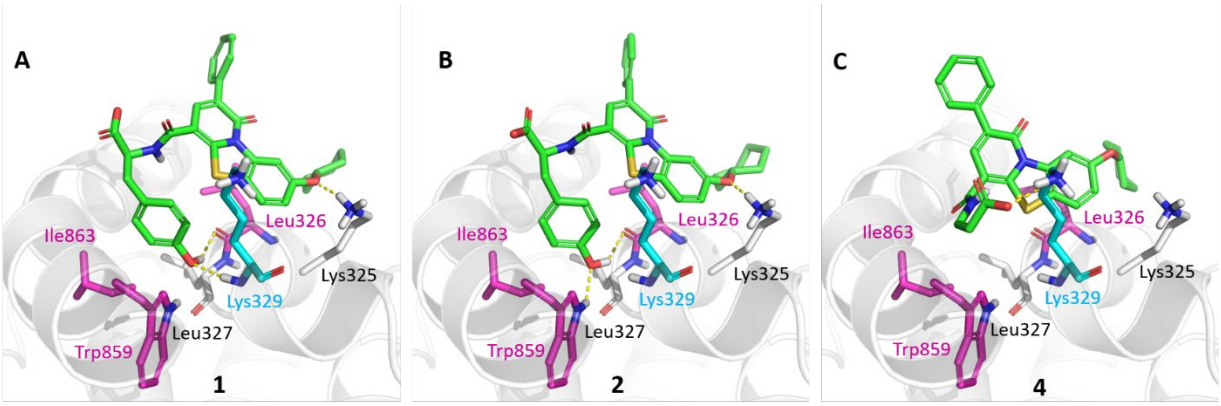

Figure S1: (A) Docking pose of compounds **1** (panel A)**, 2** (panel B) and **4** (panel C).

4. Experimental procedures for the biological evaluation

Cell lines and viruses

BHK-21 cells (baby hamster kidney fibroblast; ATCC) were grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (P/S) at 37°C in 5% CO_2. C6/36, an *Aedes albopictus* cell line (ATCC), was maintained in RPMI 1640 medium with 25mM HEPES, 10% FBS and 1% P/S

at 28°C in the absence of CO_2. HuH-7 cells (human hepatocarcinoma; ATCC) were cultured in DMEM medium (Gibco) with 4.5g/L glucose, 10% FBS and 1% P/S at 37°C in 5% CO_2. The DENV strains (GenBank accessions: DENV1, EU081230; DENV2, EU081177; DENV3, EU081190; DENV4, GQ398256) were obtained from the Early Dengue infection and outcome (EDEN) study.[10] The ZIKV strain H/PF/2013 (GenBank accession: KJ776791.2) was a gift from Cécile Baronti at Aix Marseille Université. These viruses used in this study were grown in C6/36 cells, titered in BHK-21 cells and stored at -80°C. Institutional approval has been granted by Duke-NUS Medical School to perform experiments with ZIKV.

Competitive NS3-NS5 Interaction ELISA

The ELISA was performed as published previously.[11] Maxisorb Immunoplates were coated with NS5 RdRp (5 μg/mL) in PBS overnight at 4°C and blocked plate with 5% skim milk at 37°C for 1 h. The blocked wells were then incubated with $NS2B_{CF18}$ NS3 full-length at fixed concentration and mixed with increasing concentrations of DENV3 NS3 helicase (aa 173-618)/inhibitors for 1 h at 37°C. The plates were washed 5 times with PBS-T and the bound NS2B/NS3 were detected by incubation with 3F10 IgG (1nmol/L) at room temperature for 1 h. Plates were washed again with PBS-T and incubated with an anti-human IgG HRP prior to development with the colorimetric substrate TMB (3,3', 5, 5' – tetramethylbenzidine; Sigma, St. Louis, USA). The concentrations of inhibitors at which 50% inhibition of full-length protein binding occurs was represent as IC_{50}. Data were fitted to the sigmoidal dose-response equation (variable slope) by nonlinear regression using GraphPad Prism 5.

DENV2 Polymerase assay *in vitro* assays

The *de novo* initiation/elongation assay was performed as described previously[12] with slight modification. Briefly, the initiation assay reaction comprised 100 nM DENV2 NS5, 100 nM in vitro transcribed DENV2 5'UTR- 3'UTR RNA, 20 μM ATP, 20 μM GTP, 20 μM UTP, 5 μM Atto-CTP (Trilink Biotechnologies), in a volume of 15 μL of reaction buffer comprising 50 Mm Tris-HCL, Ph7.5, 10 Mm KCl, 1mM $MgCl_2$, 0.3 mM $MnCl_2$, 0.001% Triton X-100 and 10 μM cysteine. The elongation assay reaction comprised of 100 nM DENV2 NS5, 100 nM U30-3' UTR (Integrated DNA Technologies), 3 μM Atto-ATP, in a volume of 15 μL of reaction comprised of 50 mM Tris-HCL, pH 7.5, 10 mM KCl, 0.5 mM $MnCl_2$, 0.001% Triton and 10 μM cysteine. All reactions were incubated at 37°C for 1 h and 10 μL of STOP buffer (200 mM NaCl, 25 mM $MgCl_2$, 1.5 M DEA, pH10; Promega) with 25 nM calf intestine alkaline phosphatase was added to the wells to stop the reactions. The plate was centrifuge briefly at 1200 rpm, followed by incubation at RT for 60 mins and the release AttoPhos was monitored by reading on a Tecan machine at $excitation_{max}$ and $emission_{max}$ wavelengths 422 nm and 566 nm respectively.

Cell viability test

2×10^4 HuH-7 cells were plated onto a 96-well white opaque plate (Grenier) and treated with the compounds at the indicated concentrations for 48 h. Cytotoxicity was determined by CellTiter Glo® Luminescent Assay (Promega) kit according to manufacturer's instructions. Cell viability curve is presented as percentage of luminescence derived from treated sample to that of the untreated cell control.

Virus inhibition assay

1×10^5 HuH-7 cells were infected with DENV at MOI 0.3 (Multiplicity of Infection) for 1 h, followed by treatment with the compounds at the indicated concentrations for 48 h. Supernatants were collected, clarified and subjected to plaque quantification. The efficacy of the compounds (EC_{50}: concentration at which the virus infection is reduced by 50%) was determined by the sigmoidal dose response curve of virus titer against concentration in GraphPad Prism.

Compound resistance selection and virus sequencing

The selection for compound resistance in the virus is shown as a schematic in Figure 4A. Briefly, 4×10^5 HuH-7 cells were initially infected with DENV2-3295 at MOI 5 for 1 h, followed by treatment with 0.5μM of the compound for 72 h. The supernatant collected from the initial infection is referred as passage 1 (P1). 1 mL of the supernatant from the previous passage was used for subsequent infection in HuH-7 cells until passage 10. Compound treatment for selection was done for 72 h with dosing as such: 0.5μM for P2-3; 1μM for P4-6; 5μM for P7-9 and finally 10μM for P10. The virus from the P10 supernatant was expanded once in C6/36 and tested for susceptibility towards the compound at 10μM.[13,14]

Susceptibility test of the compound 4 resistant virus to other anti-DENV compounds.

To confirm that the observed resistance by compound **4** is specific, the passaged 10 (P10) resistant virus was subjected to compound susceptibility test with other known anti-DENV compounds with different mode of actions (alpha glucosidase inhibitor: Celgosivir,[15]; nucleoside

analog inhibitor: NITD008,[16]; non-nucleoside N-pocket inhibitor: N27[17]). NITD008 was kindly provided by Novartis and the N-pocket inhibitor N27 was a generous gift from Professor Julien Lescar (Nanyang Technological University, Singapore). Proposed cavity B inhibitors PBTZs **1** and **5** reported in this study were also included for comparison. Briefly, 1×10^5 HuH7 cells were infected with wild-type DENV2 or the compound 4 resistant virus at MOI 0.3 for 1h followed by treatment with the compounds at 10μM for 48h. Supernatants were collected and clarified for plaque quantification. The level of viral reduction for each compound treatment is compared between the wild-type DENV2 and the resistant virus to determine if there is a change in the virus sensitivity towards the compound.

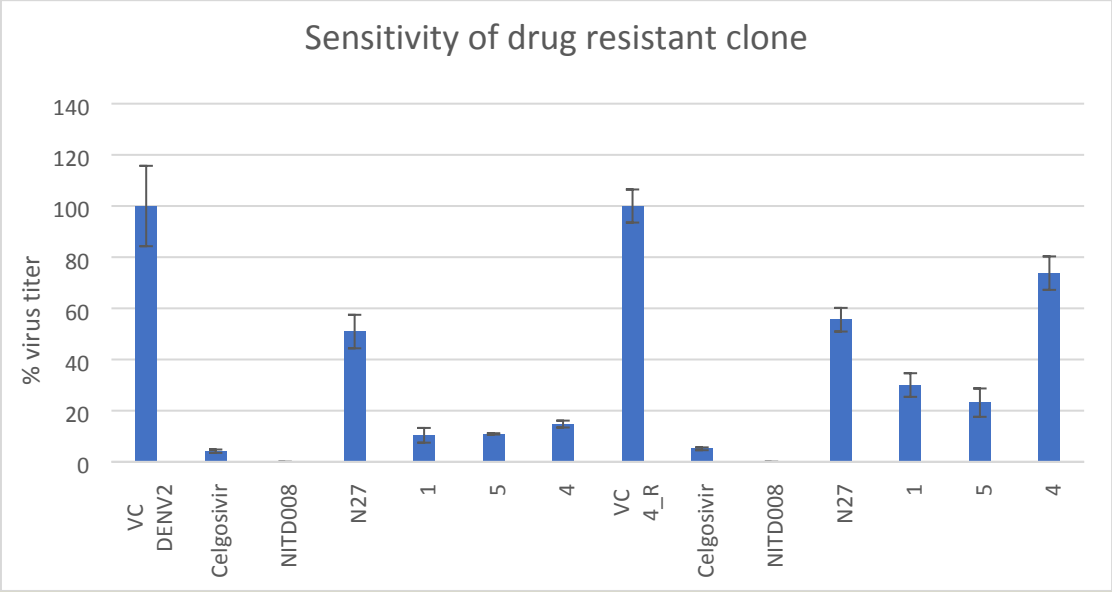

Figure S2: Susceptibility of compound **4** resistant virus clone to other anti-DENV compounds compared to wild-type DENV2.

Full genome Sanger sequencing:

Virus clone 4_R displaying reduced susceptibility towards the compound was selected for full genome Sanger sequencing to examine changes in the viral genome with respect to the wild-type genome sequence.

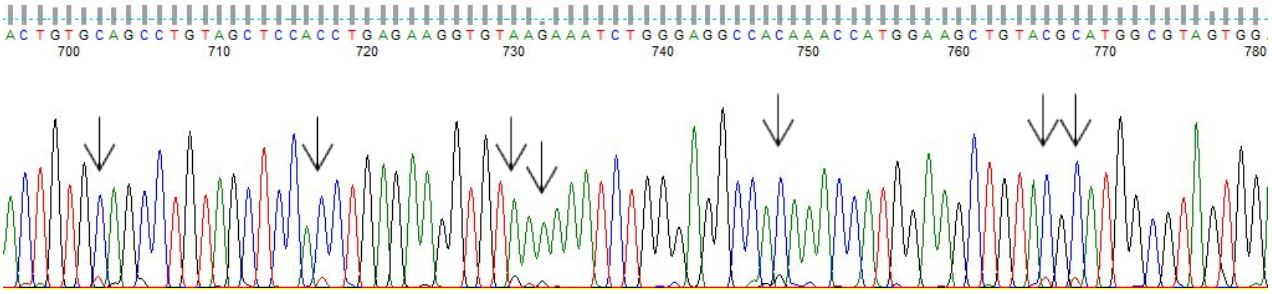

Figure S3: Sanger sequencing chromatogram of the compound **4** resistant virus showing the small traces of nucleotide changes (indicated with a black arrow) at the DENV2 3'UTR positions ranging from 10410 to 10500.

RNA Electrophoretic Mobility Shift Assay (REMSA)

The full length 3'UTR of DENV2 (GenBank accession: EU081177; nt 10270-10723) was synthesized with a T7 promoter by GenScript. The 3'UTR RNA was subsequently obtained by *in vitro* transcription using the MEGAScript T7 transcription kit (Ambion) according to manufacturer's instructions. To assess the ability of the compound to interfere the NS5-RNA complex, the RNA electrophoretic mobility shift assay was performed as described previously[18] with slight modifications. Briefly, 25 µM of the compound or mock in the binding buffer (50mM HEPES pH7.0, 150mM NaCl, 10% glycerol, 1mM TCEP & 0.5mg/ml BSA) was added to 1.28µM of NS5 and incubated at 37°C for an hour. 0.16 µM RNA was then added to the compound-NS5 mixture and incubated on ice for a further 20 mins (molar ratio of RNA to NS5 is 1:8) to allow formation of RNA-protein complex. Samples were loaded onto the 1.2% agarose gel for analysis and visualized GelRed detected using Chemidoc Imaging system.

5. PAINS Filters

The compounds **1-11** were analyzed for the presence of PAINS alert using the PAINS-remover online tool https://www.cbligand.org/PAINS/ and aggregators by using the ZINC15 remover filter (at the website http://zinc15.docking.org),[19] and it was not found as potential PAINS and/or

aggregator. Moreover, the compounds do not show high lipophilicity (calculated LogD is around 2), a warning for aggregation-prone compounds, and no reactive groups are present in their structures thus excluding possible aspecific binding to proteins. To definitely exclude any possible aspecific inhibition due to potential aggregation, we routinely carried out the DENV2 Polymerase *in vitro* assays in presence of the non-ionic detergent Triton X-100 (0.001%) that is known to break possible colloidal aggregates; thus, the observed inhibitory activity is correlated to specific inhibition and no to colloidal aggregation. Similarly, in the REMSA we use BSA (0.5 mg/ml) (recommended ≥0.1 mg/ml) as decoy protein. Moreover, we checked also for autofluorescence of the compounds in the assays in order to exclude inference with the experimental readout.

We provided concentration-response curves for different assays demonstrating that the inhibitory activity is clearly related to the concentration and also the presence of a SAR is in line with specific inhibition. Indeed, only compound with an aminoacid moiety are able to inhibit RdRp and NS5-NS3 PPI, most likely by binding NS5 cavity B and as suggested by docking the aminoacid moiety seems very important to interact with this pocket. Finally, we have previously reported[20] that a similar PBTZ analogue was able to inhibit viral replication of flaviviruses but not of other RNA viruses, therefore this finding strongly support the specific activity of our compounds against flaviviruses acting on viral NS5 RdRp."

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