Supporting Information

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

Rolando Cannalire,† Kitti Wing Ki Chan,# Maria Sole Burali,† Chin Piaw Gwee,# Sai Wang,# Andrea Astolfi,† Serena Massari, † Stefano Sabatini, † Oriana Tabarrini, † Eloise Mastrangelo,§ † Maria Letizia Barreca, † Violetta Cecchetti, † Subhash G. Vasudevan, #§ Giuseppe Manfroni†

†Dipartimento di Scienze Farmaceutiche, Università degli Studi di Perugia, Via del Liceo, 1-06123, Perugia, Italy
§ Dipartimento di Bioscienze, Università di Milano, Via Celoria 26, I-20133, Milano, Italy
# CNR-IBF, Consiglio Nazionale delle Ricerche, Istituto di Biofisica, Via Celoria 26, I-20133, Milano, Italy
#† Program in Emerging Infectious Diseases, Duke-NUS Medical School, 8 College Road 169857, Singapore
* Department of Microbiology and Immunology, National University of Singapore, 5 Science Drive 2, Singapore 117545
§ Institute for Glycomics, Griffith University, Gold Coast Campus, Queensland 4022, Australia

Table of contents

1. Chemistry pag. S2
   1.1 Synthesis of pyridobenzothiazolones 2-9 pag. S3
   1.2 Synthesis of pyridones 10 and 11 pag. S4

2. Experimental procedures: general chemistry pag. S4
   2.1. Synthesis of pyridobenzothiazoles 2-9 pag. S6
   2.2. Synthesis of pyridones 10 and 11 pag. S16

3. Experimental procedures for the in silico studies pag. S23
   3.1. Sequence Alignments pag. S23
   3.2. Docking Studies pag. S25

S1
4. Experimental procedures for the biological evaluation pag. S26

5. PAINS Filters pag. S31

6. References pag. S32
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-cyclohexyloxy analogue 16, prepared as previously reported,\textsuperscript{1} were hydrolyzed under basic conditions into carboxylates 17 and 18\textsuperscript{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\textsuperscript{1} was coupled with benzyamines to give target compounds 6 and 7 in moderate yield, while compound 8 was synthesized by refluxing ester 16\textsuperscript{1} in ethanolamine. Decarboxylation of acid 18\textsuperscript{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\(_2\)SO\(_4\) 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 $\lambda$ 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$_3$CN in 30% H$_2$O with 0.1% formic acid for compounds 2-5; 70% CH$_3$CN in 30% H$_2$O for compounds 6 and 8; 90% CH$_3$CN in 10% H$_2$O for compounds 7 and 9; 60% CH$_3$CN in 40% H$_2$O with 0.1% formic acid for compound 10; 30% CH$_3$CN in 70% H$_2$O with 0.1% formic acid for compound 11. Injection volume was of 20 µL and column temperature of 30 °C, peaks retention time (rt) 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]-beta-alaninate (20).** Following the general procedure and starting from intermediate 18 and using L-Ser methyl ester hydrochloride, compound 20 was obtained after purification by semi-automated column chromatography eluting with CHCl₃/Acetone 98:2 and subsequently triturated with EtOH giving a yellow solid in 47% yield (reaction time: 2 h): mp 158-159 °C; TLC (CHCl₃/MeOH 95:5v/v) Rₚ = 0.6. ¹H NMR (400 MHz, DMSO-d₆): 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-CH₂), 3.62 (s, 3H, CH₃), 1.90 (m, 2H, Cy-CH₂), 1.68-1.56 (m, 2H, Cy-CH₂), 1.55-1.18 (m, 6H, Cy-CH₂).

**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 181 and using β-Ala methyl ester hydrochloride, compound 21 was obtained after purification by semi-automated column chromatography eluting with CH2Cl2 100% to give a yellow solid in 27% yield: mp 218-219 °C; TLC (CHCl3/MeOH 99:1 v/v) Rf=0.5. 1H NMR (400 MHz, DMSO-d6): 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, CH3), 3.51-3.46 (m, 2H, β-Ala NHC=CH2CO2CH3), 2.58 (t, J = 6.9 Hz, 2H, β-Ala NHCH2CH2CO2CH3), 1.91-1.90 (m, 2H, Cy-CH2), 1.69-1.66 (m, 2H, Cy-CH2), 1.54-1.17 (m, 6H, Cy-CH2).

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 181 and using L-m-OH Phe methyl ester hydrochloride, compound 22 was obtained after purification by flash column chromatography CH2Cl2/acetone 98:2 giving a yellow solid in 49% yield (reaction time: 2 h): TLC (CHCl3/MeOH 98:2 v/v)RF = 0.4. 1H NMR (400 MHz, DMSO-d6): 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, CH3), 3.07-2.94 (m, 2H, Phe CH2), 1.90 (m, 2H, Cy-CH2), 1.67 (m, 2H, Cy-CH2), 1.46-1.18 (m, 6H, Cy-CH2).
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 and using 4-Hydroxybenzylamine, compound 6 was obtained after purification by flash column chromatography CH$_2$Cl$_2$/acetone 95:5 giving a yellow solid in 20% yield (reaction time: 24h): mp 244-245 °C; TLC (CH$_2$Cl$_2$/MeOH 95:5v/v) $R_f$ = 0.5. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 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-$d_6$): $\delta$ 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 $\lambda$ = 231 nm; HPLC: r$_t$ 5,630 minutes. HRMS (ESI) calculated for C$_{31}$H$_{28}$N$_2$O$_4$S [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 and using benzyamine, 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-$d_6$): $\delta$ 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-$d_6$): $\delta$

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 $\lambda$ = 237 nm; HPLC: $r_t$ 5.379 minutes. HRMS (ESI) calculated for C$_{31}$H$_{28}$N$_2$O$_3$S
$[M + H]^+$ 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-$d_6$): $\delta$
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$_2$CH$_2$OH), 3.37 (q, $J$ = 5.8 Hz, 2H, NHCH$_2$CH$_2$OH), 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-$d_6$): $\delta$
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 $\lambda$ = 234 nm; HPLC: $r_t$ 5.305 minutes. HRMS (ESI) calculated for C$_{26}$H$_{26}$N$_2$O$_4$S
$[M + H]^+$
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; [α]D = -0.15 (0.5% p/v, DMSO). 1H NMR (400 MHz, DMSO-d6): 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-CH2), 1.90 (m, 2H, Cy-CH2), 1.67 (m, 2H, Cy-CH2), 1.46-1.26 (m, 6H, Cy-CH2), 1.46-1.26 (m, 6H, Cy-CH2). 13C-NMR (100 MHz, DMSO-d6): 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 C27H26N2O6S [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. 1H NMR (400 MHz, DMSO-d6): 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, \( \beta \)-Ala NHC\( \text{H}_2\text{C}\text{H}_2\text{CO}_2\text{H} \)), 2.51 (t, \( J = 7.0 \) Hz, 2H, \( \beta \)-Ala NHCH\( \text{C}\text{H}_2\text{CH}_2\text{CO}_2\text{H} \)), 1.90 (m, 2H, Cy-CH\( _2 \)), 1.67 (m, 2H, Cy-CH\( _2 \)), 1.46-1.23 (m, 6H, Cy-CH\( _2 \)). \( ^{13} \text{C-NMR} (100 \text{ MHz}, \text{DMSO-}d_6): \delta 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 \( \lambda = 234.5 \) nm; HPLC: \( r_t \) 6.530 minutes. HRMS (ESI) calculated for \( \text{C}_{27}\text{H}_{26}\text{N}_2\text{O}_5\text{S} [\text{M + H}]^+ 491.1640 \), found 491.1637.

\( \text{N-}\{8-(\text{Cyclohexyloxy})-1-\text{oxo}-2-\text{phenyl}-1H-\text{pyrido}[2,1-b][1,3]benzothiazol-4-yl}\text{carbonyl}\}-3-\text{hydroxy-}L-\text{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 \degree\text{C}; [\alpha]_D = -0.08 (0.5\% p/v, DMSO).} \( ^1\text{H NMR} (400 \text{ MHz, DMSO-}d_6): \delta 9.26 (\text{bs, 1H, Phe-OH}), 8.81 (\text{s, 2H, H-9 and Phe-NH}), 8.48 (\text{bs,1H, H-3}), 7.83 (\text{d, } J = 8.6 \text{ Hz , 1H, H-6}), 7.78-7.76 (\text{m, 2H, Ar-H}), 7.44 (\text{t, } J = 7.1 \text{ Hz , 2H, Ar-H}), 7.34 (\text{m, 1H, Ar-H}), 7.06 (\text{d, } J = 8.5 \text{ Hz , 1H, H-7}), 6.95 (\text{t, } J = 7.9 \text{ Hz , 1H, PheAr-H}), 6.67 (\text{s, 2H, PheAr-H}), 6.48 (\text{d, } J = 7.7 \text{ Hz , 1H, PheAr-H}), 4.55-4.47 (\text{m, 1H, Phe-CH }), 4.35 (\text{m, 1H, Cy-OCH}), 3.12-3.11 (\text{m, 1H, PheCH\( _a\)CH\( _b\)}), 2.94-2.84 (\text{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} \text{C-NMR} (100 \text{ MHz, DMSO-}d_6): \delta 174.31, 164.05, 161.28, 157.13, 156.23, 151.38, 140.46, 138.71, 136.71, 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 \( \lambda = 226 \) nm; HPLC: \( r_t \) 5,948 minutes. HRMS (ESI) calculated for \( \text{C}_{33}\text{H}_{30}\text{N}_2\text{O}_6\text{S} [\text{M + H}]^+ 583.1903 \), 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 (0.1 g, 0.24 mmol) the reaction was carried out by MW irradiation $T = 250^\circ C$, $P = 10$ 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$_2$Cl$_2$/cyclohexane 50:50 to CH$_2$Cl$_2$ 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$_6$): $\delta$ 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$_6$): $\delta$ 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 $\lambda = 227$ nm; HPLC: $r_t$ 5.371 minutes. HRMS (ESI) calculated for C$_{23}$H$_{21}$NO$_2$S [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 (40 mL), 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 mineral oil (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, OCH\(_2\)CH\(_3\)), 4.11 (s, 2H, CH\(_2\)CO), 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\(_2\)CH\(_3\)).
**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 (CHCl₃/MeOH 99:1 v/v): Rf = 0.2. H NMR (400 MHz, CDCl₃): δ 7.80 (s, 1H, CHNCH₃CH₃), 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, OCH₂CH₃), 2.92 (s, 6H, CHNCH₃CH₃), 2.15-2.03 (m, 2H, Cycloheptyl CH₂), 1.82-1.71 (m, 6H, Cycloheptyl CH₂), 1.47-1.46 (m, 4H, Cycloheptyl CH₂), 1.24 (t, J = 7.0 Hz, 3H, OCH₂CH₃).

**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 Et₂O and compound 15 was obtained as yellow solid without any further purification in 58% yield: mp 167-168 °C; TLC (CHCl₃ 100%): Rf = 0.65. H NMR (400 MHz, DMSO-d₆): 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, OCH₂CH₃), 2.02-1.92 (m, 2H,
Cycloheptyl CH₂, 1.73-1.61 (m, 5H, Cycloheptyl CH₂), 1.51-1.43 (m, 5H, Cycloheptyl CH₂), 1.29 (t, J = 7.0 Hz, 3H, OCH₂CH₃).

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 (CH₂Cl₂/MeOH 95:5 v/v) Rf=0.3. ¹H NMR (400 MHz, DMSO-d₆): 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-CH₂), 1.74-1.44 (m, 10H, Cycloheptyl-CH₂).

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 CHCl₃/MeOH 98:2, as a yellow solid in 44% yield (reaction time: 2 h): mp 139-140 °C; TLC (CHCl₃/MeOH 95:5 v/v) Rf= 0.6. ¹H NMR (400 MHz, DMSO-d₆): 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$_2$CH$_3$), 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$_2$).

$\text{N-}\{8-\text{(Cycloheptyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl} \text{carbonyl}\}-\text{L-tyrosine (2).}$ 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-$d_6$): $\delta$ 12.71 (bs, 1H, CO$_2$H), 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$_a$H$_b$), 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-$d_6$): $\delta$ 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 $\lambda = 229.5$ nm; HPLC: $r_t$ 5.985 minutes.

2.2. Synthesis of pyridones 10 and 11.

$\text{Methyl 5-bromo-1-(3-methoxyphenyl)-6-oxo-1,6-dihydropyridine-3-carboxylate}$
To a mixture of starting material \(23\) (0.2 g, 0.86 mmol) in dry \(\text{CH}_2\text{Cl}_2\), (3-methoxyphenyl)boronic acid (0.16 g, 1.03 mmol), \(\text{Cu(OAc)}_2\cdot\text{H}_2\text{O}\) (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 \(\text{CH}_2\text{Cl}_2\), washed with brine, dried and concentrated under vacuum to provide a crude white residue which was purified by flash column chromatography eluting with \(\text{CHCl}_3\) 100% followed by \(\text{CHCl}_3/\text{acetone} 99:1\), to give the desired derivative \(24\) (218 mg, 75%) as pale brown solid: mp 108-109 °C; TLC (\(\text{CH}_2\text{Cl}_2/\text{MeOH}, 99:1\ v/v\)): \(R_f = 0.55\). \(^1\text{H NMR} (400 \text{ MHz, CDCl}_3): \delta 8.31 (d, J = 2.4 \text{ Hz, 1H, H-2}), 8.20 (d, J = 2.3 \text{ 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), \(\text{PhB(OH)}_2\) (0.043 g, 0.35 mmol), \(\text{Pd(PPh}_3)_4\) (0.017 g, 0.015 mmol), DME (1.6 mL), and 2M \(\text{K}_2\text{CO}_3\) solution (0.3 mL, 0.59 mmol) were added successively. The resulting mixture was put in the MW at \(T = 100^\circ\text{C, P = 8 bar, cooling on,}\) 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 \(\text{CH}_2\text{Cl}_2\) (100%), giving the title compound \(25\) in 74% yield as white solid: mp 114-115 °C; TLC (Cyclohexane/EtOAc, 80:20 v/v): \(R_f = 0.5\). \(^1\text{H NMR} (400 \text{ MHz, CDCl}_3): \delta\)
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, OCH₃), 3.85 (s, 3H, CH₃).

**Methyl 1-(3-hydroxyphenyl)-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylate (26).** Under N₂ atmosphere, to a stirred solution of 25 (0.10g, 0.30 mmol) in dry CH₂Cl₂ (3 mL) at 0 °C, BBr₃ 1M in CH₂Cl₂ (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 CHCl₃/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): Rf = 0.4. ¹H NMR (400 MHz, CDCl₃): 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, CH₃).

**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% CHCl₃ obtaining 27 as white solid (240 mg, 68% yield): mp 94-95 °C; TLC (Cyclohexane/EtOAc, 80:20 v/v): Rₜ = 0.4. ¹H NMR (400 MHz, CDCl₃): 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 CH₃), 2.04-1.94 (m, 2H, Cy-CH₂), 1.85-1.75 (m, 2H, Cy-CH₂), 1.56-1.47 (m, 2H, Cy-CH₂), 1.40-1.19 (m, 4H, Cy-CH₂).

**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(PPh₃)₄ (0.046 g, 0.05 mmol), and 2M K₂CO₃ solution (2 mmol) were added successively. The resulting mixture was degassed and replaced with N₂ 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 CHCl₃. The filtrate was extracted with water and CHCl₃ 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 CH₂Cl₂/acetone (97:3) followed by CH₂Cl₂/acetone (95:5). Derivative 29 was obtained in 81% yield as white solid: TLC (Cyclohexane/EtOAc, 50:50 v/v): mp 113-114 °C; Rₜ = 0.5. ¹H NMR (400 MHz, DMSO-d₆): 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 CH₃), 3.56 (s, 3H NCH₃).

**1-[3-(Cyclohexyloxy)phenyl]-6-oxo-5-phenyl-1,6-dihydropyridine-3-carboxylic acid (30).** To a solution 0.1M of compound 27, (0.10 g, 0.30 mmol) 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 (CHCl₃/MeOH, 90:10 v/v): Rₑ = 0.25. ¹H NMR (400 MHz, CDCl₃): 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-CH₂), 1.78 (m, 2H, Cy-CH₂), 1.57-1.50 (m, 2H, Cy-CH₂), 1.43-1.22 (m, 4H, Cy-CH₂).

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 (CHCl₃/MeOH, 90:10 v/v): Rₑ = 0.25. ¹H NMR (400 MHz, DMSO-d₆): δ 12.88 (bs, 1H, CO₂H), 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, NCH₃).

(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 CHCl₃/Acetone (98:2) as a light yellow solid in 50% yield: mp 189-190 °C; TLC (CH₂Cl₂/MeOH, 95:5 v/v): Rₑ = 0.5. ¹H NMR (400 MHz, CDCl₃): 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 and Ar-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$_a$H$_b$), 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$_2$Cl$_2$/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; [α]$_D$ = -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<sub>a</sub>H<sub>b</sub>), 2.82-2.70 (m, 1H, Tyr-CH<sub>a</sub>H<sub>b</sub>), 2.07-2.02 (m, 2H, Cy-CH<sub>2</sub>), 1.75-1.64 (m, 2H, Cy-CH<sub>2</sub>), 1.46-1.11 (m, 6H, Cy-CH<sub>2</sub>). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>): 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<sub>t</sub> 4,676 minutes. HRMS (ESI) calculated for C<sub>33</sub>H<sub>32</sub>N<sub>2</sub>O<sub>6</sub> [M + H]<sup>+</sup> 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<sub>2</sub>O/CH<sub>3</sub>CN (80:20) as white solid, yield: 27%; mp 163-164 °C; [α]<sub>D</sub> = -0.35 (0.5% p/v, DMSO). 1H NMR (400 MHz, DMSO-d<sub>6</sub>): 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<sub>3</sub>), 3.00-2.97 (m, 1H Tyr-CH<sub>a</sub>H<sub>b</sub>), 2.90-2.84 (m, 1H, Tyr-CH<sub>a</sub>H<sub>b</sub>). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>): 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<sub>t</sub> 4.862 minutes. HRMS (ESI) calculated for C<sub>22</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub> [M + H]<sup>+</sup> 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.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Taxonomy</th>
<th>UniProt ID</th>
<th>UniProt Name</th>
<th>Residues (Dengue 2 numeration)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue virus type 1</td>
<td>DEN1W</td>
<td>P17763</td>
<td>POLG_DEN1W</td>
<td>327 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 1</td>
<td>DEN1B</td>
<td>P27909</td>
<td>POLG_DEN1B</td>
<td>328 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 1</td>
<td>DEN1S</td>
<td>P33478</td>
<td>POLG_DEN1S</td>
<td>329 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN26</td>
<td>P29990</td>
<td>POLG_DEN26</td>
<td>330 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN27</td>
<td>P29991</td>
<td>POLG_DEN27</td>
<td>331 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN28</td>
<td>P14337</td>
<td>POLG_DEN28</td>
<td>858 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN2J</td>
<td>P07564</td>
<td>POLG_DEN2J</td>
<td>859 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN2N</td>
<td>P14340</td>
<td>POLG_DEN2N</td>
<td>860 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN2P</td>
<td>P12823</td>
<td>POLG_DEN2P</td>
<td>861 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 2</td>
<td>DEN2Q</td>
<td>Q9WDA6</td>
<td>POLG_DEN2Q</td>
<td>862 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 3</td>
<td>DEN3C</td>
<td>Q99D35</td>
<td>POLG_DEN3C</td>
<td>863 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 3</td>
<td>DEN3I</td>
<td>Q5UB51</td>
<td>POLG_DEN3I</td>
<td>864 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 3</td>
<td>DEN3M</td>
<td>Q6YMS3</td>
<td>POLG_DEN3M</td>
<td>865 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 3</td>
<td>DEN3P</td>
<td>P27915</td>
<td>POLG_DEN3P</td>
<td>866 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 3</td>
<td>DEN3S</td>
<td>Q6YMS4</td>
<td>POLG_DEN3S</td>
<td>867 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 4</td>
<td>DEN4D</td>
<td>P09866</td>
<td>POLG_DEN4D</td>
<td>868 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 4</td>
<td>DEN4H</td>
<td>Q2YHF2</td>
<td>POLG_DEN4H</td>
<td>869 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 4</td>
<td>DEN4P</td>
<td>Q58HT7</td>
<td>POLG_DEN4P</td>
<td>870 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 4</td>
<td>DEN4S</td>
<td>Q5UCB8</td>
<td>POLG_DEN4S</td>
<td>871 L L T K T W N I A</td>
</tr>
<tr>
<td>Dengue virus type 4</td>
<td>DEN4T</td>
<td>Q2YHF0</td>
<td>POLG_DEN4T</td>
<td>872 L L T K T W N I A</td>
</tr>
<tr>
<td>Tick-borne encephalitis virus</td>
<td>TBEVS</td>
<td>P07720</td>
<td>POLG_TBEVS</td>
<td>873 L L S K E W N I A</td>
</tr>
<tr>
<td>West Nile virus</td>
<td>WNV</td>
<td>P06935</td>
<td>POLG_WNV</td>
<td>874 L L S K T W N I A</td>
</tr>
<tr>
<td>Yellow fever virus</td>
<td>YEFVT</td>
<td>Q9YRV3</td>
<td>POLG_YEFVT</td>
<td>875 L L T Y T W N I A</td>
</tr>
<tr>
<td>Zika virus</td>
<td>ZIKVF</td>
<td>A0A024B7W1</td>
<td>POLG_ZIKVF</td>
<td>876 L L S K T W N I A</td>
</tr>
</tbody>
</table>

*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 Bank6 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LBE (Kcal/mol)</th>
<th>NiC</th>
<th>Interacting Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-9.56</td>
<td>45</td>
<td>322;325;326;327;328;329;332;859;862;863;866;869</td>
</tr>
<tr>
<td>1</td>
<td>-9.24</td>
<td>40</td>
<td>322;325;326;327;328;329;859;862;863;865;866;869</td>
</tr>
<tr>
<td>2</td>
<td>-9.05</td>
<td>28</td>
<td>322;325;326;327;328;329;332;859;862;863;865;866;869</td>
</tr>
<tr>
<td>4</td>
<td>-7.78</td>
<td>22</td>
<td>322;325;326;329;858;859;862;863;865;866;869</td>
</tr>
</tbody>
</table>

*aLigand 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% CO2. 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 CO₂. 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% CO₂.

The DENV strains (GenBank accessions: DENV1, EU081230; DENV2, EU081177; DENV3, EU081190; DENV4, GQ398256) were obtained from the Early Dengue infection and outcome (EDEN) study. 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. 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<sub>CF18</sub> 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<sub>50</sub>. 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\textsuperscript{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\textsubscript{2}, 0.3 mM MnCl\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{max} and emission\textsubscript{max} wavelengths 422 nm and 566 nm respectively.

**Cell viability test**

2 × 10\textsuperscript{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 0.5 μM 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: 0.5 μM for P2-3; 1 μM for P4-6; 5 μM for P7-9 and finally 10 μM for P10. The virus from the P10 supernatant was expanded once in C6/36 and tested for susceptibility towards the compound at 10 μM.\textsuperscript{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,\textsuperscript{15}; nucleoside
analog inhibitor: NITD008,\textsuperscript{16}; non-nucleoside N-pocket inhibitor: N27\textsuperscript{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, $1 \times 10^5$ 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.

![Sensitivity of drug resistant clone](image)

**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\textsuperscript{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),\textsuperscript{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\textsuperscript{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.”

6. References


