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## Supplementary Material

# Expanding on the plecstatin anticancer agent class: exchange of the chlorido ligand for N-heterocyclic ligands

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## Experimental

*Materials and Equipment*

Chemicals and solvents were purchased from suppliers and no further purifications were performed prior to usage. RP-HPLC solvents were of HPLC grade and used without further purification apart from DCM and MeOH which were dried in a solvent purification system (LC Technology Solutions Inc., SP-1 solvent purifier) and transferred under vacuum to Schlenk flasks following purging with $N_2$ gas. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Acetonitrile (MeCN) (HPLC grade) and dichloromethane (DCM) (AR) were ordered from JT Baker (Radnor, PA, USA), methanol (MeOH)(HPLC grade) from Macron Fine Chemicals (Radnor, PA, USA), trifluoroacetic acid (TFA) from Oakwood Chemicals (Estill, SC, USA), and diethyl ether ($Et_2O$) (AR), n-hexane (technical), pyridine, silver nitrate and toluene from ECP (Auckland, New Zealand). Milli-Q high purity deionised water (MQ $H_2O$) (18 Ω) was available from a Sartorius Arium Pro Ultrapure Water System from Sartorius Stedim Biotech (Gottingen, Germany). 1-Methylimidazole and silver triflate were purchased from AK Scientific (Union City, CA, USA), $Ag_2O$ from Oakwood Chemicals (Estill, SC, USA), and $RuCl_3 \cdot H_2O$ from Precious Metals Online (Wollongong, NSW, Australia). $NH_4PF_6$ and 1-methylbenzimidazole were ordered from Sigma–Aldrich (Saint Louis, MO, USA). $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$,[1] *N*-(4-fluorophenyl)pyridine-2-carbothioamide (*p*-F-PCA),[2] [chlorido(η$^6$-*p*-cymene)(*N*-(4-fluorophenyl)pyridine-2-carbothioamide)ruthenium(II)] chloride (plecstatin-1),[2] [dichlorido(η$^6$-*p*-cymene)(*N*-methylimidazole)ruthenium(II)] (**1**),[3] [dichlorido(η$^6$-*p*-cymene)(*N*-methylbenzimidazole)ruthenium(II)] (**2**),[3] and [dichlorido(η$^6$-*p*-cymene)(*N*-pyridine)ruthenium(II)] (**3**)[3] were prepared according to literature procedures.

$^1$H NMR, $^{13}$C{$^1$H} and 2-D NOESY NMR spectra were recorded on Bruker (Billerica, MA, USA) DRX 400 MHz NMR spectrometers at ambient temperature at 400.13 ($^1$H) or 100.57 ($^{13}$C) MHz with chemical shifts (δ, ppm) reported relative to the residual solvent peaks of the deuterated solvents. Elemental analyses were conducted on a vario EL cube (Elementar Analysensysteme GmbH, Hanau, Germany) using CHN mode. Electrospray ionisation mass spectra were recorded on a Bruker micrOTOF-QII instrument in positive ion mode or on an Agilent Technologies (Santa Clara, CA, USA) 1260 Infinity LC connected to an Agilent Technologies 6120 quadrupole MSD

spectrophotometer at a flow rate of 0.3 mL $min^{-1}$ of 0.1% formic acid in water/MeCN (1/1, v/v).

Analytical HPLC was performed on a Dionex UltiMate 3000 equipped with a Gemini NX-C18 110 Å column (5 µm, 250 × 4.6 mm) at a flow rate of 1.0 mL/min and using a linear gradient of 1% B to 75% B at an increase of 1% B $min^{-1}$ (solvent A = $H_2O$ + 0.1% TFA, solvent B = MeCN + 0.1% TFA). UV absorbance was traced at 210, 225, 254 and 280 nm.

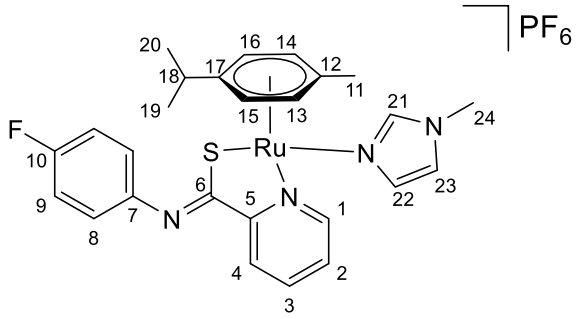
X-ray diffraction measurements of single crystals of **4**, grown from $d_6$-acetone in an NMR tube, were performed on a Rigaku Oxford Diffraction XtaLAB-Synergy-S single-crystal diffractometer (Rigaku Corp., Tokyo, Japan) with a PILATUS 200K hybrid pixel array detector using Cu Kα radiation [λ = 1.54184 Å]. The data were processed with the SHELXT[4] and Olex2[5,6] software packages (Table S1). All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were inserted at calculated positions and refined with a riding model or without restrictions. Mercury 2022.1.0 was used to visualize the molecular structures.[7]

## Synthesis

General protocol

Compound **2** (53 mg, 0.10 mmol) was dissolved in MeOH (2 mL) and diluted with MilliQ water (4 mL). *N*-Heterocyclic compound (1.5 eq.) was added, and the reaction mixture stirred overnight at room temperature under $N_2$ atmosphere. $NH_4PF_6$ (163 mg, 1.0 mmol) was added, and the resulting yellow-orange precipitate was collected, washed with $Et_2O$ (2 × 10 ml) and dried *in vacuo* to give the product as a yellow to orange powder.

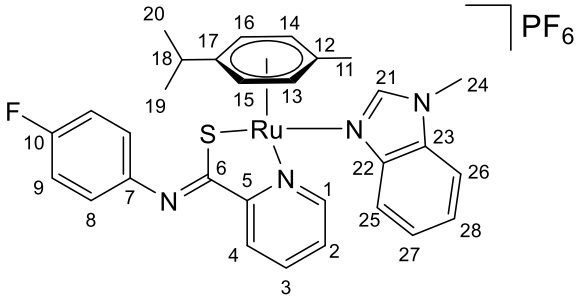
[(η$^6$-*p*-Cymene)(*N*-(4-fluorophenyl)pyridine-2-carbothioamide)(1-methylimidazole)ruthenium(II)] hexafluorophosphate (**4**)




Compound **4** was synthesised following the general protocol using 1-methylimidazole (12.0 µL, 0.15 mmol). Yield: 51 mg (74%).

$^1$H NMR (400.13 MHz, *d*$_6$-acetone) δ 9.78–9.75 (m, 1H, H-1), 8.15 – 8.20 (m, 1H, H-4), 8.13 – 8.07 (m, 1H, H-3), 7.97 (s, 1H, H-23/24), 7.70 (ddd, *J* = 7, 6, 2 Hz, 1H, H-2), 7.44 (d, *J* = 7 Hz, 1H, H-21), 7.11–7.18 (m, 5H, H-8/9, H-23/24), 6.10 – 6.15 (m, 3H, H-13/15/16), 5.65 (d, *J* = 6 Hz, 1H, H-14), 3.75 (s, 3H, H-24), 2.61 (m, 1H, H-8), 1.89 (s, 3H, H-11), 1.13 (d, *J* = 7 Hz, 3H, H-19), 1.04 (d, *J* = 7 Hz, 3H, H-20) ppm. $^{13}$C{$^1$H} NMR (100.57 MHz, *d*$_6$-acetone) δ 173.52 (C-6), 160.56 (C-10), 158.94 (C-5), 157.60 (C-1), 142.11 (C-22/23), 141.56 (C-7), 139.96 (C-3), 131.60 (C-22/23), 128.47 (C-2), 124.90 (C-4), 123.88 (C-8/9), 123.81 (C-8/9), 123.37 (C-21), 116.13 (C-8/9), 115.90 (C-8/9), 105.41 (C-12), 103.79 (C-17), 90.75 (C-13), 89.13 (C-15/16), 88.95 (C-15/16), 82.26 (C-14), 34.90 (C-24), 31.65 (C-18), 23.05 (C-19/20), 21.93 (C-19/20), 18.11 (C-11) ppm. Elemental analysis calculated for $C_{26}H_{28}F_7N_4PRuS \cdot 0.95H_2O \cdot 0.1NH_4PF_6$: C 42.95; H 4.20; N 7.90%. Found C 43.37; H 3.77; N 7.45%. MS (ESI$^+$) *m/z* 467.0564 [M – methylimidazole]$^+$ (*m/z*$_{calculated}$ 467.0531).

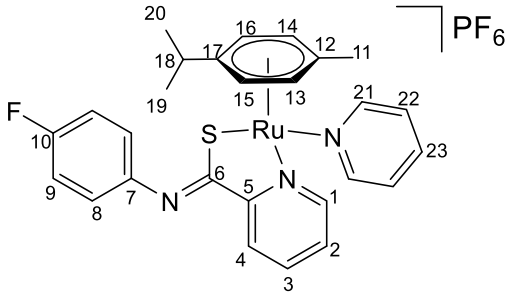
[(η$^6$-*p*-Cymene)(*N*-(4-fluorophenyl)pyridine-2-carbothioamide)(1-methylbenzimidazole)ruthenium(II)] hexafluorophosphate (**5**)




Compound **5** was synthesised following the general protocol using 1-methylbenzimidazole (19.8 mg, 0.15 mmol). Yield: 47 mg (61%).

$^{1}$H NMR (400.13 MHz, $d_6$-acetone) δ 10.06 (d, *J* = 6 Hz, 1H, H-1), 8.33 (s, 1H, H-21), 8.13 – 7.98 (m, 2H, H-3/4), 7.76 – 7.66 (m, 3H, H-2, H-25–28), 7.44 (d, *J* = 2 Hz, 2H, H-25–28), 7.17 – 7.07 (m, 2H, H-8/9), 7.02 – 6.93 (m, 2H, H-8/9), 6.42 (d, *J* = 5 Hz, 1H, H-13), 6.33 (d, *J* = 6 Hz, 1H, H-16), 6.26 (d, *J* = 6 Hz, 1H, H-15), 5.67 (d, *J* = 6 Hz, 1H, H-14), 3.95 (s, 3H, H-24), 2.70 (m, 1H, H-18), 1.75 (s, 3H, H-11), 1.18 (d, *J* = 7 Hz, 3H, H-20), 1.02 (d, *J* = 7 Hz, 3H, H-19) ppm. $^{13}$C{$^{1}$H} NMR (100.57 MHz, $d_6$-acetone) δ 173.09 (C-6), 160.71 (C-10), 158.96 (C-5), 158.33 (C-1), 147.23 (C-21), 141.58 (C-22/23), 140.20 (C-3/4), 135.39 (C-22/23), 128.31 (C-2), 124.58 (C-3/4), 123.52 (C-25 – 28), 123.35 (C-8/9), 123.26 (C-8/9), 117.60 (C-25–28), 117.38 (C-25–28), 116.16 (C-8/9), 115.94 (C-8/9), 112.56 (C-25–28), 108.71 (C-12), 103.69 (C-17), 90.31 (C-16), 89.46 (C-13), 88.88 (C-15), 81.32 (C-14), 32.43 (C-18), 31.72 (C-24), 23.33 (C-20), 21.64 (C-19), 18.35 (C-11) ppm. Elemental analysis calculated for $C_{30}H_{30}F_7N_4PRuS \cdot 0.7H_2O \cdot 0.25NH_4PF_6$: C 45.21; H 4.10; N 7.47%. Found C 45.31; H 3.96; N 7.33%. MS (ESI$^+$) *m/z* 467.0549 [M – methylbenzimidazole]$^+$ ($m/z_{calculated}$ 467.0531).

[(η$^6$-*p*-Cymene)(*N*-(4-fluorophenyl)pyridine-2-carbothioamide)(*N*-pyridine)ruthenium(II)] hexafluorophosphate (**6**)




Compound **6** was synthesised following the general protocol using pyridine (12.1 µL, 0.15 mmol). Yield: 49 mg (70%).

$^{1}$H NMR (400.13 MHz, $d_6$-acetone) δ 9.77 (d, *J* = 5 Hz, 1H, H-1), 8.63 (dt, *J* = 1, 5, Hz, 2H, H-21), 8.11 – 7.97 (m, 2H, H-3–4), 7.93 – 7.81 (m, 1H, H-23), 7.70 – 7.62 (m, 1H, H-2), 7.43 – 7.33 (td, *J* = 1, 6 Hz, 2H, H-22), 7.10 – 6.88 (m, 4H, H-8–9), 6.21 (d, *J* = 6 Hz 1H, H-13/14), 6.15 – 6.08 (m, 2H, H-15–16), 5.48 (d, *J* = 6 Hz, 1H, H-13/14), 2.55 (sept, *J* = 17 Hz, 1H, H-18), 1.70 (s, 3H, H-11), 1.05 (d, *J* = 7 Hz, 3H, H-20/19), 0.90 (d, *J* = 7 Hz, 3H H-20/19) ppm. $^{13}$C{$^{1}$H} NMR (100.57 MHz, $d_6$-acetone) δ 160.38 (C-5), 158.94 (C-10), 157.82 (C-1), 155.53 (C-21), 148.30 (C-7), 140.46 (C-3/4), 140.05 (C-23), 129.10 (C-2), 125.04 (C-3/4), 123.67 (C-8–9), 123.59 (C-8–9), 116.21 (C-8–

9), 115.98 (C-8–9), 106.59 (C-17), 103.32 (C-12), 92.03 (C-13/14), 91.13 (C-15/16), 90.35 (C-13/14), 89.75 (C-15/16), 31.68 (C-18), 23.29 (C-19/20), 21.66 (C-19/20), 17.96 (C-11) ppm. Elemental analysis calculated for $C_{27}H_{27}F_7N_3PRuS \cdot 0.3NH_4Cl$: C, 45.91; H, 3.89; N, 6.64%. Found C, 46.03; H, 4.14; N, 6.81%. MS ($ESI^+$) *m/z* 467.0552 $[M - pyridine]^+$ ($m/z_{calculated}$ 467.0531).

**Aqueous stability studies**

Complex **6** (1.6 mg) was dissolved in DMF (50 µL) and diluted with MeCN/MilliQ water (300/650 µL) and was agitated at 25°C for the duration of the experiment. For the studies in 60-mM HCl solution, the latter solution was acidified with 1 M HCl solution to reach the desired HCl concentration. Aliquots of 50 µL were taken at 0-, 4- and 24-h time points and analysed by RP-HPLC.

**Antiproliferative activity assay**

HCT116, SW480, and NCI-H460 cells were supplied by ATCC, while SiHa cells were supplied from Dr D. Cowan, Ontario Cancer Institute, Canada. The cells were grown in α-MEM (Life Technologies) supplemented with 5% fetal calf serum (Moregate Biotech) at 37°C in a humidified incubator with 5% $CO_2$. The antiproliferative activity was determined by the sulforhodamine B assay as described previously.[8]

## NMR spectra

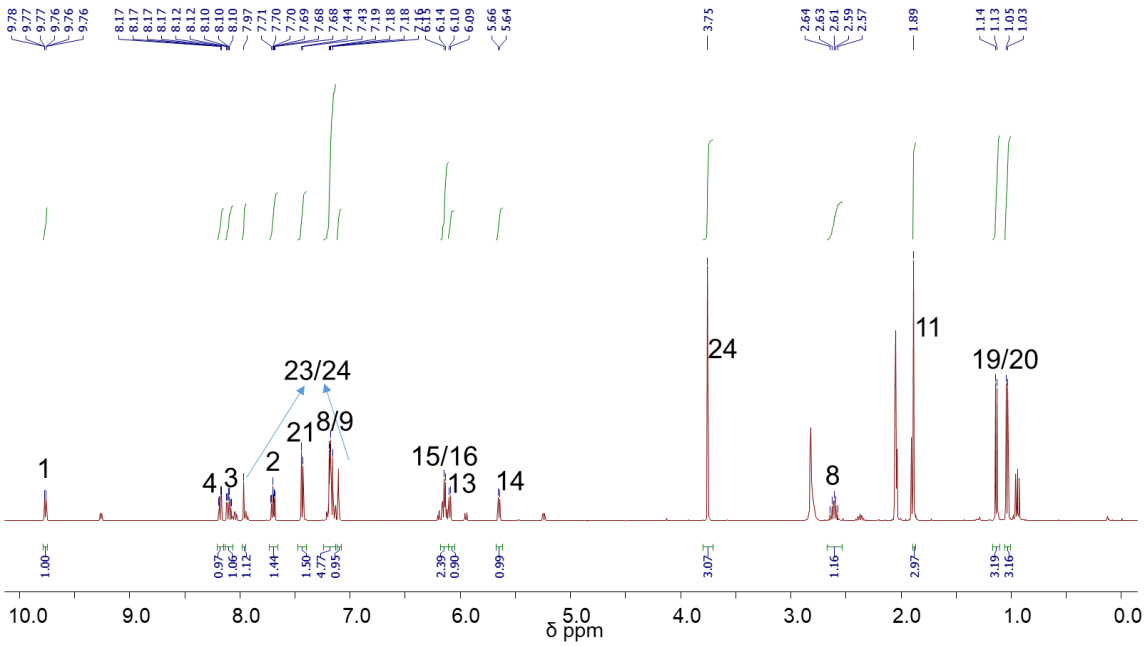



**Figure S1.** $^{1}$H NMR spectrum of **4** in $d_6$-acetone.

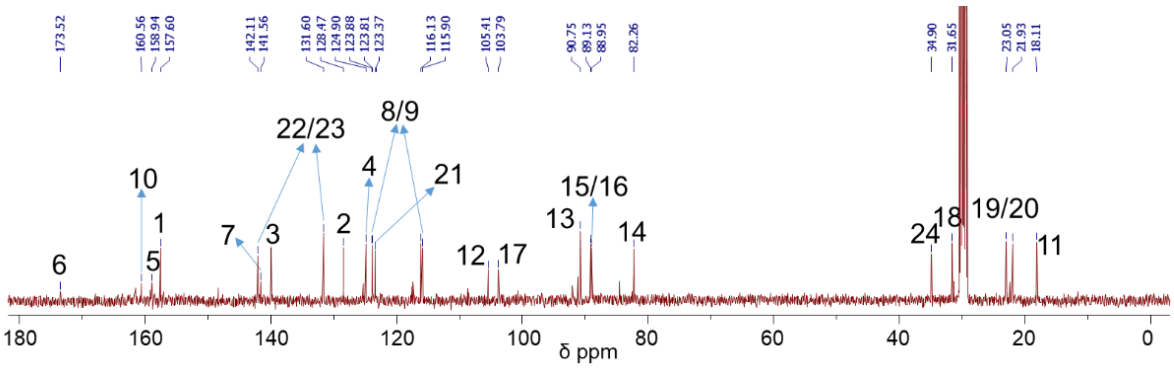



**Figure S2.** $^{13}$C{$^{1}$H} NMR spectrum of **4** in $d_6$-acetone.

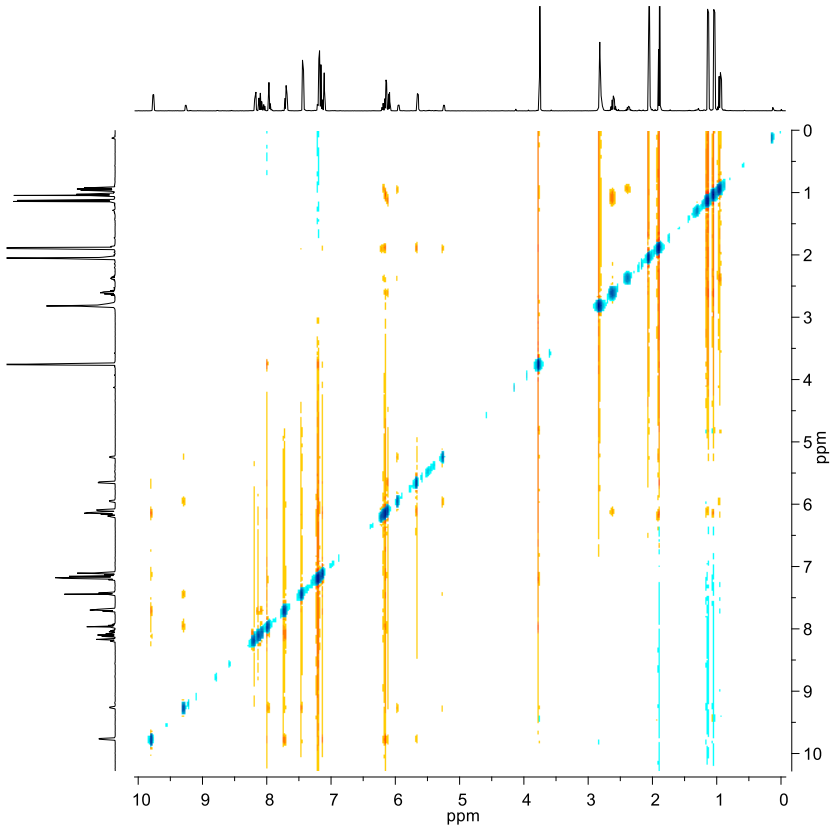



**Figure S3.** $^{1}$H-$^{1}$H NOESY NMR spectrum of **4** in $d_6$-acetone.

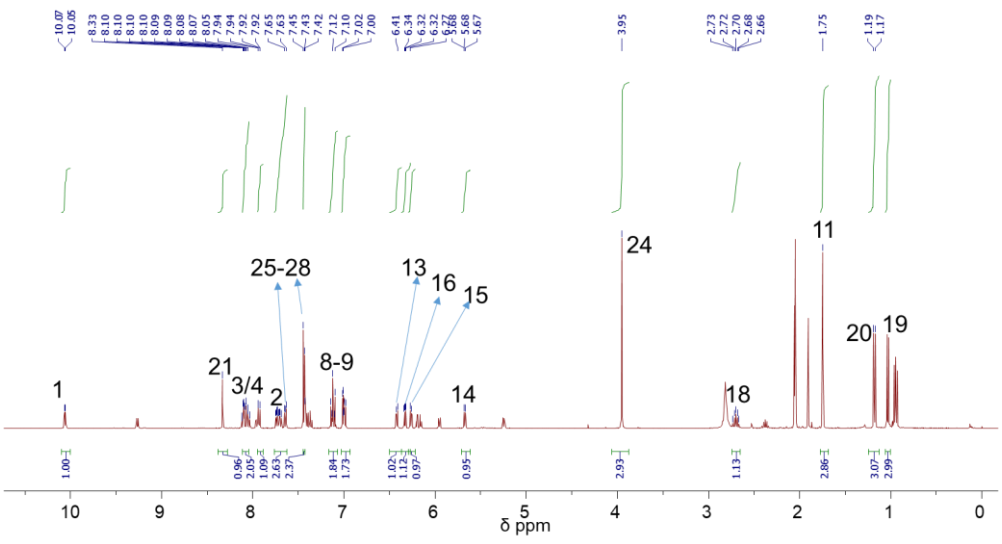



**Figure S4.** $^{1}$H NMR spectrum of **5** in $d_6$-acetone.

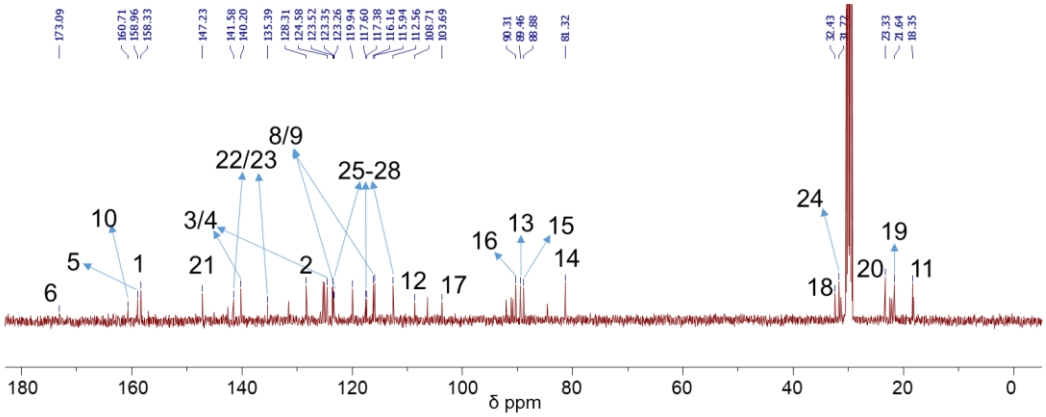



**Figure S5.** $^{13}C\{^{1}H\}$ NMR spectrum of **5** in $d_6$-acetone.

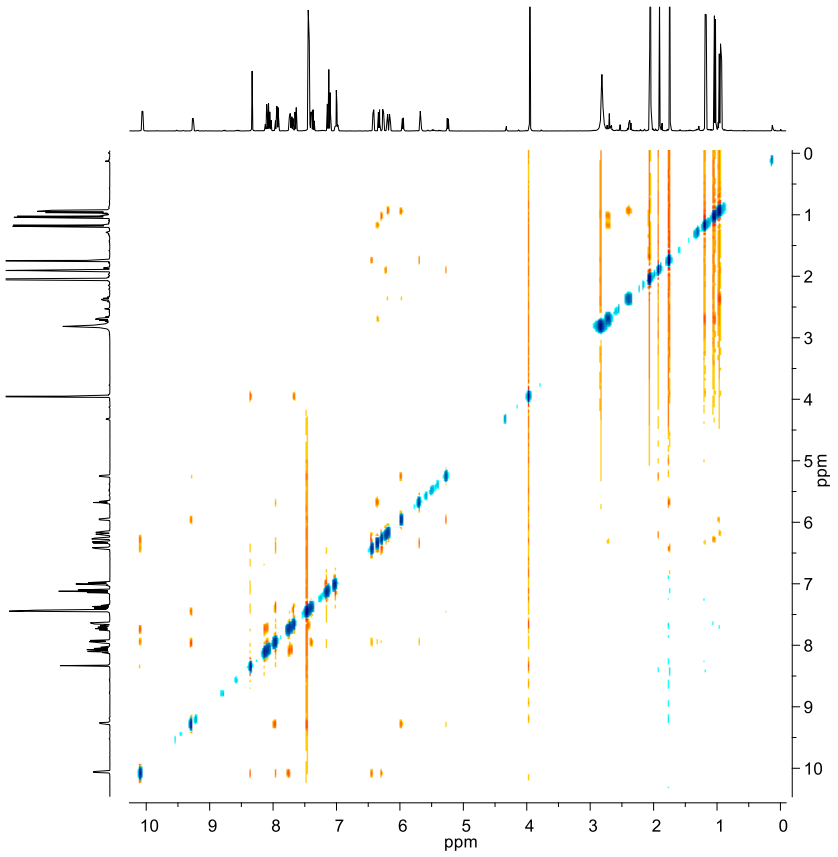



**Figure S6.** $^{1}H$-$^{1}H$ NOESY NMR spectrum of **5** in $d_6$-acetone.

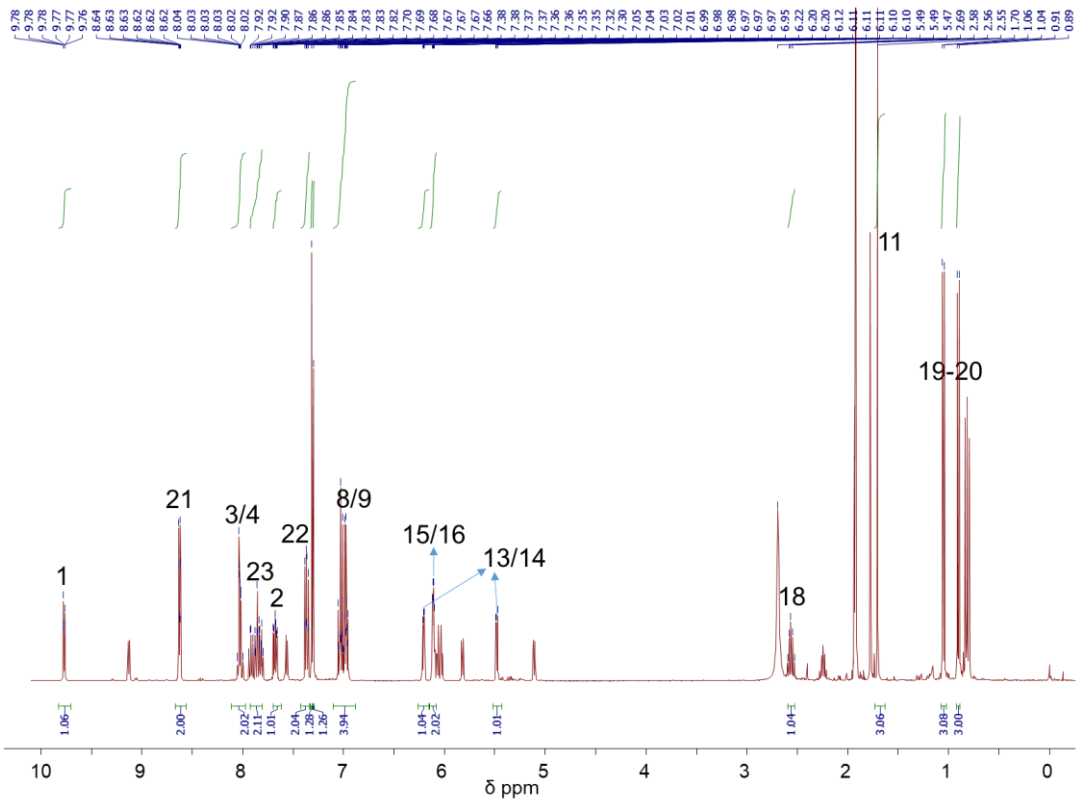



**Figure S7.** $^{1}$H NMR spectrum of **6** in $d_6$-acetone.

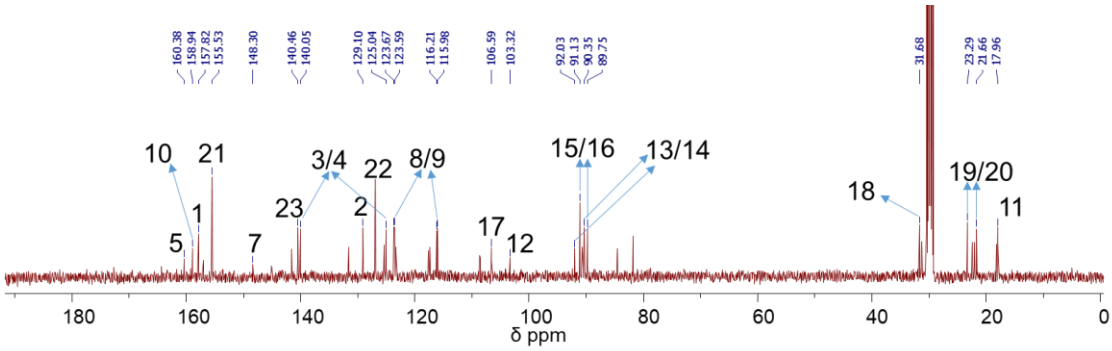



**Figure S8.** $^{13}$C{$^{1}$H} NMR spectrum of **6** in $d_6$-acetone.

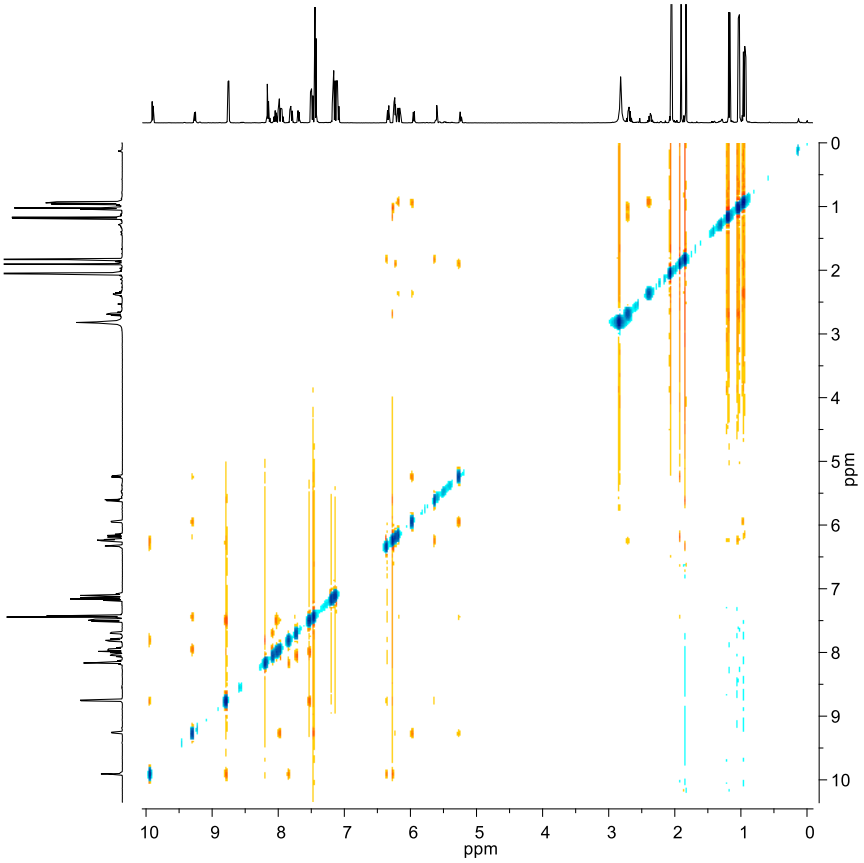



**Figure S9.** $^{1}$H-$^{1}$H NOESY NMR spectrum of **6** in $d_6$-acetone.

## Mass Spectra

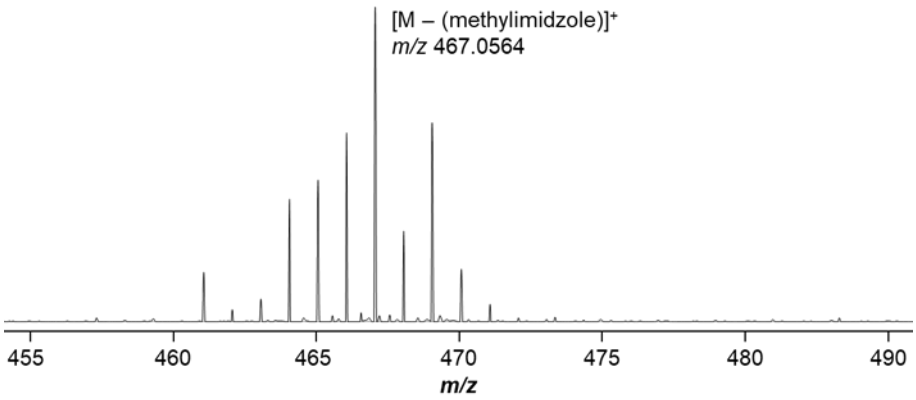



**Figure S10.** ESI-QToF mass spectrum of **4**.

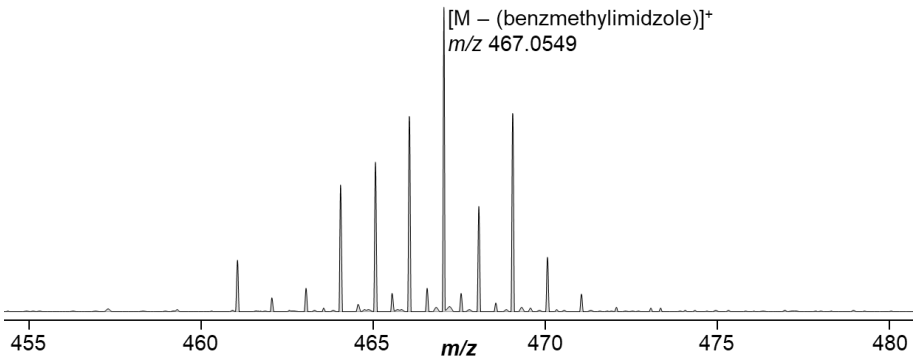



**Figure S11.** ESI-QToF mass spectrum of **5**.

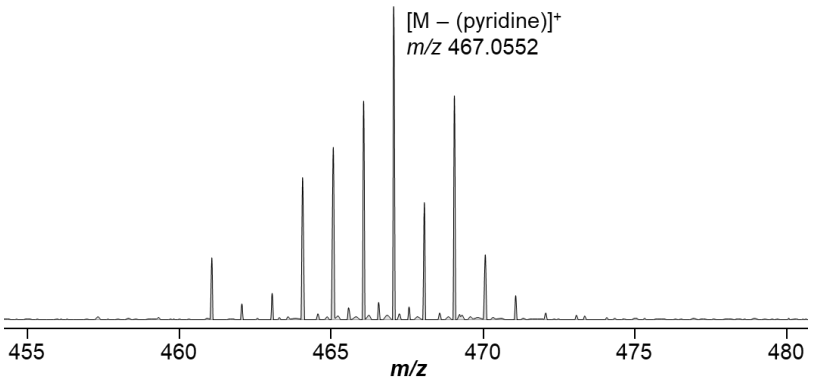



**Figure S12.** ESI-QToF mass spectrum of **6**.

## HPLC Chromatograms

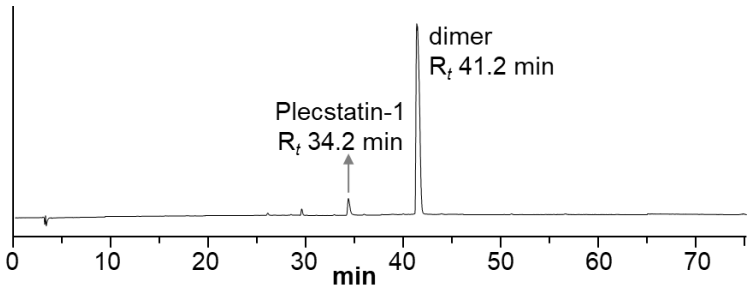



**Figure S13.** RP-HPLC chromatogram of plecstatin-1. Flow rate of 1.0 mL/min, 1% B per minute with a linear gradient of 1% B to 75% B (A = $H_2O$ + 0.1% TFA, B = MeCN + 0.1% TFA) on a Gemini NX-C18 110-Å column (5 µm, 250 × 4.6 mm).

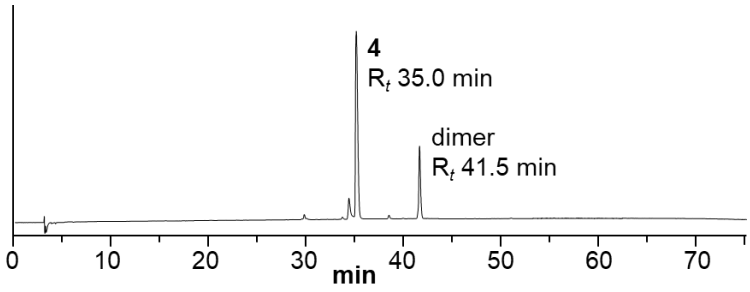



**Figure S14.** RP-HPLC chromatogram of complex 4. Flow rate of 1.0 mL/min, 1% B per minute with a linear gradient of 1% B to 75% B (A = $H_2O$ + 0.1% TFA, B = MeCN + 0.1% TFA) on a Gemini NX-C18 110-Å column (5 µm, 250 × 4.6 mm).

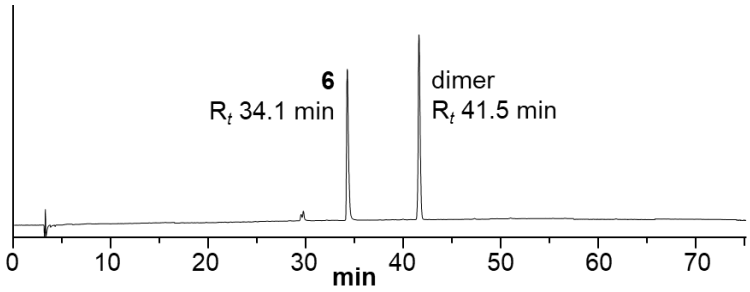



**Figure S15.** RP-HPLC chromatogram of complex **6**. Flow rate of 1.0 mL/min, 1% B per minute with a linear gradient of 1% B to 75% B (A = $H_2O$ + 0.1% TFA, B = MeCN + 0.1% TFA) on a Gemini NX-C18 110-Å column (5 µm, 250 × 4.6 mm).

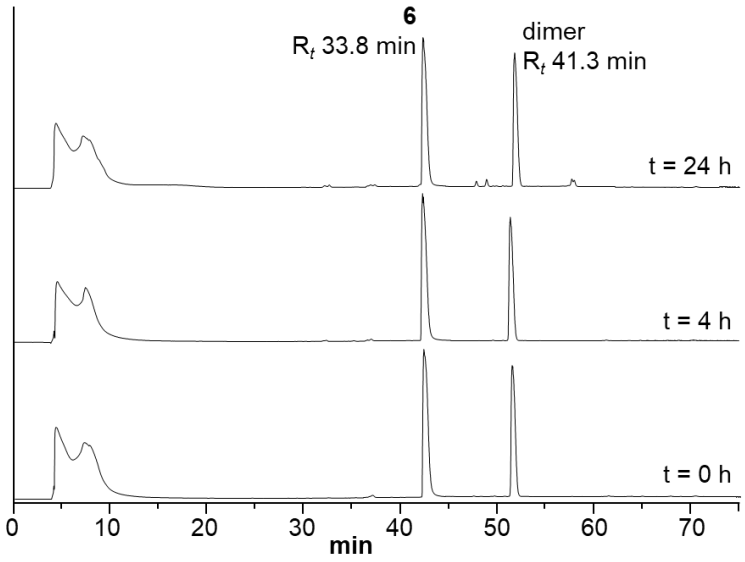



**Figure S16.** RP-HPLC chromatograms of complex **6** in aqueous solution with an aliquot taken at 0, 4 and 24 h. Flow rate of 1.0 mL/min, 1% B per minute with a linear gradient of 1% B to 75% B (A = $H_2O$ + 0.1% TFA, B = MeCN + 0.1% TFA) on a Gemini NX-C18 110-Å column (5 µm, 250 × 4.6 mm).

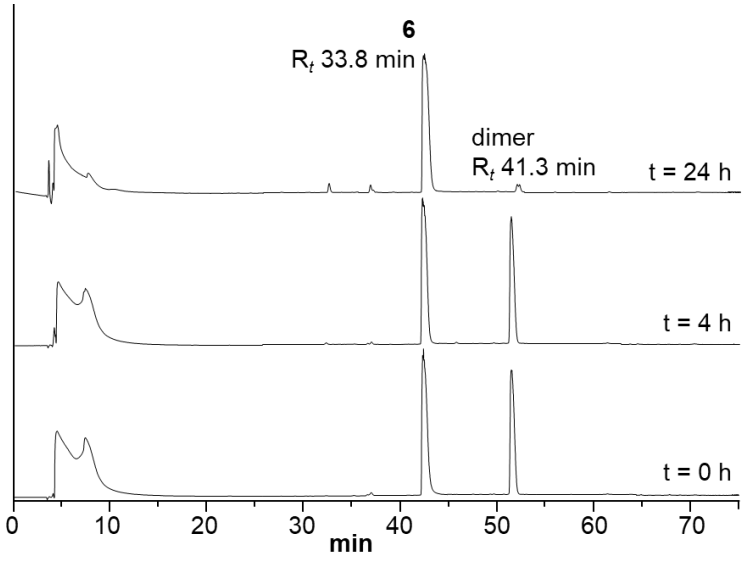



**Figure S17.** RP-HPLC chromatograms of complex **6** in acidified (HCl) solution with an aliquot taken at 0, 4 and 24 h. Flow rate of 1.0 mL/min, 1% B per minute with a linear gradient of 1% B to 75% B (A = $H_2O$ + 0.1% TFA, B = MeCN + 0.1% TFA) on a Gemini NX-C18 110-Å column (5 µm, 250 × 4.6 mm).

## XRD analysis

**Table S1.** Crystal data and measurement parameters for the analysis of **4**.

| **Compound** | **4**·$(CH_3)_2CO$ |
|---|---|
| CCDC | 2352856 |
| Chemical Formula | $C_{29}H_{34}F_7N_4OPRuS$ |
| *M* (g $mol^{-1}$) | 751.70 |
| Temperature (K) | 112.6(4) |
| Crystal size (mm) | 0.2 × 0.05 × 0.05 |
| Crystal system | triclinic |
| Space group | *P*-1 |
| *a* (Å) | 9.1964(2) |
| *b* (Å) | 13.5173(3) |
| *c* (Å) | 13.7396(4) |
| *α* (º) | 69.088(2) |
| *β* (º) | 89.115(2) |
| *γ* (º) | 79.794(2) |
| V ($A^3$) | 1568.10(7) |
| *Z* | 2 |
| $D_c$ (g $cm^{-3}$) | 1.592 |
| μ ($mm^{-1}$) | 5.798 |
| F (000) | 764.0 |
| Θ range (°) | 7.934 to 135.472 |
| *h* range | -11 ≤ 10 |
| *k* range | -16 ≤ 16 |
| *l* range | -16 ≤ 13 |
| Reflections collected | 19971 |
| Data/restraints/parameters | 5652/17/417 |
| Independent reflections ($R_{int}$) | 5652 [$R_{int}$ = 0.0254, $R_{sigma}$ = 0.0178] |
| $R_1$, $wR_2$ (obs., I ≥ 2σ (I)) | $R_1$ = 0.0243, $wR_2$ = 0.0637 |
| $R_1$, $wR_2$ (all data) | $R_1$ = 0.0245, $wR_2$ = 0.0639 |
| Goodness of fit on $F^2$ | 1.055 |

## References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