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Development of a new protein labeling strategy, oxidation labeling. part 1: Preliminary evaluation and synthesis of tautomycin containing a metal coordinating unit

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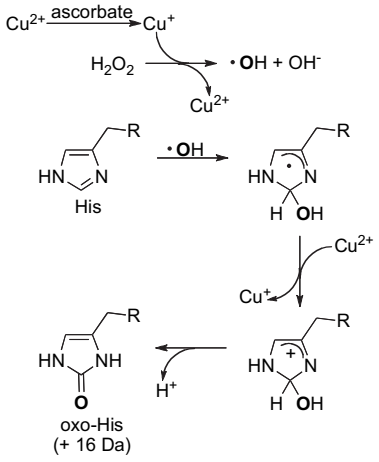
ABSTRACT

In the current work we present our preliminary evaluation of a new protein labeling strategy, namely oxidation labeling. We found that a bis(2-picolyl) amine analogue coordinating Cu^+ was able to oxidize histidine to oxo-histedine in a small peptide by generating reactive oxygen species upon exposure to hydrogen peroxide. The bis(2-picolyl) amine unit was then incorporated into the natural product tautomycin via an oxime linker. The compound, which showed good activity toward protein phosphatase 1γ (PP1γ), will be used in oxidation labeling studies with PP1γ.

1. Introduction

Protein labeling, by means of covalently attaching a 'foreign' unit to the protein, has become one of the methods of choice for elucidating the site of interaction between natural products/drugs and proteins.[1–3] X-ray crystallography[4] and NMR analysis[5] are the two other methods making up the remaining techniques for such studies. Depending on the labeling method chosen the technique used for elucidating the labeled amino acid residue varies. One of the most common strategies for such labeling is photoaffinity labeling (PAL) with various photochemically active groups available.[1] The position that has been labeled can then be elucidated by utilizing various protein degradation techniques followed by, for example, fluorescence or MS and MS/MS analysis.

Our group have for some time been involved in studies of metallopeptides and metalloproteins.[6] From our more recent pin–point oxidation studies[7] and others work[8] with superoxide dismutase (SOD), we knew that Cu^+ in combination with H_2O_2 facilitates the formation of reactive oxygen species (ROS) by Fenton-like reactions.[9] The hydroxy radical generated during the reaction between Cu^+ and H_2O_2 facilitates the oxidation of histidine to oxo-histidine via the mechanism outlined in Scheme 1.[8a,10] The generated ROS also has the capability to oxidize a few other amino acid residues, e.g., cysteine, methionine, and tryptophan.[7,9] This protein oxidation strategy has also successfully been used by us[10] and others[11] to study other metalloproteins.



Scheme 1. Mechanism for Cu^+ generated hydroxy radical (ROS) from H_2O_2 and the mechanism for oxidation of histidine to oxo-histidine.[10]

Another long standing research program in this laboratory has been to unravel the binding site of tautomycin (**1**, Scheme 2) with

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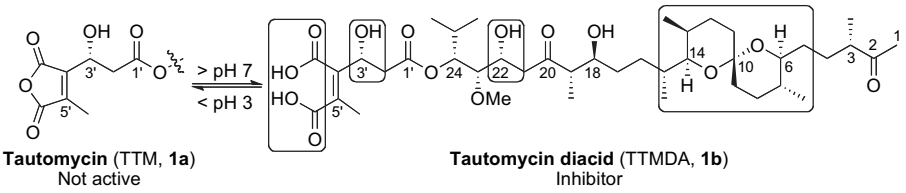
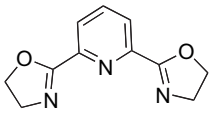


Scheme 2. Structure of tautomycin (TTM, **1a**) and tautomycin diacid (TTMDA, **1b**). TTM exists in a pH dependent equilibrium between the dicarboxylic acid form **1b** (TTMDA) and the anhydride form **1a** where only the diacid form possesses inhibitory activity toward PP1 and PP2A.[18] The black boxes indicate functional groups that are important for biological activity. Position 2 is remote from the essential groups needed for activity and can therefore be modified with moderate loss of activity as previously demonstrated.[12a,12e]

protein phosphatase (PP) 1γ (PP1γ) using various strategies.[12,13] Inspired by our previous work with metalloproteins[7,10] we envisaged that tautomycin diacid (TTMDA, **1b**),[14] a specific inhibitor of PP1[15,16] and PP2A,[16,17] modified with a metal coordinating unit, such as pyridine bis(oxazoline) (Pybox, **2**) or bis(2-picolyl) amine (BPA, **3a**), attached via a linker to position 2 of TTMDA (**1b**), would facilitate transportation of Cu^{+} to the vicinity of the active site. Upon exposure to H_2O_2, Cu^{+} will facilitate the generation of ROS, which can oxidize amino acid residues that are in close proximity to the metal, thus resulting in a mass increase of $16\times n$ Da (n=number of oxidations that has taken place). Since the process regenerates Cu^{+} (Scheme 1) it is possible to oxidize more than one amino acid residue when an excess amount of H_2O_2 is used. Herein, we report on the preliminary evaluation of this labeling strategy, the synthesis of tautomycin-BPA (TTMDA-BPA, **10b**), and TTMDA-BPA's activity toward PP1γ and PP2A.

2. Results and discussion

In order to determine a suitable metal coordination ligand for this work a few preliminary questions needed to be addressed. Two ligands were in mind for this work, namely Pybox[19] and BPA. Firstly we wanted to confirm that these ligands could coordinate copper and that copper simultaneously could promote the formation of ROS, thus resulting in the oxidation of histidine. Secondly, the acid stability of Pybox also needed to be confirmed due to literature reports stating that the oxazoline ring undergoes ring opening under acidic conditions.[20] At the final stage of the coupling reaction with TTM (vide infra) the reaction mixture is stirred at pH 3 for several hours, so acid stability is important. For the first preliminary tests we decided to prepare the simple Pybox **2**, which could be prepared in three steps from pyridine-2,6-dicarboxylic acid using literature procedures,[21] and BPA analogue **3b** prepared in one step from bis(2-picolyl)amine using the method reported by Leigh et al.[22]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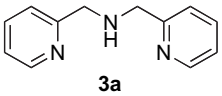
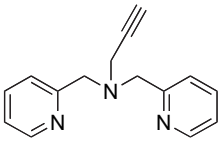
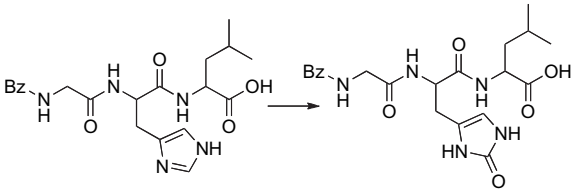

3a


3b

A simple three amino acid residue peptide, viz. Bz-Gly-His-Leu (**4**) (Table 1), containing histidine in the center was looked upon as suitable for testing the desired oxidation reaction. The experiments conducted are summarized in Table 1, and they show that indeed the combination of Pybox **2**, copper plus sodium ascorbate and BPA **3b**, copper plus sodium ascorbate results in oxidation of the histidine amino acid residue, thus generating the oxidized peptide **5** in 9 and 2% yield, respectively (entries 1 and 5). The control experiments show that no oxidation takes place when there is no copper present in the reaction mixture (entries 2, 4, and 6) and, not surprisingly, when only copper and sodium ascorbate is in the reaction mixture we observe some oxidation taking place (entry 3, oxidation yield 3%). A plausible explanation as to why the oxidation proceeds in a higher yield when pybox **2** is used rather than BPA **3b** might be due to pybox having a higher affinity toward Cu^{+}/Cu^{2+} than BPA **3b**. Although the oxidation yields are modest they were still looked upon as suitable for detection by MS analysis, as recently demonstrated by us where we relatively easily could determine the oxidation site when the oxidation yield was 3%.[23] We also expect that the oxidation yields will improve when the real substrate and PP1γ are used due to the affinity of the natural product to the protein.

Table 1
Proof of principal: Oxidation of histidine in Bz-Gly-His-Leu (**4**)

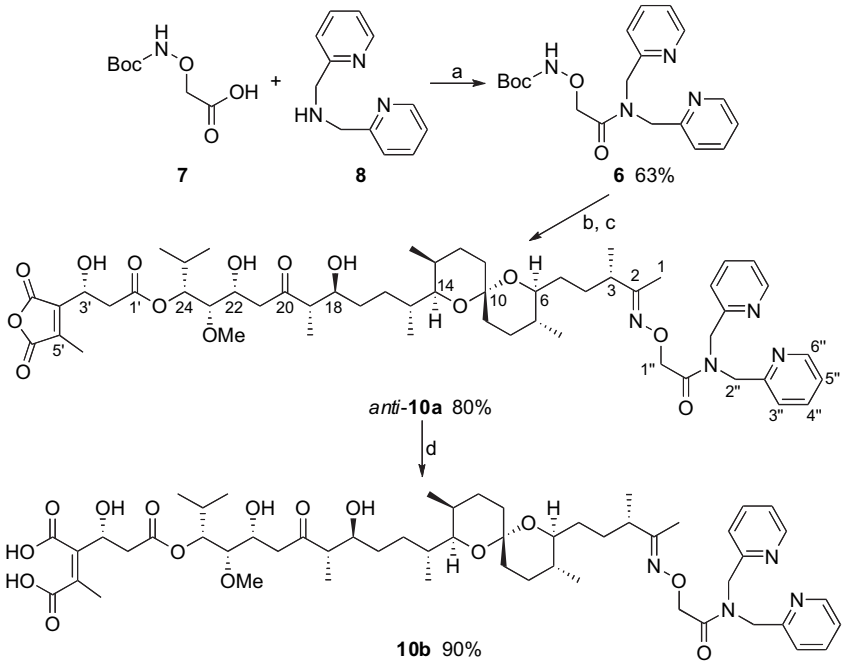

4 *m/z* 430 $(M+H)^{+}$ **5** *m/z* 446 $(M+H)^{+}$

Entry	Ligand	$CuSO_4$	Sodium ascorbate	H_2O_2	Oxidation yield[a] (%)
1	**2**	+	+	+	9
2	**2**	−	−	+	0
3	—	+	+	+	3
4	—	−	−	+	0
5	**3b**	+	+	+	2
6	**3b**	−	−	+	0

[a] Estimated yield based on peak intensity in the respective MS spectra; +=included; −=not included.

In order to determine if copper was coordinated to the ligand during the above oxidation experiments we conducted some UV experiments. In these experiments we used Pybox **2** and BPA analogue **6**. Substrate **6** was chosen since we in the final compound wanted to attach the ligand via a linker to TTM. BPA analogue **6** was therefore prepared as outlined in Scheme 3 by coupling acid **7** with bis(2-picolyl) amine (**8**). Compounds **2** and **6** were then dissolved in water/MeOH/CH_2Cl_2 97:2.25:0.75 and water/MeOH 99:1, respectively, and the amount of copper was gradually increased by 0.1 equiv (see Experimental part for details) at the time and UV spectra were recorded and plotted as shown in Figures 1 and 2. By such means we could confirm that both Pybox **2** and BPA **6** bound copper with a ca. 1:1 ratio as evident from the isosbestic point occurring after ca. 1 equiv of copper was added.

Pybox **2** was then subjected to acid stability testing utilizing the same conditions as used for converting diacid to anhydride, viz. 1 M HCl aq solution/MeCN 1:1 (see Section 4.1.2 for details), which revealed that upon exposure to acidic conditions over a prolong period of time the oxazoline ring underwent ring opening (the



Scheme 3. Synthesis of TTMDA-BPA **10b**. Reagents and conditions: (a) EDC·HCl, CH_2Cl_2/THF (9:1), room temperature, 19 h; (b) TFA, CH_2Cl_2, 0 °C—room temperature, 4.5 h; (c) TTMDA (**1b**),[25] DMA/H_2O (1:1), pH 6, room temperature, 44 h then HCl (1 N aq solution)/CH_3CN (1:1), 0 °C—room temperature, 5 h, then HPLC purification; (d) $NaHCO_3$, CH_3CN, room temperature, 8 h.

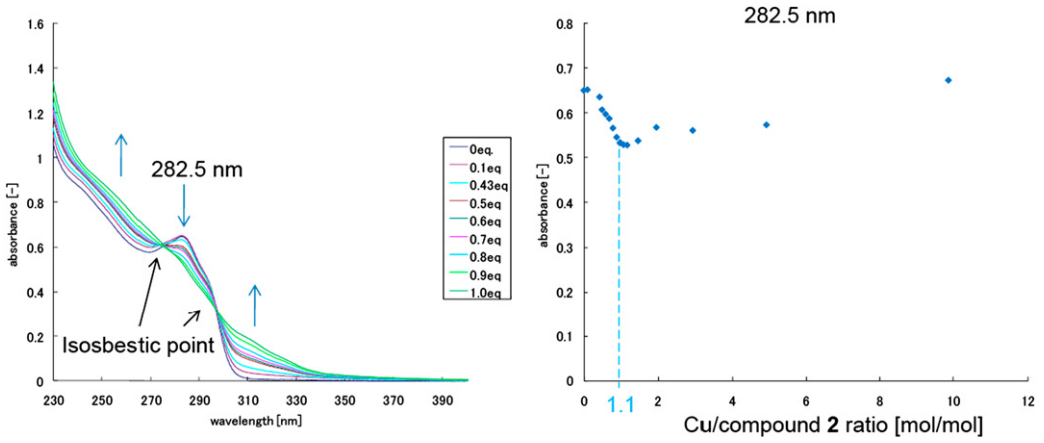


Figure 1. UV spectra of Pybox **2** when copper is gradually added (0–1.0 equiv).

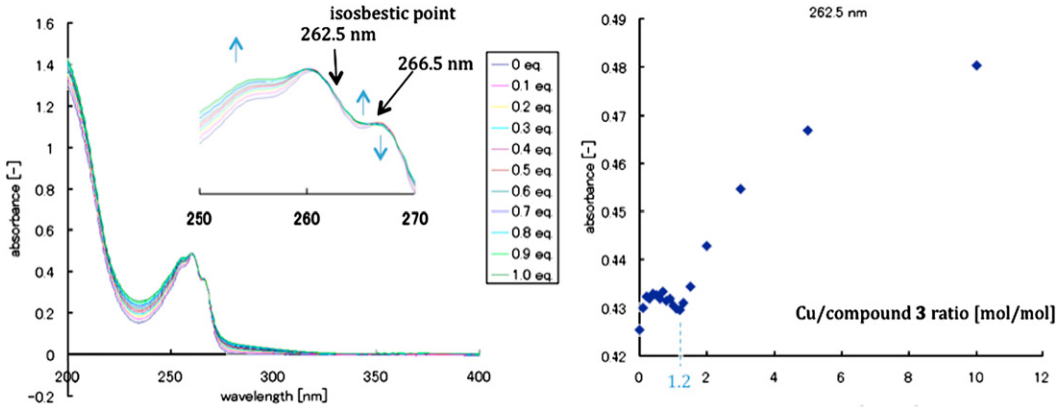
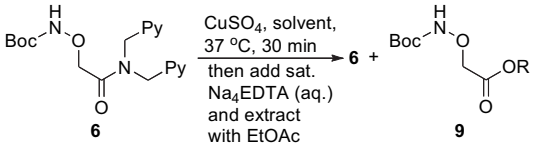


Figure 2. UV spectra of BPA analogue **6** when copper is gradually added (0–1.0 equiv).

yield of the ring opened product was approaching 50% in the course of 5 h). Pybox **2** was therefore found not to be suitable for our purpose due to the fact that the oxazoline ring most likely would undergo ring opening during the acidic work-up in the penultimate reaction (vide infra).

Since Pybox was found to be acid sensitive the ligand of choice seemed to be BPA. However, before embarking on the synthesis of the TTMDA analogue containing BPA one final potential issue needed to be investigated. Literature reports stating that coordination of Cu^{2+}/Cu^{+} to BPA facilitates the slow methanolysis of the amide bond[24] were worrisome, in particular if a similar process takes place in other solvents/solvent systems. The stability of compound **6** was therefore tested in two different solvent systems, namely methanol/water 1:1 and DMSO/water 1:1. These tests showed that BPA analogue **6** undergoes methanolysis in methanol/water in the presence of copper (Table 2, entry 1). However, to our satisfaction under conditions resembling the conditions we would use during our oxidation experiments, viz. DMSO/buffer, no hydrolysis was detected as evident from a 97% recovery of compound **6** (entry 2). Thus BPA attached via an amide linker seemed to be a good option for our further work.

Table 2
Stability testing of compound **6**

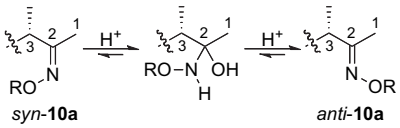


Entry	Solvent	Recovery of compound **6**	Ester or carboxylic acid **9**
1	$MeOH/H_2O$	34%	55% (R=Me)
2	$DMSO/H_2O$	97%	—

Py=pyridine.

With confidents that the chosen ligand would be stable under our reaction conditions and during our oxidation experiments we pursued with the synthesis of TTMDA-BPA **10b**. The Boc protection group within compound **6** was first removed upon treatment with TFA in dichloromethane and the crude product was used directly in the coupling with TTMDA (**1b**).[25] The coupling was conducted by using our standard reaction conditions,[12a,12d] which implemented stirring TTMDA (**1b**) with the primary amine at pH 6 in a solvent mixture of DMA/water 1:1 followed by addition to acid after 44 h (Scheme 3), thus generating the corresponding anhydride (**10a**).

Interestingly, compound **10a** was initially separated by HPLC in what is thought to be a *syn*/*anti* ratio of ca. 1:2 based on previous experience.[12a,12d] However, upon concentration of the fractions containing *syn*-**10a** the compound was fully converted to *anti*-**10a** most likely due to its readily isomerizable nature in acidic medium as outlined in Scheme 4 (the mobile phase used during HPLC purification contained 0.1% TFA, see experimental part for details). Thus giving compound *anti*-**10a** as pure *anti* in a combined yield of 80%. Substrate *anti*-**10a** was then converted



Scheme 4. Mechanism for the conversion of *syn*-**10a** to *anti*-**10a**.[26]

to the active form, namely inhibitor **10b**, using our previously reported method.[12a,12e]

The inhibitory activity of compound **10b** against phosphatase activities was then tested using the *p*-nitrophenyl phosphate method[27] and it was found to have a K_i of 5.4±0.1 nM toward PP1γ (Fig. 3). The K_i for compound **10b** compares favorable with the activity of the natural product **1b**, which was found to have a K_i of 1.4±0.1 nM in the same test round. Inhibitor **10b** also retains its selectivity for PP1γ over PP2A with a K_i of 474±5 nM when tested against PP2A [TTMDA (**1b**) was in the same test round found to have a K_i of 75.8±0.2 nM against PP2A] (see Fig. 4). With the active inhibitor **10b** in hand work is now focusing on conducing oxidation labeling of PP1γ. Results from these studies will be reported in due course.

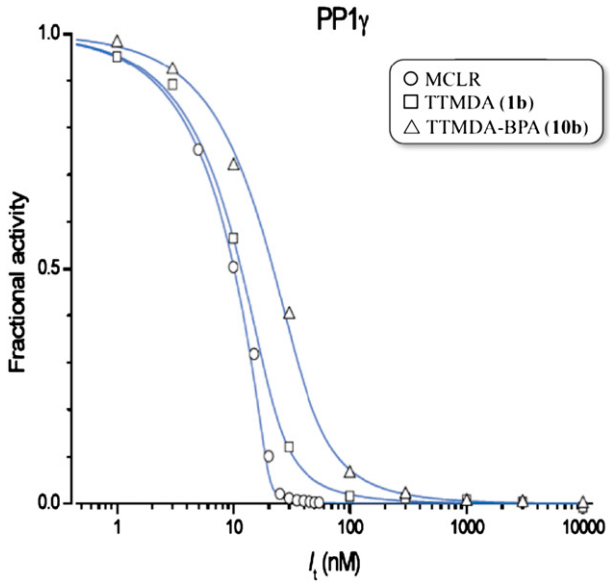


Figure 3. Inhibition curve for PP1γ. Microcystin-LR (MCLR) is used as internal standard in these tests.

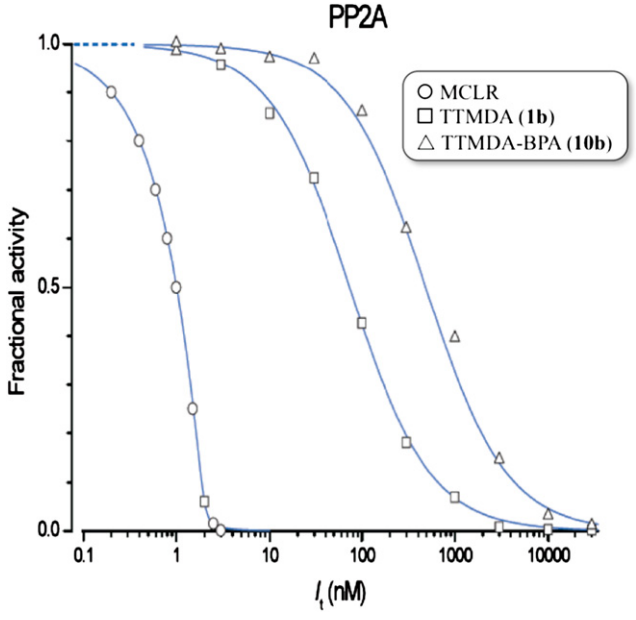


Figure 4. Inhibition curve for PP2A. Microcystin-LR (MCLR) is used as internal standard in these tests.

3. Conclusion

The synthesis of an active inhibitor, viz. compound **10b**, of PP1γ containing a copper coordinating ligand is reported. Preliminary results using a model compound showed that the ligand is capable of coordinating copper and that copper simultaneously can generate ROS capable of oxidizing histidine to oxy-histidine in a model peptide. Although the oxidation yield for our model system is modest we anticipate that the oxidation yields will improve once TTMDA-BPA (**10b**) and PP1γ is used in the experiments due to the affinity of the substrate to the protein.

4. Experimental

4.1. General experimental

Melting points were measured on a Yanaco MP-S3 melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a JASCO FT/IR-6100 spectrophotometer and are reported in wave number (cm^{-1}). Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on either a Bruker ARX-400 operating at 400 MHz for proton and 101 MHz for carbon, a JEOL JNM-A600 operating at 600 MHz for proton, or a Bruker AMX2600 operating at 151 MHz for carbon. Chemical shifts were recorded as δ values in parts per million (ppm) using tetramethylsilan (δ=0.00 ppm) and residual chloroform (δ=77.0 ppm) as internal standard for proton and carbon NMR, respectively. Low- and high-resolution mass spectra, EI and FAB, were recorded on a JEOL JMN-700. ESI mass spectra were recorded on a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a *Z*-spray type ESI source. Optical rotations were measured with a Jasco P-1010-GT polarimeter at the sodium-D line (589 nm) and the concentrations (*c*) (g/100 mL) indicated using spectroscopic grade $CHCl_3$. Elemental analyses were performed by Mr. S. Kitamura in the Analytical Laboratory at Bioagricultural Sciences, Nagoya University. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel coated glass plates $60F_{254}$ using UV light visualizing agent and 12molybdo(VI)phosphoric acid *n*-hydrate, *p*-anisaldehyde or basic $KMnO_4$ solution followed by heating as developing agents. Silica gel 60 (particle size 0.063–0.2 mm ASTM) was used for flash chromatography. Tautomycin (1) was purified according to our previously reported method.[28] The progress of the coupling reaction between tautomycin and the ligand with linker were monitored by HPLC using a Develosil C30-UG-5 column (4.6×250 mm i.d.), CH_3CN/H_2O 4:1 with a flow rate of 1.0 mL/min., detected at 254 nm.

*4.1.1. N-Boc-aminoxy bis(2-picolyl)amide (**6**).* EDC·HCl (259.4 mg, 1.35 mmol) was added to a stirred solution of *N*-*tert*-butoxycarbonyl-aminooxy acetic acid **7**[12a] (154.4 mg, 0.807 mmol) and bis(2-picolyl)amine (**8**) (0.160 mL, 0.89 mmol) in CH_2Cl_2/THF=9:1 (20 mL) at room temperature. The resulting reaction mixture was then stirred at room temperature for 19 h before being diluted with CH_2Cl_2 (20 mL), washed with water (1×15 mL) and brine (1×15 mL) before being dried over Na_2SO_4. Filtration and concentration under reduced pressure gave a dark yellow oil, that was subjected to flash chromatography (CH_2Cl_2/MeOH/Et_3N=95:4.9:0.1). Concentration of the relevant fractions (R_f 0.3 in CH_2Cl_2/MeOH 95:5) gave compound **6** (188.5 mg, 63%) as a yellow oil and as a ca. 1:1 mixture of rotamers as judged by ^{13}C NMR analysis. IR ν_{max} 2973, 2932, 1737, 1658, 1591, 1471, 1433, 1366, 1255, 1168, 1114, 1020, 800, 764 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.47 (9H, s, H-Boc), 4.70 (4H, d, *J*=6.0 Hz, H-2″), 4.79 (2H, s, H-1″), 7.22–7.15 (2H, m, H-3″, H-4″), 7.29 (1H, d, *J*=8.0 Hz, H-3″), 7.62 (1H, dt, *J*=2.0 and 8.0 Hz, H-4″), 7.65 (1H, dt, *J*=2.0 and 8.0 Hz, H-4″), 8.50 (1H, dd, *J*=0.8 and 5.0 Hz, H-6″), 8.55 (1H, dd, *J*=0.8 and 5.0 Hz, H-6″), 9.34 (1H, s, NH); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 28.1, 50.6, 51.8, 73.9, 81.2, 122.0, 122.2, 122.5, 122.7, 136.6, 136.8, 148.9, 149.7, 155.5, 156.1, 156.4, 169.4; MS (FAB+) *m*/*z* 373 ($[M+H]^+$, 69%), 347 (24), 273 (40), 165 (42); HRMS (FAB+) Found: $[M+H]^+$, *m*/*z* 373.1866. $C_{19}H_{25}N_4O_4^+$ requires $[M+H]^+$, *m*/*z* 373.1876; Anal. Found: C 61.02, H 6.44, N 14.84%. $C_{19}H_{24}N_4O_4$ requires: C 61.28, H 6.50, N 15.04%.

*4.1.2. TTM-BPA (**anti-10a**).* Trifluoroacetic acid (1.0 mL) was added dropwise over a 10 min. period to a stirred solution of compound **6** (20.9 mg, 0.0561 mmol) in CH_2Cl_2 (1.0 mL) maintained at 0 °C. The resulting reaction mixture was then stirred at 0 °C for 4.5 h before being concentrated under reduced pressure. In order to remove trace amount of TFA the residue was dissolved in water (5.0 mL) and concentrated in vacuo thus providing the corresponding primary amine (15.2 mg) as a light-yellow oil. The crude product was used directly in the coupling reaction with tautomycin diacid **1b** without further purification. ^1H NMR ($CDCl_3$ with a few drops of CD_3OD, 400 MHz) δ 4.70 (2H, s, H-1″), 4.85 (4H, s, H-2″), 7.29 (1H, t, *J*=6.0 Hz, H-5″), 7.33 (1H, d, *J*=8.0 Hz, H-3″), 7.60 (1H, t, *J*=5.6 Hz, H-5″), 7.78–7.71 (2H, m, H-3″, H-4″), 8.13 (1H, t, *J*=7.4 Hz, H-4″), 8.47 (1H, d, *J*=4.4 Hz, H-6″), 8.62 (1H, d, *J*=3.6 Hz, H-6″), the signal due to NH_2 could not be discerned; ^{13}C NMR ($CDCl_3$ with a few drops of CD_3OD, 101 MHz) δ 51.6, 71.0, 123.1, 123.9, 124.8, 125.7, 139.2, 142.9, 143.9, 148.1, 153.1, 153.5, 170.2; MS (FAB+) *m*/*z* 273 ($[M+H]^+$, 69%), 240 (15), 200 (28); HRMS (FAB+) Found: $[M+H]^+$, *m*/*z* 273.1370. $C_{14}H_{17}N_4O_2^+$ requires $[M+H]^+$, *m*/*z* 273.1352.

A solution of the amine in DMA/H_2O (2.0 mL of a 1:1 solution), which carefully had been adjusted to pH 6 with NaOH solution (0.1 M aq solution), was added to a stirred solution of tautomycin diacid **1b**[25] (24.0 mg, 0.0313 mmol) in DMA/H_2O (2.8 mL of a 1:1 solution) pH 6 at room temperature. The resulting reaction mixture was stirred at room temperature for 44 h before being poured into an ice cooled solution of HCl (1 M aq solution)/MeCN 1:1 (2.0 mL) and stirred vigorously for 10 min. The ice-bath was then removed and stirring was continued at room temperature for 4.5 h. The resulting reaction mixture was extracted with $CHCl_3$ (3×15 mL) and the combined organic fractions were concentrated under reduced pressure. The crude light-yellow oil was subjected to HPLC purification [Develosil C30-UG-5 (250×10 mm i.d.), MeCN:H_2O 4:1 containing 0.1% TFA, 254 nm] thus affording three fraction, A–C.

Concentration of fraction A and B (t_R 4.3 min and t_R 5.6 min, respectively) gave rise to exactly the same ^1H NMR,^{13}C NMR, MS, and IR spectra as well as the same optical rotation (see results and discussion part for details), thus clearly showing that they are the same compound. The combined fractions afforded [25.6 mg, 80% (89% at 90% conversion)] of the desired compound TTM-BPA (***anti*-10a**) as a clear oil and as a ca. 1:1 mixture of rotamers about the terminal bis(2-picolyl)amide unit. $[\alpha]_D^{27}$ +2.7 (*c* 0.066, $CHCl_3$); IR ν_{max} 2932, 2875, 1766, 1682, 1460, 1435, 1374, 1256, 1200, 1134, 1098, 986, 915 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 0.80 (3H, d, *J*=6.6 Hz, 7-CH_3), 0.87 (3H, d, *J*=6.6 Hz, 13-CH_3), 0.98–0.93 (9H, m, 15-CH_3, 25-CH_3, 26-CH_3), 1.01 (3H, d, *J*=6.6 Hz, 3-CH_3), 1.05 (3H, d, *J*=7.2 Hz, 19-CH_3), 1.62 (3H, s, H-1), 1.68–1.26 (17H, m, H-4, H-5, H-7, H-8, H-9, H-11, H-12a, H-15, H-16, H-17), 1.81 (1H, br s, H-13), 2.00–1.94 (1H, m, H-12b), 2.09 (1H, app. sextet, *J*=6.6 Hz, H-25), 2.21 (3H, s, 5′-CH_3), 2.34 (1H, m, H-3), 2.62 (1H, dd, *J*=2.7 and 17.1 Hz, H-21a), 2.66 (1H, partly obscured quintet, *J*=7.2 Hz, H-19), 2.78 (1H, dd, *J*=9.6 and 16.2 Hz, H-2′a), 2.86 (1H, dd, *J*=3.3 and 16.2 Hz, H-2′b), 2.96 (1H, dd, *J*=8.7 and 17.1 Hz, H-21b), 3.15 (1H, app. t, *J*=9.3 Hz, H-6), 3.27–3.24 (2H, m, H-14, H-23), 3.44 (3H, s, 23-OCH_3), 3.73 (1H, app. br t, *J*=7.2 Hz, H-18), 4.32 (1H, br d, *J*=6.0 Hz, H-22), 4.80 (2H, s, H-1″), 5.08–5.00 (3H, m, H-24, H-2″), 5.17 (3H, br s, H-3′, H-2″), 7.62 (1H, app. br s, H-5″), 7.65 (1H, d, *J*=7.4 Hz, H-3″), 7.74 (1H, app. br s, H-5″), 7.91 (1H, d, *J*=7.4 Hz, H-3″), 8.14 (1H, t, *J*=7.4 Hz, H-4″), 8.27 (1H, t, *J*=7.4 Hz, H-4″), 8.72 (2H, br d, 10.2 Hz, H-6″) (signals for the three OH protons could not be discerned); ^{13}C NMR ($CDCl_3$,

150 MHz) δ 9.9, 10.9, 11.0, 13.5, 16.7, 17.2, 17.5, 17.9, 19.5, 26.7, 27.1, 27.6, 28.1, 28.6, 29.6, 30.1, 30.5, 31.1, 34.7, 34.9, 36.0, 39.1, 40.6, 45.3, 48.4, 50.7, 52.4, 59.3, 63.5, 66.4, 71.9, 73.8, 74.0, 74.8, 76.5, 80.9, 95.7, 123.9, 124.8, 125.2, 126.4, 142.1, 142.4, 142.9, 144.7, 145.5, 153.2, 153.7, 164.6, 164.8, 165.7, 169.7, 171.7, 214.9 (one carbon obscured or overlapping); MS (ESI$^+$) 1022 ([M+H]$^+$, 100%), 1004 (5), 622 (20), 604 (27); HRMS (ESI$^+$) Found: [M+H]$^+$, m/z 1021.5747. $C_{55}H_{81}N_4O_{14}^+$ requires m/z 1021.5744.

Concentration of fraction C (t_R 16.6 min) gave (2.5 mg, 10% recovery) of TTM (**1a**), which was identical, in all respects, with an authentic sample.

*4.1.3. Conversion of TTM-BPA (**anti-10a**) to TTMDA-BPA (**10b**).* A solution of $NaHCO_3$ (30.2 μL of a aq solution, 20 mg/mL) was added to a stirred solution of TTM-BPA (***anti*-11**) (3.3 mg, 3.23 mmol) in MeCN/H_2O 4:1 (1.0 mL) at room temperature. The resulting reaction mixture was stirred for 7 h before being neutralized with HCl (0.1 N aq solution) and purified by HPLC [Develosil ODS-UG-5 column (250×4.6 mm i.d.), MeCN/H_2O 4:1, 0.5 mL/min, 254 nm]. Concentration of the relevant fraction (t_R 4.33 min) in vacuo gave TTMDA-BPA (**10b**) (3.15 mg, 90%) as a clear oil. MS (ESI$^+$): m/z 1039 ([M+H]$^+$, 100%), 1021 (26), 530 (18).

*4.1.4. General procedure for peptide oxidation using Pybox **2** and BPA **3b**.* To a solution of Pybox **2** or BPA **3b** in CH_2Cl_2 (150 μL, 21.2 mM) was added sodium ascorbate in water (76 μL, 42.2 mM, 1 equiv), $CuSO_4\times5H_2O$ in water (73 μL, 43.3 mM, 1 equiv) and MeOH (400 μL) and the resulting solution was incubated at 37 °C for 30 min. The peptide dissolved in MeOH (150 μL, 21.9 mM, 1 equiv) was then added and the resulting solution was incubated at 37 °C for 30 min before H_2O_2 (150 μL of a 1% solution, 15 equiv) was added and the solution incubated at 37 °C for 30 min. The resulting reaction mixture was then concentrated under reduced pressure and redissolved in MeOH followed by filtration prior to MS analysis.

*4.1.5. Procedure for determination of isosbestic point for Pybox **2**.* To a solution of Pybox **2** (0.1 mM solution in water/MeOH/CH_2Cl_2 97:2.25:0.75) was added 0.1 equiv of $CuSO_4\times5H_2O$ (10 mM solution in water) followed by recording a UV spectrum. The procedure was repeated until 10 equiv of $CuSO_4\times5H_2O$ (10 mM solution in water) was added.

*4.1.6. Procedure for determination of isosbestic point for BPA **6**.* To a solution BPA **6** (0.1 mM in water/MeOH 99:1) was added 0.1 equiv of $CuSO_4\times5H_2O$ (50 mM solution in water) followed by recording a UV spectrum. The procedure was repeated until 10 equiv of $CuSO_4\times5H_2O$ (50 mM solution in water) was added.

*4.1.7. Stability check of BPA (**6**) in MeOH/H_2O.* To a mixture of compound **6** (8.3 mg, 22.2 μmol) in MeOH (1.0 mL) and H_2O (0.5 mL), was added $CuSO_4$ (0.5 mL of a 44.5 mM solution, 1 equiv) and the resulting solution was incubated at 37 °C for 30 min. Na_4EDTA solution (10 mL of a satd aq solution) was then added and the mixture was extracted with EtOAc (3×10 mL). The combined organic fractions were washed with Na_4EDTA solution (2×10 mL of a satd aq solution), $NaHCO_3$ solution (1×10 mL of a satd aq solution), NH_4Cl solution (1×10 mL of a satd aq solution) and brine (1×10 mL) before being dried over Na_2SO_4. Filtration and concentration under reduced pressure afforded a white solid (5.3 mg), which was a 1:1.6 mixture of starting material **6** and methyl ester **9** (R=Me) as determined by ^{1}H NMR analysis. The amount of recovered starting material was 2.8 mg (34%) based on calculation.

*4.1.8. Stability check of BPA (**6**) in DMSO/H_2O.* To a mixture of compound **6** (7.3 mg, 19.6 μmol) in DMSO (1.0 mL) and H_2O (0.5 mL), was added $CuSO_4$ (0.5 mL of a 39.1 mM solution) and the resulting solution was incubated at 37 °C for 30 min. Na_4EDTA solution (10 mL of a satd aq solution) was then added and the mixture was extracted with EtOAc (3×10 mL). The combined organic fractions were washed with Na_4EDTA solution (2×10 mL of a satd aq solution), $NaHCO_3$ solution (1×10 mL of a satd aq solution), NH_4Cl solution (1×10 mL of a satd aq solution) and brine (1×10 mL) before being dried over Na_2SO_4. Filtration and concentration under reduced pressure afforded a white solid (7.5 mg), which was a 1:0.27 mixture of starting material **6** and DMSO as determined by ^{1}H NMR analysis. The amount of recovered starting material was 7.1 mg (97%) based on calculation.

4.1.9. Assay of protein phosphatase activity. The protein phosphatase activities were measured by the spectrophotometric method using *p*-nitrophenyl phosphate (the final concentration in the reaction mixture was 5 mM), as described previously.[27]

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Supplementary data

Copies of ^{1}H and ^{13}C NMR spectra are included as Supplementary datas. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.12.036.

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