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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

Magne O. Sydnes [a], Atsushi Miyazaki [a], Minoru Isobe [b,*], Hiroshi Ohinata [c], Motoi Miyazu [c], Akira Takai [c]

[a] Laboratory of Organic Chemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan
[b] Institute for Advanced Research, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan
[c] Asahikawa Medical College, Midorigaoka Higashi 2-1-1-1, Asahikawa 078-8510, Japan

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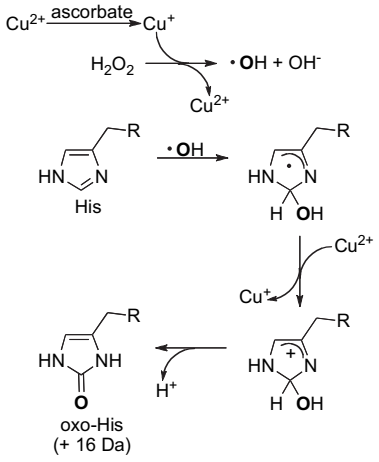
ABSTRACT

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

* Corresponding author at present address: Chemistry Department, National Tsing Hua University, 101, Sec.2, Kuang-Fu Road, Hsinchu, Taiwan 30013. Tel.: +81 52 789 4109; fax: +81 52 789 4111.
E-mail address: minoru@mx.nthu.edu.tw (M. Isobe).

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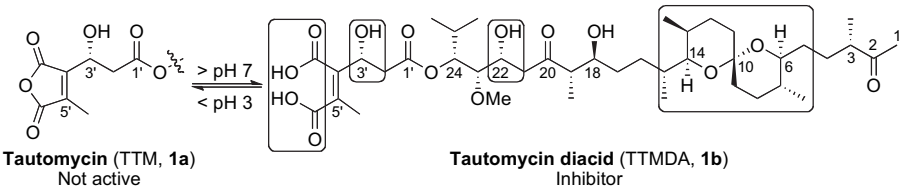
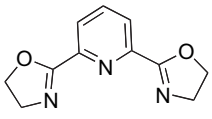


Scheme 2. Structure of tautomycin (TTM, **1a**) and tautomycin diacid (TTMDA, **1b**). TTM exists in a pH dependent equilibrium between the dicarboxylic acid form **1b** (TTMDA) and the anhydride form **1a** where only the diacid form possesses inhibitory activity toward PP1 and PP2A.[18] The black boxes 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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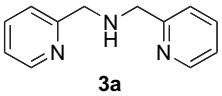
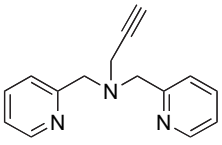
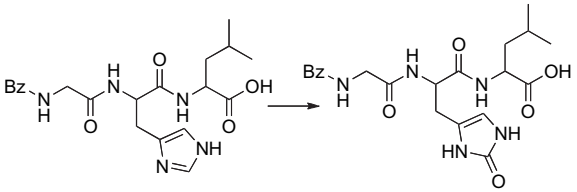

3a


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