Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 5, pp. 705-710, 1994

0960-894X/94 $6.00+0.00

0960-894X(94)E0033-B

ON THE MECHANISM OF ENZYMATIC INACTIVATION OF BLEOMYCIN. THE β-AMINOALANINAMIDE MOIETY AS AN ENZYME-DEPENDENT MOLECULAR SWITCH

Toru Sugiyama,† Masaji Ohno,†† and Masakatsu Shibasaki
Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Masami Otsuka* and Yukio Sugiura
Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

Susumu Kobayashi
Sagami Chemical Research Center, Nishi-ohnuma, Sagamihara-shi, Kanagawa 229, Japan

Kenji Maeda
Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Abstract. Structure-function study on the β-aminoalaninamide moiety of bleomycin models revealed that the axial amino group is essential for efficient oxygen activation. Treatment of a model PYML-6 with mouse liver extract resulted in the hydrolysis of the carbamoyl group of the axial ligand moiety to give an inactive product, demonstrating the β-aminoalaninamide moiety to be an enzyme-dependent switch of activity.

It has been well documented that antitumor antibiotic bleomycin (BLM) forms iron-complex by the β-aminoalaninamide-pyrimidine-β-hydroxyhistidine region to activate molecular oxygen (Figure 1A).[1,2] The antitumor activity of BLM has been believed to be due to the DNA cleavage with the oxygen radical species generated by BLM-Fe(II)-O_2 complex. It has been reported that BLM is inactivated by an enzyme BLM hydrolase which hydrolyzes the carbamoyl group of the β-aminoalaninamide moiety.[3] The resulting deamido-BLM shows markedly lowered dioxygen activation because axial coordination site of the iron complex of deamido-BLM is presumably occupied by the carboxylate under physiological conditions (Figure 1B).[4] The enzymatic inactivation is particularly significant in relation to the tissue specificity of the drug based on the varied abundance of BLM hydrolase in each tissue. However, further detailed investigation has been hampered by the limitation in the availability of deamido-BLM. The objective of this study is to examine and confirm the above mechanism of enzymatic inactivation of BLM by a synthetic model approach.

In our continuing synthetic effort toward man-designed BLMs, we have prepared several models including PYML-6 (Figure 2), which exhibited metal-binding and dioxygen-activating properties well in accordance with those of BLM.[2e,5-8] Herein, aiming at the elucidation of the mechanism of the enzymatic inactivation of BLM, we report a new entry, deamido-PYML-6, a model corresponding to deamido-BLM, possessing amino and carboxyl groups in the apical side chain.

---

† Present address: Department of Chemistry, College of Arts and Sciences, The University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan.

†† Present address: Eisai Co., Ltd., Research and Development, 1-3, Tokodai 5-chome, Tsukuba-shi, Ibaraki 300-26, Japan.

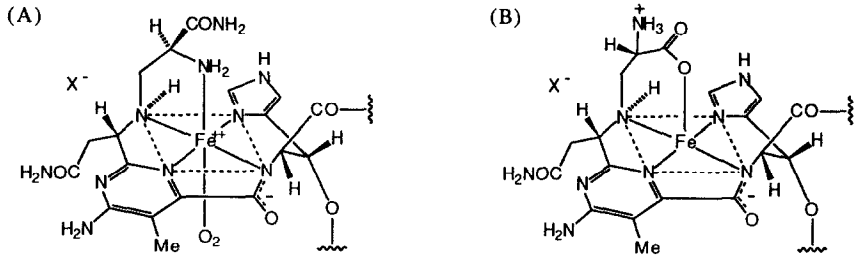


Figure 1. Proposed structure for the iron complexes of bleomycin (A) and deamido-bleomycin (B).[4]

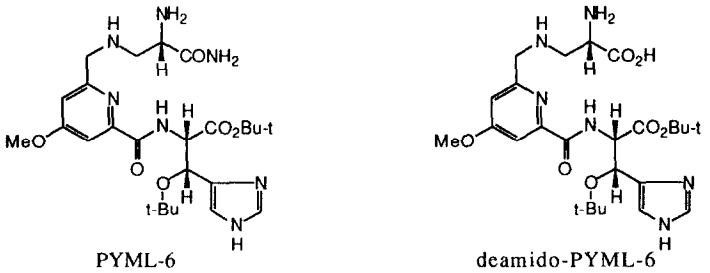
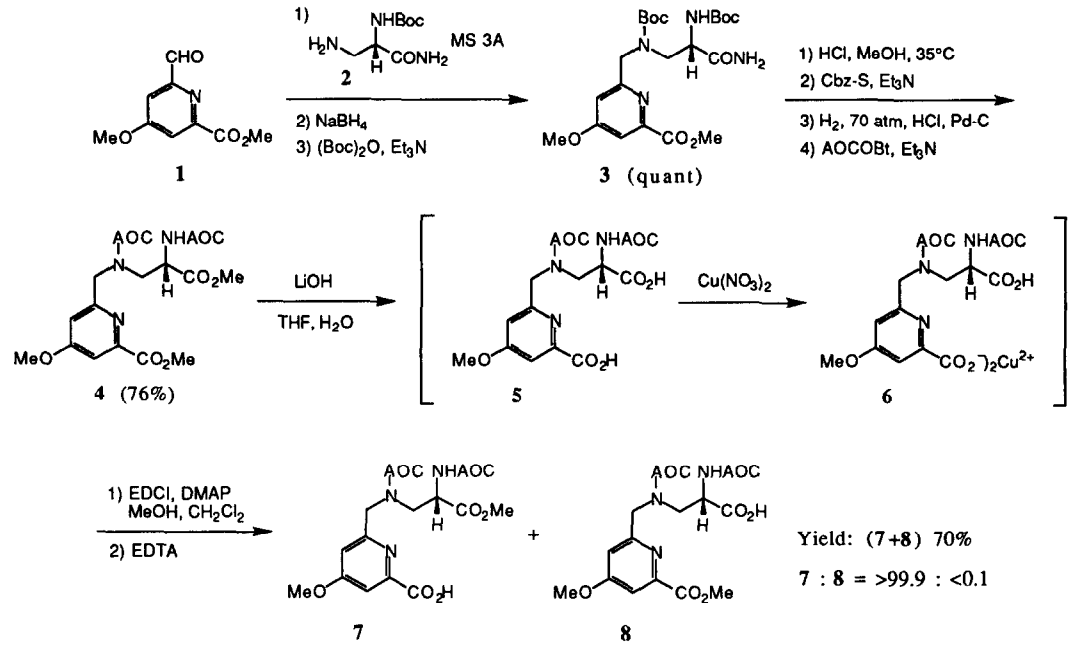


Figure 2. Synthetic models of the metal-binding site of bleomycin.

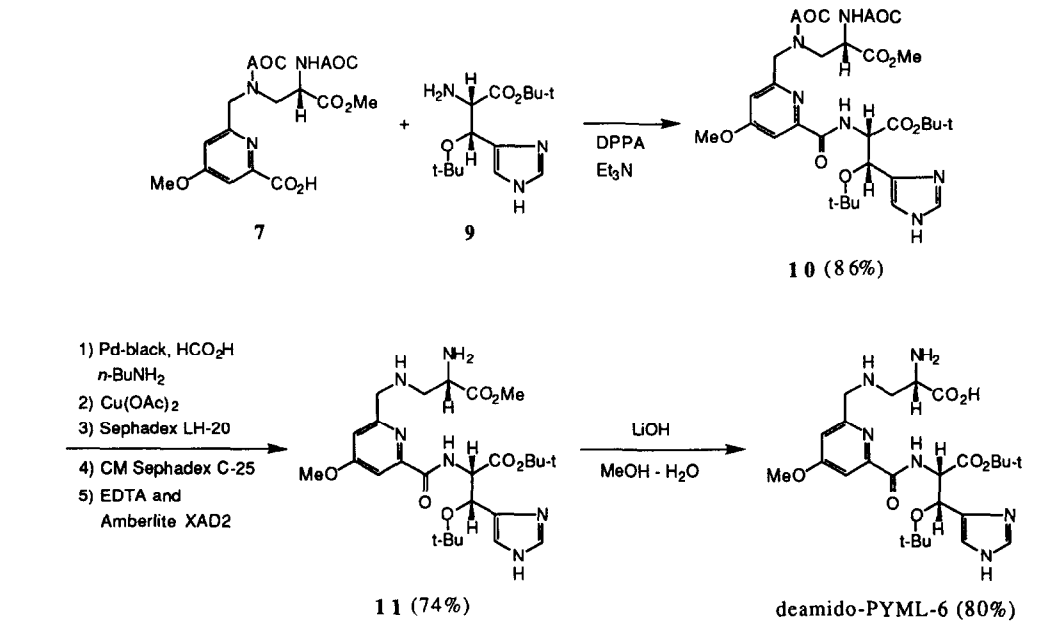
First we attempted a synthesis of deamido-PYML-6 by the same strategy as that for PYML-6. However, severe difficulty was encountered mainly due to the inherent instability of β-aminoalanine methyl ester. Therefore we examined the use of more stable β-aminoalaninamide and developed an alternate synthetic route by taking advantage of the unique metal-chelating characteristics of the molecule as shown in Schemes 1 and 2. Thus, aldehyde **1**[8] was treated with amine **2**[9] in the presence of molecular sieves 3A to give Schiff base, which was then reduced with sodium borohydride. The resulting secondary amine was treated with $(Boc)_2O$,[10] affording bis(Boc) derivative **3** quantitatively. The carboxamide **3** was smoothly transformed into the corresponding methyl ester under mild condition (hydrogen chloride - methanol, 35°C) concomitant with the removal of the Boc group. The resulting diamine was isolated in a good purity by converting into the corresponding bis(Z) derivative and subsequently transformed into bis(AOC) derivative **4**[11] by hydrogenation followed by treatment with AOCOBt.[12] It was gratifying that differentiation of the two ester groups of **4** was successfully accomplished as follows. Both the esters of **4** were quantitatively hydrolyzed with lithium hydroxide to give diacid **5**. To a solution of **5** was added equimolar $Cu(NO_3)_2$ to afford copper complex **6** in which the carboxyl group on the pyridine ring appeared to be coordinated to the cupric ion. Treatment of this complex with methanol and EDCI[13] afforded the desired monoester **7** exclusively in 70% yield from the ester **4**. When this reaction was carried out without copper, both of the regioisomeric monoester **7** and **8** were produced in a ratio 6:1 and the separation of the two was difficult.

Monoester **7** thus obtained was further transformed into deamido-PYML-6 (Scheme 2). Coupling of the acid **7** and the amine **9** was effected by DPPA[14] to afford peptide **10** in 86% yield. The final deprotection of the AOC group was not straightforward. Treatment of bis(AOC) derivative **10** with palladium black, formic acid,

Scheme 1



Scheme 2



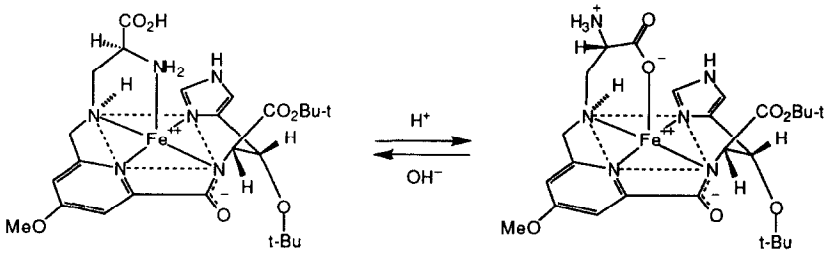
and *n*-butylamine generated free diamine which immediately formed a ninhydrin-negative product, probably *N*-formyl derivative, under the reaction condition. This problematic formylation was found to be suppressed by the copper chelate formation. Thus, the copper-chelated ester was purified by Sephadex LH-20 followed by CM-Sephadex C-25 and the cupric ion was removed with EDTA. The resulting copper-free material was purified by Amberlite XAD-2 to give methyl ester **11** in 74% yield. Deamido-PYML-6[15] was obtained in 80% yield by alkaline hydrolysis.

Table 1 shows the ESR parameters for the Fe(III) complexes of BLM, deamido-BLM, and deamido-PYML-6. BLM showed a transient and a stable Fe(III) complex species in the course of oxygen activation.[4] At pH 6.2, deamido-BLM-Fe(III) complex exhibited a signal assigned to high-spin ferric ion and carboxy-coordinated structure (Figure 1B) has been proposed.[4] On the other hand, at pH 9.6, deamido-BLM-Fe(III) showed a signal characteristic of rhombic low-spin Fe(III) close to the stable BLM-Fe(III) complex.[4] Interestingly, deamido-PYML-6 successfully reproduced the pH-dependent duality in the iron complex formation of deamido-BLM. Whereas the catalytically inactive high-spin species (g = 4.4) was observed at pH 7.4, a stable low-spin species of deamido-PYML-6 was detected (g_1 = 2.360, g_2 = 2.183, g_3 = 1.920) at pH 9.8.

Table 1. ESR parameters for Fe(III) complexes of bleomycin, deamido-bleomycin, and deamido-PYML-6.

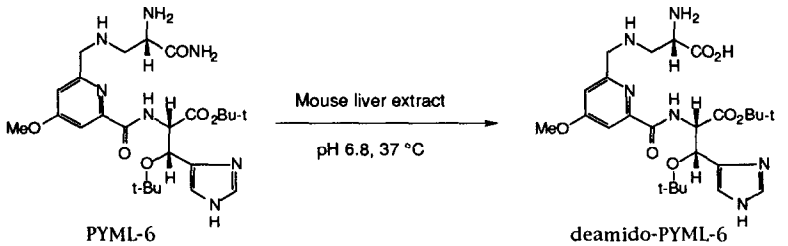
	Transient Fe(III) complex			Stable Fe(III) complex		
Ligand	g_1	g_2	g_3	g_1	g_2	g_3
BLM[4]	2.254	2.171	1.937	2.431	2.185	1.893
deamido-BLM (pH 6.2)[4]			g = 4.4			
deamido-BLM (pH 9.6)[4]		_		2.472	2.181	1.874
deamido-PYML-6 (pH 7.4)			g = 4.4			
deamido-PYML-6 (pH 9.8)		_		2.360	2.183	1.920

Scheme 3. A possible explanation for the pH-dependence in complex formation based on equilibrium between hypothetical amino- and carboxy-complexes.



In the ESR spin trapping experiments, pH-dependence in oxygen activation was again observed for deamido-PYML-6. Whereas the efficiency of oxygen activation of deamido-PYML-6 was 38% of that of BLM at neutral pH region, the oxygen-activating efficiency of deamido-PYML-6 increased up to 90% of that of BLM at alkaline pH. Among the donor atoms contained in deamido-PYML-6, the primary amino and the carboxyl groups seem to be most susceptible to the change of pH of the solution. The pH-dependent duality of the oxygen activation can be explained by assuming the equilibrium between hypothetical amino- and carboxy-complexes (Scheme 3). Thus, low oxygen activation of deamido-PYML-6 at neutral pH is presumably due to the high population of inert carboxylate-coordinated species. Enhanced oxygen activation at alkaline pH is probably the result of the predominance of the amino-coordination.

Next, we examined enzymatic hydrolysis of our synthetic model PYML-6. PYML-6 was incubated with mouse liver extract (SPF mouse, BALB/C, female) at 37°C for 2 hours. HPLC analysis of the reaction mixture revealed exclusive formation of a substance which was identical to the synthetic specimen of deamido-PYML-6.[16] This unambiguously showed that the carbamoyl group of the β-aminoalaninamide moiety was hydrolyzed with the mouse liver extract. As this hydrolysis of PYML-6 with the liver extract was found to be inhibited by leupeptin, a specific inhibitor of BLM hydrolase,[17] there is no doubt that the hydrolysis was indeed caused by BLM hydrolase present in the mouse liver extract. The same liver extract was shown to hydrolyze BLM to afford a single product. Dose dependence was observed in both cases (Figure 3). Thus, we found PYML-6, devoid of the acetamide group contained in the pyrimidine side chain of BLM,[18] to be a good substrate of BLM hydrolase.



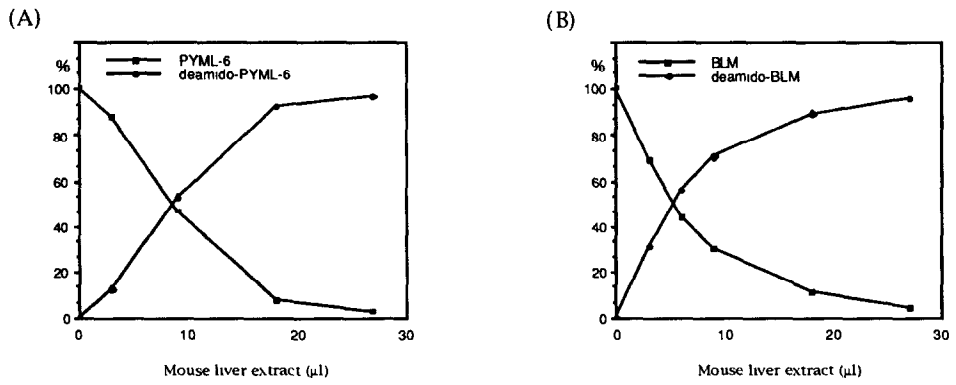


Figure 3. Dose dependent hydrolysis of PYML-6 (A) and bleomycin (B) with mouse liver extract.

We demonstrated that deamido-PYML-6 is a model relevant to deamido-BLM and obtained a supporting evidence for the molecular mechanism of inactivation of BLM, i. e., the β-aminoalaninamide moiety of BLM is hydrolyzed by BLM hydrolase and the resulting carboxylic acid is inferior to BLM in oxygen activation under physiological condition. It can be said that the antibiotic possesses a switch-off device in the β-aminoalaninamide moiety depending on the abundance of BLM hydrolase in each tissue. The present results provide a basis for rational design of tissue-specific BLM analogues.

Acknowledgment. The authors thank Professor Keizo Inoue and Dr. Junken Aoki for the experiment of mouse liver extract and Dr. Chiaki Nishimura for pertinent advice on bleomycin hydrolase. The present study was financially supported in part by a Grant-in-Aid for Scientific Research on Priority Areas No. 03242104 (to S. K.) from Ministry of Education, Science, and Culture, Japan.

References and Notes

1. Umezawa, H.; Maeda, K.; Takeuchi, T.; Okami, Y., *J Antibiot.*, Ser. A, **1966**, *19*, 200.
2. (a) Sugiura, Y.; Takita, T.; Umezawa, H. *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1985; pp81-108. (b) Hecht, S. M. *Acc. Chem Res.* **1986**, *19*, 383. (c) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. (d) Otsuka, M.; Sugiura, Y. *Handbook in Metal-Ligand Interaction in Biological Fluids*; Berthon, G., Ed.; Marcel Dekker, in press. (e) Ohno, M. Otsuka, M. *Stereocontrolled Organic Synthesis*; Trost, B. M., Ed.; Blackwell, in press.
3. Umezawa, H.; Hori, S.; Sawa, T.; Yoshioka, T.; Takeuchi, T. *J Antibiot*, **1974**, *27*, 419.
4 Sugiura, Y. *J Am Chem Soc*, **1980**, *102*, 5208.
5. Kittaka, A.; Sugano, Y.; Otsuka, M.; Sugiura, Y.; Umezawa, H. *Tetrahedron Lett.*, **1986**, *27*, 3631.
6. Sugano, Y.; Kittaka, A.; Otsuka, M.; Ohno, M.; Sugiura, Y.; Umezawa, H. *Tetrahedron Lett.* **1986**, *27*, 3635.
7. Kittaka, A.; Sugano, Y.; Otsuka, M.; Ohno, M. *Tetrahedron* **1988**, *44*, 2811.
8. Kittaka, A.; Sugano, Y.; Otsuka, M.; Ohno, M. *Tetrahedron* **1988**, *44*, 2821.
9. Otsuka, M.; Kittaka, A.; Iimori, T.; Yamashita, H.; Kobayashi, S.; Ohno, M. *Chem. Pharm. Bull.*, **1985**, *23*, 509.
10. Tarbell, D. S.; Yamamoto, Y.; Pope, B. M. *Proc. Natl. Acad Sci., U. S A.* **1972**, *69*, 730.
11. Bis(AOC) derivative **4** was found to be superior to the corresponding bis(Z) derivative in selectivity.
12. Hayakawa, Y.; Kato, H.; Uchiyama, M.; Kajino, H.; Noyori, R. *J Org. Chem*, **1986**, *51*, 2400.
13. Sheehan, J. C.; Preston, J.; Cruickshank, P. A. *J. Am. Chem. Soc.*, **1965**, *87*, 2492.
14. Shioiri, T.; Ninomiya, K.; Yamada, S. *J Am Chem Soc*, **1972**, *94*, 6203.
15. Deamido-PYML-6. $[\alpha]_D^{21}$ = +38.3° (c = 0.81, $CHCl_3$); Rf 0.69 (*n*-PrOH : AcOH : Pyridine : H_2O = 15 : 3 : 10 : 12); IR (KBr) 3430, 2976, 1734, 1653, 1603, 1521, 1457, 1368, 1151, 1073, 847, 668 cm^{-1}; ^1H NMR (D_2O) δ 1.16 (9H, s, *t-Bu*O), 1.36 (9H, s, *t-Bu*OCO), 3.11 (1H, dd, *J* = 7.0, 13.2 Hz, NC*H*$_2$CH), 3.17 (1H, dd, *J* = 5.5, 13.2 Hz, NC*H*$_2$CH), 3.80 (1H, dd, *J* = 5.5, 7.0 Hz, NCH$_2$C*H*), 3.89 (3H, s, PyOC*H*$_3$), 4.04 (2H, s, PyC*H*$_2$), 5.15 (1H, d, *J* = 6.2 Hz, C$_\alpha$*H* of His), 5.21 (1H, d, *J* = 6.2 Hz, C$_\beta$*H* of His), 7.13 (1H, d, *J* = 2.2 Hz, Py), 7.20 (1H, s, Im), 7.44 (1H, d, *J* = 2.2 Hz, Py), 7.81 (1H, s, Im); FABMS m/z 535 (MH^+); HRMS (FAB) calcd for $C_{25}H_{39}O_7N_6$ (MH^+): 535.2880; found: 535.2883.
16. HPLC analysis was carried out using YMC-Pack C_8-AP column, eluted with MeOH : 4%NH_4OAc = 35 : 65 (flow rate: 1.0 ml/min, detector: UV290 nm). Retention times of PYML-6 and deamido-PYML-6 were 13 min and 11 min, respectively.
17. Nishimura, C. *Dissertation*, University of Tokyo, **1989**.
18. For stereoselective introduction of the acetamide group for the more advanced models, see Boger, D. L.; Menezes, R. F.; Honda, T. *Angew. Chem Int. Ed. Engl.*, **1993**, *32*, 273.

(*Received in USA* 3 *September* 1993; *accepted* 6 *January* 1994)