Isolation, Structure Determination, and Proposed Mechanism of Action for Artifacts of Maduropeptin Chromophore

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Maduropeptin,[1] isolated from the broth filtrate of *Actinomadura madurae*, consists of a 1:1 complex of an acidic, water soluble carrier protein (32 kD)[2] and a 9-membered ring enediyne chromophore possessing potent antibacterial and antitumor properties. Maduropeptin apoprotein represents a new protein class showing no sequence homology[2] to the related chromoproteins[3] neocarzinostatin, auromomycin, actinoxanthin, C-1027, or kedarcidin. In contrast, the chromophore[4] appears to be biogenetically related to the enediyne chromophores that have been characterized thus far,[5-7a] having the same 14 unbranched core carbons.

Maduropeptin has a tightly bound chromophore that required the development of a new process to effect the dissociation. Substantially pure holoantibiotic was bound to DEAE–cellulose. With the chromoprotein bound to the anion exchanger, cold methanol was allowed to percolate slowly through the bed. The methanol apparently denatured the protein and released the chromophore, leaving the apoprotein bound. The methanol extract contained the bioactivity as determined by cytotoxicity and microbial and *Escherichia coli* SOS chromotest bioassays. HPLC–UV analysis of the extract revealed four predominant peaks, three of which (**1**–**3**) had UV spectra reminiscent of holoantibiotic, while the fourth (**7**) indicated extended conjugation. The isolates were purified with bioassay guidance, using conventional techniques including Sephadex LH-20 and chromatography on silica gel. The increased stability and the decreased potency of these materials as compared to holoantibiotic and the presence of three compounds differing by substitution at only one position suggested that they were artifacts of the isolation process, and, indeed, if ethanol was substituted for methanol in the above process, a new isolate (**4**) was obtained with the corresponding ethoxy group on the C-5 position. Formation of the chlorine-containing artifact **2** was attributable to exposure to Tris buffer (containing Tris·HCl) used during the isolation of maduropeptin. When using a modified isolation scheme devoid of chloride ion sources was used, **2** was not detected. The hydroxy analog **3** was always detectable in minor amounts. The cycloaromatization product **7** was the major isolate if maduropeptin was bound to DEAE–cellulose that had not been thoroughly rinsed with fresh water. The eluant from commercially available DEAE–cellulose is slightly basic (pH 8.5). When the DEAE–cellulose was rinsed to neutrality before use, the methanol adduct **1** was predominantly formed. If aprotic solvents were substituted in the extraction process, the chromophore was not released.

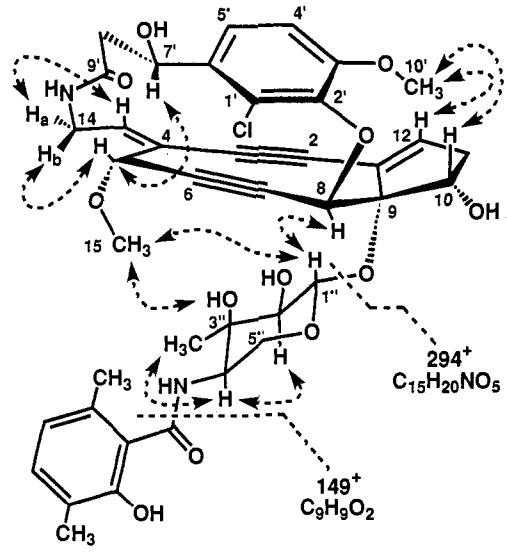

Figure 1. Proposed conformation and relative stereochemistry of the methanol adduct **1**. Selected NOE interactions from single irradiation experiments are shown (DMSO-d_6, 500 MHz). Prominent fragment ions of $(M + H)^+$ m/z 779 are indicated from MS/MS and HRFABMS studies.

Structures were determined[8] by a series of low-temperature[9] 1D and 2D NMR experiments in conjunction with UV, IR, and FABMS measurements. The UV spectrum of **1** showed two maxima: λ_{max} (MeOH) 212, 278 nm (log ϵ 4.56, 4.14). The IR spectrum had bands (ν_{max} 1640, 2170 cm^{-1}) characteristic of amide and alkyne groups, respectively. The molecular formula for **1** was established as $C_{40}H_{43}N_2O_{12}Cl$: HRFABMS $(M + H)^+$ m/z 779.2603, calcd 779.2583. All of the isolates showed prominent fragment ions at m/z 294, consistent with the entire ribopyranose side chain, as well as m/z 149 ions, indicating the terminal benzamide portion only (Figure 1). The 4-amino-4-deoxy-3-*C*-methyl-ribopyranose moiety is apparently a new sugar, now designated as madurosamine. The anomeric proton coupling constant (J = 6.7 Hz) indicated β linkage of the sugar. A three-bond coherence in the HMBC experiment between H-1″ and C-9 placed the sugar on the core. HMBC coherences between H-8 and C-2′ and between both H-14 protons and C-9′ established the ether and amide linkages, respectively, of the bridging atoms to the core. The observance of all expected two- and three-bond correlations in the long-range heteronuclear experiments allowed us to bridge the many quaternary carbons and determine connectivity.

The conformation of **1** became evident through ^1H–^1H coupling constants and NOE studies.[10] Interactions between the aromatic methoxy (H-10′) and the methine protons at H-10 and H-12

(1) Hanada, M.; Ohkuma, H.; Yonemoto, T.; Tomita, K.; Ohbayashi, M.; Kamei, H.; Miyaki, T.; Konishi, M.; Kawaguchi, H.; Forenza, S. *J. Antibiot.* **1991**, *44*, 403–414.

(2) Personal communication from Dr. Hans Marquardt, Bristol-Myers Squibb, Seattle, WA 98121. In contrast, the other apoproteins are in the 11.5 kD range and show approximately 40% sequence homology.

(3) For a comprehensive listing of references in the chromoprotein area, see ref 5a.

(4) Schroeder, D. R.; Lam, K. S.; Veitch, J. M. U.S. Patent 5,281,417, Jan 25, 1994. This contains physical and biological data and spectra for the major isolate (**1**).

(5) Kedarcidin chromophore: (a) Leet, J. E.; Schroeder, D. R.; Langley, D. R.; Colson, K. L.; Huang, S.; Klohr, S. E.; Lee, M. S.; Golik, J.; Hofstead, S. J.; Doyle, T. W.; Matson, J. A. *J. Am. Chem. Soc.* **1993**, *115*, 8432–8443. (b) Leet, J. E.; Schroeder, D. R.; Hofstead, S. J.; Golik, J.; Colson, K. L.; Huang, S.; Klohr, S. E.; Doyle, T. W.; Matson, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 7946–7948.

(6) C1027 chromophore: (a) Minami, Y.; Yoshida, K.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2633–2636. (b) Yoshida, K.; Minami, Y.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2637–2640.

(7) Neocarzinostatin chromophore: (a) Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Otake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331–334. (b) Edo, K.; Akiyama, Y.; Saito, K.; Mizugaki, M.; Koide, Y.; Ishida, N. *J. Antibiot.* **1988**, *41*, 1272–1274. (c) Lee, S. H.; Goldberg, I. H. *Mol. Pharm.* **1988**, *33*, 396–401.

(8) HRFABMS indicated $C_{39}H_{40}N_2O_{11}Cl_2$ ($[M + H]^+$ m/z 783.2085, calcd 783.2087) as the molecular formula for the chlorine containing artifact **2**. The NMR of **2** showed splitting patterns very close to that of **1** and chemical shift differences centered around C-5. The molecular formula for the hydroxy analog **3** was established as $C_{39}H_{41}N_2O_{12}Cl$ by HRFABMS: $(M + H)^+$ m/z 765.2405, calcd 765.2425. NMR spectra for **3** were nearly identical to those of **1** except for the absence of the aliphatic methoxy group (C-15).

(9) As for kedarcidin,[5] use of a 1:1 mixture of DMSO-d_6/CD_3CN at 273 K greatly prolonged the chromophore half-life for 2D NMR work.

(10) An in-depth NMR study of a tetraacetate derivative of **1** aided in this determination.

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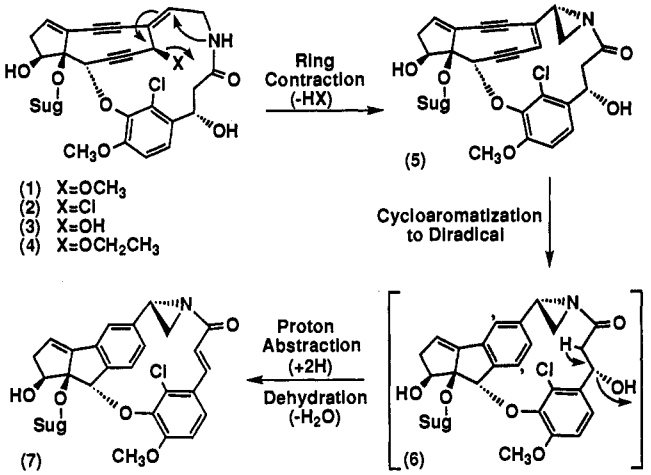
Scheme 1. Proposed Mechanism of Action for Artifacts **1–4** of Maduropeptin Chromophore in a Planar Representation[a]



[a] **Sug** denotes the 4-amino-(2-hydroxy-3,6-dimethylbenzoyl)-4-deoxy-3-*C*-methylribopyranose side chain, which remains intact (Figure 1).

indicated that the bridging system was stacked on the core rings and established the relative stereochemistry at C-10 (Figure 1). The H-13 vinyl proton showed large couplings to the adjacent geminal protons (10.7 Hz to H-14b, 6.2 Hz to H-14a) that could be accommodated only by a C-14 to nitrogen bond that was nearly perpendicular to the plane of the 9-membered ring. NOE interactions between H-5 and H-7′ on the bridge established the stereochemistry of the C-7′ hydroxyl, while interactions between H-5 and H-14b determined the geometry of the exocyclic double bond. Observed NOEs between H-8 and the anomeric proton H-1″ and between the aliphatic methoxy (H-15) and the sugar moiety (especially 3″-OH, a sharp singlet in dry DMSO-d_6), established the trans arrangement of the C-8 and C-9 substituents. The chlorine group at C-1′ is sufficiently large to prevent the bridging system from rotating through. As depicted (Figure 1), the proposed relative stereochemistry for the methanol adduct **1** is 5*R*,8*S*,9*R*,10*S*,7′*S* with a β-D-amino sugar side chain. The absolute stereochemistry has not been determined.

Compound **7** differed substantially from the other isolates. The molecular formula was established as $C_{39}H_{39}N_2O_{10}Cl$: HRFABMS $(M + K)^+$ m/z 769.1925, calcd 769.1930. The absence of the acetylene resonances by ^{13}C-NMR and the presence of a new ABX system in the ^1H-NMR indicated that cycloaromatization had occurred. Completely unexpected was the fact that **7** still displayed both antibacterial and antitumor properties, with a unique biological profile. NMR analysis revealed a new transsubstituted double bond at C-7′,C-8′ (J_{H-H} = 15.7 Hz) which was consistent with the UV spectrum: λ_{max} (MeOH) 216, 237 sh, 278 sh, 308 nm (log ε 4.23, 4.15, 3.68, 3.48). The presence of the aziridine ring was supported by $^1J_{C-H}$ couplings of 175 Hz for both the C-13 and C-14 carbons in the fully coupled ^{13}C-NMR spectrum and an observed H-14 geminal coupling of 0.5 Hz. NOE studies indicated a relative stereochemistry for the aziridine center (13*R*) that would result from the exocyclic methylene geometry (C-4,C-13) depicted in **1** (Figure 1).

Efforts to isolate intact maduropeptin chromophore, which may be more deeply imbedded in this larger protein, were unsuccessful. Conditions necessary to disrupt the protein and release the chromophore also lead to addition at C-5. The fact that compounds **1–4** selectively cleave DNA[11] suggests that rearrangement of the C-4,C-13 double bond must occur to form an enediyne system. The isolation and characterization of the cycloaromatization product **7** allows us to propose a concerted intramolecular ring contraction process to form **5**, whereby the amide nitrogen adds to C-13, causing double bond migration and the antielimination of the group that initially combined at C-5. Upon formation of the enediyne ring **5**, no constraints would remain to inhibit cycloaromatization to a highly reactive diradical species (**6**) capable of proton abstraction and DNA strand cleavage. Modeling studies indicate that dehydration of the C-7′ bridge hydroxyl is not energetically favored in **1–4**, and the elimination may follow a conformational change after cycloaromatization. The compound **7** generated *in situ* from these transformations is capable of further damage, possibly by alkylation of DNA or a repair enzyme.

Unlike most enediyne antitumor antibiotics, maduropeptin is not bioreductively activated. Drug–DNA studies[11] using supercoiled DNA and restriction fragments indicated inhibition of scission upon addition of β-mercaptoethanol, but strand cleavage was significantly enhanced under basic conditions (optimal pH 8). This was true for both holoantibiotic and compound **1**, which showed the same high sequence specificity. The poor nucleophilicity of an amide nitrogen and the equally poor leaving group ability of a methoxy group (**1**) suggest binding site catalysis in the ring contraction step. The chlorine adduct **2** showed considerably increased potency as compared to **1**, which is most easily rationalized by the superior leaving group ability of chlorine as compared to that of a methoxy group.

Several possibilities exist for the structure of the parent chromophore. We are considering a C-4,C-5 α-epoxide as in neocarzinostatin, where nucleophilic addition to the epoxide at C-5 might occur. Methanol and chlorine adducts at the C-5 position of neocarzinostatin were formed,[7b,c] albeit under acidic conditions. If attachment of the bridging system were trans on the core ring (C-4 to C-8), ring strain might facilitate dehydration of the C-4 tertiary hydroxyl intermediate to the less strained arrangement in **1–4** (now planar at C-4). Alternatively, proposed intermediate **5** might be maduropeptin chromophore,[12] where ring expansion via aziridine opening follows addition to C-5 (the exact reverse of transformations **1–4** to **5**, Scheme 1). The protein may stabilize the reactive enediyne system, as is apparently the case for C-1027 chromophore, and catalyze the addition to C-5. Other possibilities exist, including the presence of some unknown leaving group at C-5, even a covalent linkage to apoprotein, that is displaced, leading to artifacts **1–4**. Studies continue in order to ascertain the correct structure.

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Supplementary Material Available: Tables listing complete ^1H- and ^{13}C-NMR chemical shift assignments, ^1H–^1H coupling constants, and long-range ^1H–^{13}C correlations for the methanol adduct **1** and the cycloaromatization product **7** of maduropeptin chromophore (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(11) Zein, N.; Solomon, W.; Schroeder, D. R.; Colson, K. L., manuscript in preparation. Recent studies also indicate that maduropeptin has proteolytic activity and cleaves histones selectively, as was demonstrated for kedarcidin apoprotein. Zein, N.; Casazza, A. M.; Doyle, T. W.; Leet, J. E.; Schroeder, D. R.; Solomon, W.; Nadler, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8009–8012.

(12) Reviewer P renewed our interest in this possibility. We had considered the addition to the ene at C-5 to be an unlikely event, and without protein catalysis it might be.