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Reductive and Nucleophilic Activation Products of Dynemicin A with Methyl Thioglycolate. A Rational Mechanism for DNA Cleavage of the Thiol-Activated Dynemicin A†

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ABSTRACT: The reaction products of methyl thioglycolate with dynemicin A, dynemicin H and dynemicin S, were isolated by HPLC purification and identified spectroscopically. The major product, dynemicin H ($C_{30}H_{23}NO_9$), was determined to be a C-8 hydrogen analogue of dynemicins L and N in which the enediyne core is aromatized. The minor product, dynemicin S ($C_{33}H_{27}No_{11}S$), is an adduct of methyl thioglycolate at the C-8 position. By using NADPH instead of methyl thioglycolate, the reaction with dynemicin A also gives the same major product (dynemicin H). The nucleotide-specific cleavage of dynemicin A induced by addition of methyl thioglycolate is remarkably similar to that induced by addition of NADPH, whereas dynemicins H and S show no DNA cleavage activities. The formation of dynemicins H and S provides a rationale for the reductive and nucleophilic activations of dynemicin A.

Enediyne-class antibiotics have an exceptional antitumor potency, unusual chemical structure, and marked ability to cause DNA strand scission. Dynemicin A, recently isolated from *Micromonospora chersina*, is a novel antitumor antibiotic consisting of enediyne and anthraquinone components (Konishi et al., 1990). The antineoplastic action of dynemicin A has been suggested to be associated with the intercalation and cleavage of DNA by the antibiotic (Sugiura et al., 1990). In the dynemicin A mediated DNA breakage, the preferential cutting sites are clearly different from those of a family of other enediyne antitumor antibiotics such as esperamicin (Sugiura et al., 1989), calicheamicin (Zein et al., 1988), and neocarzinostatin (Goldberg, 1987). For DNA strand scission by dynemicin A, we previously presumed a reaction scheme in which NADPH, thiol, or visible light activates the antibiotic into a reactive 1,4-dehydrobenzene diradical species formed from the enediyne core (Sugiura et al., 1990; Shiraki & Sugiura, 1990). In addition, we proposed epoxide opening as a triggering mechanism for the action of dynemicin A (Sugiura et al., 1990). With respect to the activation of the antibiotic in the DNA cleavage, however, the reaction products of dynemicin A with thiols or NADPH have never been isolated and identified experimentally.

Herein, we report the isolation and characterization of two products from the reaction of dynemicin A with methyl thioglycolate, dynemicin H and dynemicin S. The results provide useful information on a molecular that has captured the interest of a variety of research scientists interested in the chemistry of antitumor drugs and DNA cutting reagents.

Materials and Methods

Drugs and Chemicals. Dynemicin A was isolated from the fermentation broth of *M. chersina* and purified as described (Konishi et al., 1989, 1990). G4 DNA obtained from phage R199/G4 *ori* replicative form DNA was a generous gift of T. Komano (Kyoto University, Japan). Purified pBR322 plasmid DNA and restriction enzymes *Hae*III and *Eco*RI were purchased from Takara Shuzo (Kyoto, Japan). Methyl thioglycolate and NADPH were obtained from Wako Pure Chemicals (Osaka, Japan) and Sigma, respectively. All other chemicals used were of commercial reagent grade.

Monitoring by HPLC for Reaction of Dynemicin A with Methyl Thioglycolate or NADPH. The reaction of dynemicin A (0.5 mg, 0.95 μM) with methyl thioglycolate (0.5 mg, 5 μM)

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or NADPH (3.7 mg, 5 μM) was performed in DMF (0.3 mL) and 0.1 M ethanolic acetic acid (0.2 mL) at 20 °C for 42 h. The reaction mixture was separated by HPLC on an A301-3S-3-120A ODS column with methanol–0.15 % KH_2PO_4 (pH 3.5)(75:25 v/v) as the solvent. The eluate was monitored at 254 nm.

Isolation and Purification of Dynemicins H and S. To a solution of dynemicin A (10 mg, 19 μM) in DMF (1 mL) was added dropwise a solution of methyl thioglycolate (10 mg, 100 μM) in 0.1 M ethanolic acetic acid (3 mL) with stirring at 20 °C. The decrease of dynemicin A (R_f 9.2 min) and increase of the new compounds, dynemicin H (R_f 7.4 min, major product) and dynemicin S (R_f 9.8 min, minor product) were monitored by HPLC [column, A301-3S-120A ODS (4.5 mm i.d. × 100 mm, Yamamura Chemical Lab); mobile phase, methanol–0.15% potassium phosphate (pH 3.5) buffer (75:25 v/v)]. After the reaction mixture was stirred at 20 °C for 42 h, the solution was poured into water (30 mL) and extracted twice with ethyl acetate (30 mL). The organic layers were combined, washed twice with water (60 mL), dried over anhydrous sodium sulfate, and then evaporated in vacuo. The residue (10.2 mg) was purified by preparative TLC [Kaesel gel 60 F254, Merck, xylene–methyl ethyl ketone–methanol (5:5:1 v/v/v); R_f 0.24 (dynemicin H) and 0.18 (dynemicin S)], followed by Sephadex LH-20 column chromatography [2 cm i.d. × 30 cm, methylene chloride–methanol (2:2 v/v)] to yield dynemicin H and dynemicin S as homogeneous blue solids. Finally, 1.9 mg of dynemicin H and 0.3 mg of dynemicin S were obtained as samples recrystallized from a CH_2Cl_2–CH_3OH solvent system.

Spectroscopic Measurements of Dynemicins H and S. Electronic absorption and 400-MHz ^1H NMR spectra were recorded with a JASCO UVIDEC-610 double-beam spectrophotometer and a JEOL-JNM-GX400 spectrometer, respectively. DMSO-d_6 was used as a solvent, and tetramethylsilane was utilized as an internal standard in the NMR studies. Fast atom bombardment mass spectra (FAB-MS) and high-resolution FAB-MS were measured on a JEOL-JMS-AX505H spectrometer. These spectroscopic properties of dynemicins H and S were compared with those of dynemicin A and its aromatized analogues (Konishi et al., 1990).

DNA Cleavage Assay. Analysis of dynemicin-induced damage to supercoiled, covalently closed, circular (form I) pBR322 DNA was performed in the presence of methyl thioglycolate. The reaction samples (total volume 20 μL) contained 0.5 μg of pBR322 plasmid DNA, 10 mM Tris-HCl buffer (pH 7.5), 20 μM dynemicin A (or dynemicin H), and 20 μM–2 mM methyl thioglycolate, and they were incubated at 37 °C for 30 min. The reactions were stopped by addition of cold ethanol (60 μL) and 0.3 M sodium acetate. The samples were immediately chilled at −70 °C in a dry ice/ethanol bath, and the DNA was recovered by ethanol precipitation. Electrophoresis was performed by using 1% agarose gels containing ethidium bromide (0.5 μg/mL). In the nucleotide sequence cleavage experiments, a 5′-end-labeled 100-base-pair G4 gene F/G space fragment was used as DNA substrate. DNA cleavage reactions were carried out by dynemicin A (20 μM) and methyl thioglycolate (or NADPH, 1 mM) at 37 °C for 5 h. Electrophoresis was performed on a 10% polyacrylamide/7 M urea slab gel, and DNA sequencing was carried out by the Maxam–Gilbert method (Maxam & Gilbert, 1980).

Results and Discussion

Identification of Dynemicins H and S. As shown in Figure 1, HPLC analysis of the reaction sample of dynemicin A with methyl thioglycolate clearly indicated the formation of one predominant product (dynemicin H, 7.4 min) together with minor products (dynemicin S and unknown compounds). On the other hand, the reaction of dynemicin A with NADPH presented one major product, which is chromatographically identical with dynemicin H (Figure 1). According to the procedure described under Materials and Methods, therefore, dynemicins H and S were isolated from the reaction of dynemicin A with methyl thioglycolate.

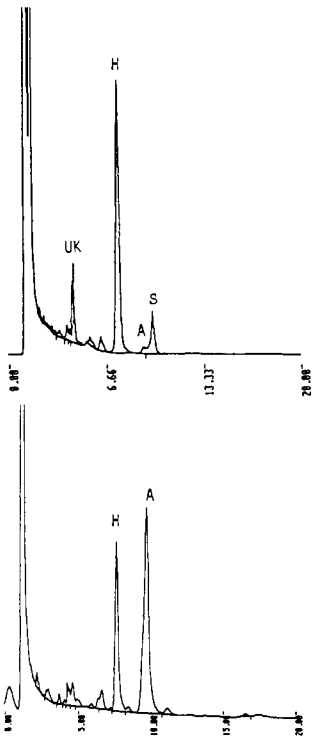

FIGURE 1: HPLC analyses after reaction of dynemicin A with methyl thioglycolate for 42 h (upper panel) and with NADPH for 72 h (lower panel). UK represents an unknown product.

Table I: Some Physicochemical Properties of Dynemicins H and S

	dynemicin H	dynemicin S
nature	blue amorphous powder	blue amorphous powder
mp (dec) (°C)	221–223	213–215
UV λ_{max}^a (ε) in MeOH	240(43 400) 455(2300) 592(14 700) 639(16 100)	267(48 500) 470(2400) 602(16 800) 650(18 300)
FAB-MS *m/z* (positive)	541$(M)^+$	645$(M)^+$
HRFAB-MS *m/z*	for $C_{30}H_{23}NO_9$	for $C_{33}H_{27}NO_{11}S$
calcd	541.1369	645.1298
found	541.1371	645.1301
TLC (R_f)[b]	0.24	0.18
HPLC (R_t, min)[c]	7.4	9.8

[a] λ_{max} in nanometers. [b] SiO_2, xylene–methyl ethyl ketone–MeOH 5:5:1 (v/v/v). [c] Column, A301-3S-3-120A ODS (4.6 mm i.d. × 100 mm, Yamamura Chemical Lab); mobile phase, MeOH–0.15% KH_2PO_4 (pH 3.5) buffer 75:25 (v/v); flow rate, 1 mL/min; detection, UV absorption at 254 nm.

Table I summarizes some properties of dynemicin H and dynemicin S. The molecular formulas of dynemicins H and S were established as $C_{30}H_{23}NO_9$ and $C_{33}H_{27}NO_{11}S$, respectively, on the basis of high-resolution fast atom bombardment mass and ^1H NMR spectroscopic analyses. Their electronic absorption spectra are markedly similar to those of dynemicins L, M, and N, containing a 1,4,6-trihydroxy 8,9-disubstituted

Table II: ^{1}H NMR Spectral Data of Dynemicins H and S[a]

proton	dynemicin H	dynemicin S
4-CH_3	0.95 (3 H, d, J = 7 Hz)	0.96 (3 H, d, J = 7 Hz)
4-H	3.23 (1 H, q, J = 7 Hz)	3.27 (1 H, q, J = 7 Hz)
8-H	3.50 (1 H, d, J = 3 Hz)	
$-S-CH_2-$		3.46 (2 H, dd, J = 10, 5 Hz)
$-COOCH_3$		3.61 (3 H, s)
6-OCH_3	3.84 (3 H, s)	3.82 (3 H, s)
7-H	3.96 (1 H, d, J = 3 Hz)	4.10 (1 H, s)
2-H	4.78 (1 H, d, J = 5 Hz)	4.72 (1 H, d, J = 5 Hz)
3-OH	5.46[b] (1 H, s)	5.39[b] (1 H, s)
16-H, 17-H, 24-H	7.13 } (6 H, m)	6.98 } (6 H, m)
25-H, 26-H, 27-H	7.56 }	7.54 }
10-H	7.63 (1 H, s)	8.43 (1 H, s)
5-COOH, 11-OH	11.3[b] } (4 H, br)	11.3[b] } (4 H, br)
15-OH, 18-OH	14.0[b] }	14.0[b] }

[a] 400 MHz in DMSO-d_6 [b] The signal disappeared by addition of D_2O.

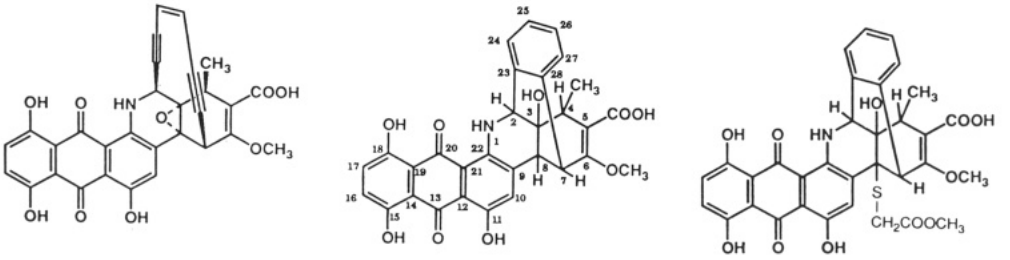

FIGURE 2: Chemical structures of dynemicin A (left), dynemicin H (middle), and dynemicin S (right).

anthraquinone and a 1,2-disubstituted benzene ring in the molecule (Konishi et al., 1990). As shown in Table II, the ^{1}H NMR features of dynemicins H and S indicate (1) the presence of four contiguous protons (δ 7.0–7.6) of a 1,2-disubstituted benzene together with the signals of an anthraquinone and (2) the lack of two double bond protons (δ 6.60 and 6.09) due to the 1,5-diyne-3-ene moiety observed in dynemicin A. Furthermore, the epoxide unit was not detected. The present data suggest that the 1,5-diyne-3-ene system of dynemicin A has aromatized to yield dynemicins H and S. A proton (δ 3.50, C-8) and a hydroxyl group (δ 5.46, C-3) of dynemicin H were assigned by comparison with the ^{1}H NMR spectra of dynemicin A and its aromatized analogues (Konishi et al., 1990). In dynemicin S, new methyl (δ 3.61) and methylene (δ 3.40) signals were observed, and also, the proton signal at C-7 was detected as a singlet. Therefore, dynemicin H was assigned as a C-8 hydrogen analogue of dynemicins L and N, and dynemicin S was assigned as an adduct of methyl thioglycolate at the C-8 position (Figure 2).

By using ascorbate instead of methyl thioglycolate or NADPH, the present reaction with dynemicin A also gave the same major product (dynemicin H) chromatographically (data not shown).

DNA Cleavage Activity of Dynemicins in the Presence of Methyl Thioglycolate. In the presence of methyl thioglycolate, dynemicin A evidently converted covalently closed, supercoiled (form I) pBR322 DNA to nicked circular (form II) and linear duplex (form III) DNAs. On the other hand, dynemicin H revealed no DNA cleavage activities. Similar experiment also showed inertness of dynemicin S in the DNA cutting (data not shown).

Figure 3 demonstrates that the DNA cutting potency and the sequence-specific cleavage of DNA by dynemicin A are appreciably similar with methyl thioglycolate or NADPH as activator. The result may suggest that (1) the activation mechanism of dynemicin A by methyl thioglycolate is similar to that by NADPH and (2) the site selectivity of dynemicin cleavage is caused by the preferential intercalation of the anthraquinone chromophore with the different base sequences.

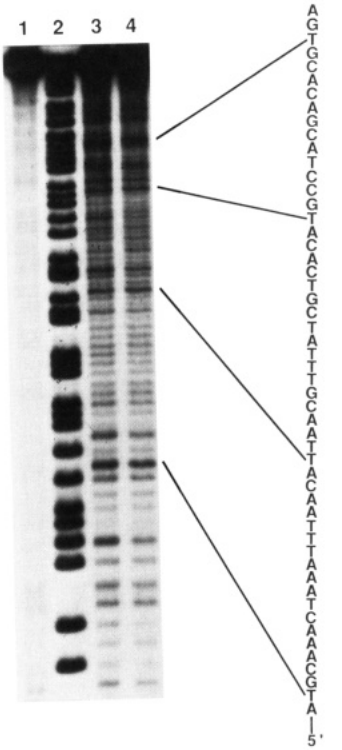

FIGURE 3: DNA cutting mode of 5′-end-labeled G4 gene F/G space DNA fragment by dynemicin A in the presence of methyl thioglycolate (lane 3) or NADPH (lane 4). DNA cleavage reactions were carried out by dynemicin A (20 μM) and methyl thioglycolate (or NADPH; 1 mM) at 37 °C for 5 h. Lanes 1 and 2 show intact DNA and the Maxam–Gilbert sequencing reaction for G+A, respectively.

Probable Mechanism for DNA Cleavage of the Thiol-Activated Dynemicin A. In the absence of DNA, dynemicin A reacted irreversibly with methyl thioglycolate to form products that do not cleave DNA. In this reaction, dynemicin H was isolated and identified as predominant product. The obser-

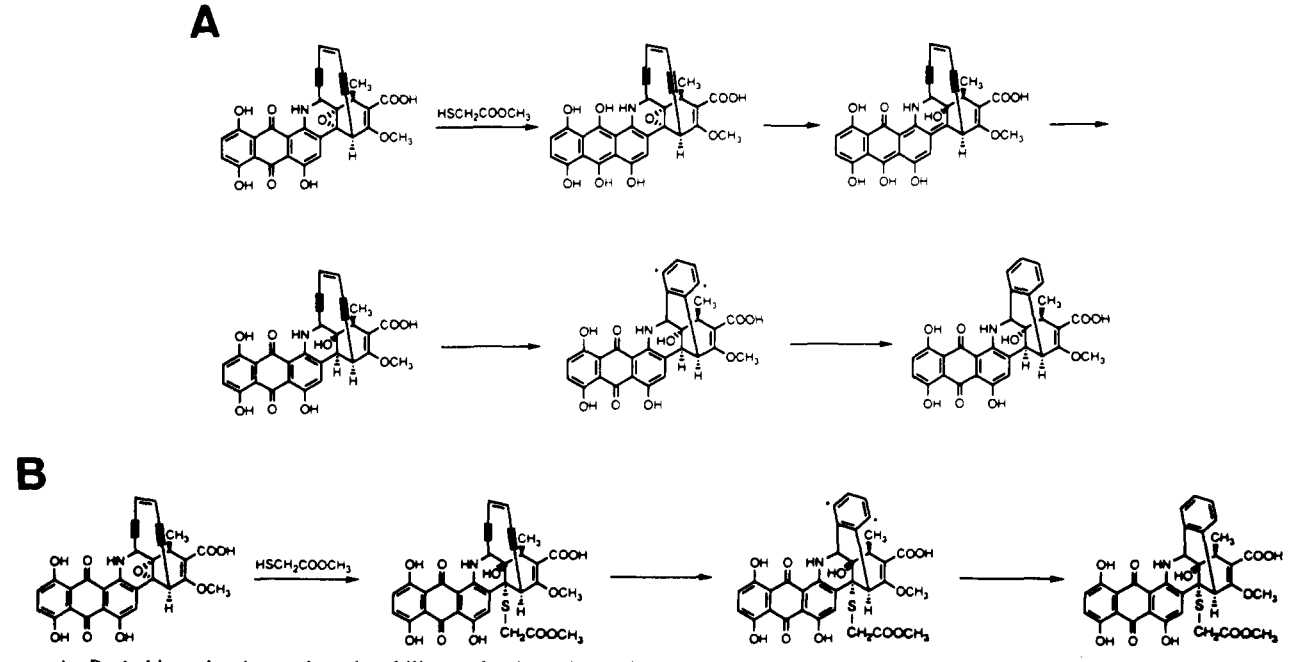

FIGURE 4: Probable reductive and nucleophilic mechanisms for activation of dynemicin A with methyl thioglycolate.

vation that dynemicin A gave dynemicin H on NADPH (or ascorbate) reduction is also important. Further, we previously demonstrated that the photoproduct of dynemicin A is chromatographically identical with dynemicin H (Shiraki & Sugiura, 1990). These results suggest that a reductive mechanism operates when dynemicin A cleaves DNA in the presence of thiols. Recent molecular mechanics calculations (Semmelhack et al., 1990; Snyder & Tipsword, 1990) also support a similar reductive mechanism for the activation of dynemicin A. One may write a reasonable mechanism based upon anthracycline chemistry for formation of both products via reduction of the quinone (Egholm, 1989; Gaudiano et al., 1990). Thiols could contribute an electron to the anthraquinone to yield a semiquinone radical or two electrons to yield a hydroquinone and in the process cause tautomeric ring opening to give the semiquinone methide or quinone methide. These species could pick up a hydrogen atom from thiol or from solvent to yield dynemicin H after reoxidation (either by O_2 or metathesis with excess unreduced quinone). In addition, nucleophilic or radical recombination events of thiol or thiol radical could account for the presence of dynemicin S (Figure 4). Indeed, the reaction product of thiol with neocarzinostatin chromophore established nucleophilic activation of neocarzinostatin (Myers, 1987). In the reaction of methyl thioglycolate (Myers, 1987) or glutathione (Chin et al., 1988) with the neocarzinostatin chromophore, the product in which one thiol added to the C-12 position of the chromophore provides a rationale for spontaneous nucleophile-induced biradical formation, a key step in the nucleophilic activation mechanism for DNA cleavage by the neocarzinostatin chromophore (Kappen & Goldberg, 1985). On the other hand, one may write a nucleophilic mechanism for formation of both products with dynemicin S as a precursor to dynemicin H. However, time course HPLC analyses of the reaction of dynemicin A with methyl thioglycolate do not indicate the definitive conversion of dynemicin S to dynemicin H.

In conclusion, the reaction products of dynemicin A with methyl thioglycolate were isolated and identified. The major product, dynemicin H, was determined to be a C-8 hydrogen analogue of dynemicins L and N. The minor product, dynemicin S, is an adduct of methyl thioglycolate at the C-8 position. Dynemicin A also gave dynemicin H on reduction with NADPH or ascorbate. The results indicate that reductive and nucleophilic mechanisms operate when dynemicin A cleaves DNA. This information makes a contribution to a rapidly expanding field of interest.

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