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A New Non-Protein Enediyne Antibiotic N1999A2: Unique Enediyne Chromophore Similar to Neocarzinostatin and DNA Cleavage Feature

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Abstract: The present NMR and X-ray structural studies demonstrated that the new antibiotic N1999A2 isolated from the broth filtrate of *Streptomyces* sp. AJ9493, possesses a novel 9-membered ring enediyne chromophore similar to neocarzinostatin, but is not chromoprotein. Of special interest is the fact that stable N1999A2 exists as enediyne chromophore alone as well as dynemicin A, esperamicin A_1 and calicheamicin γ_1. The major difference between N1999A2 and neocarzinostatin chromophore is lack of the amino sugar in N1999A2. The antibiotic N1999A2 revealed more random DNA cutting profile than neocarzinostatin chromophore.

Keywords: Antitumour compounds, Diynes, Natual products

N1999A2 (**1**), isolated recently from the broth filtrate of *Streptomyces* sp. AJ9493, strongly inhibits the growth of tumor cell lines and bacteria. Indeed, its IC_{50} of cytotoxicity to human colon carcinoma HCT116 cell was 6.0×10^{-12} M.[1] The antibiotic N1999A2 is a new 9-membered ring enediyne compound, similar to neocarzinostatin chromophore (Figure 1).[2, 3] In contrast to neocarzinostatin, however, N1999A2 contains no macromolecular peptides and is enough stable during isolation procedure in spite of non-protein chromophore.[4]

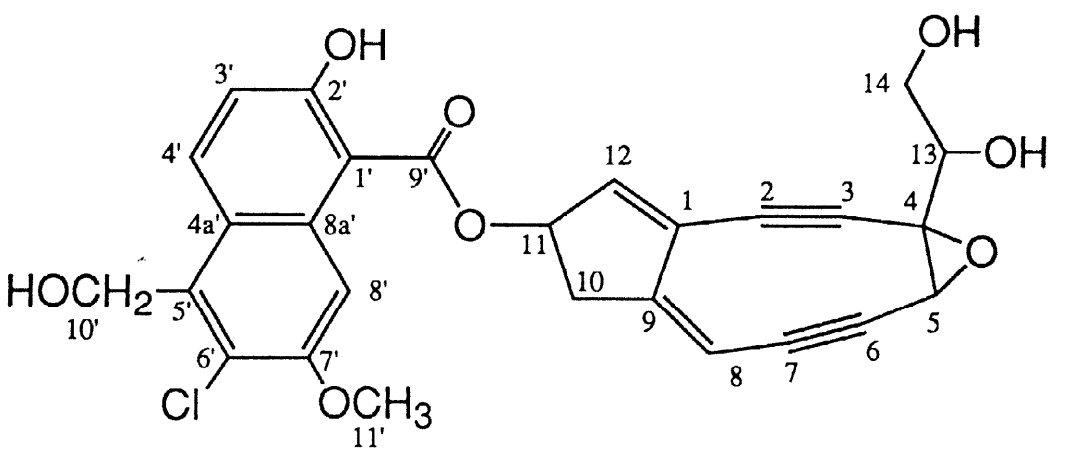

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Figure 1. Chemical Structure of N1999A2 (**1**).

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N1999A2 gave two decomposed substances, A2dec1 and A2dec2, in methanol solution at room temperature. These substances were purified by HPLC with ODS column (Waters Novapak C18 8 mm i.d. x 100 mm) using H_2O/CH_3CN gradient elution. The powder of A2dec2 was dissolved in methanol and crystallized to obtain colorless unaggregated crystals. The molecular formula of A2dec2 was established as $C_{14}H_{13}O_5Cl$: HR-FABMS $(M-H)^-$ m/z 298.0563, calcd 298.0562. The X-ray analysis of the crystal established the structure of A2dec2 as methyl naphthoate derivative. The free naphthoate structure of A2dec1 was deduced by comparison with A2dec1 and A2dec2 on ^1H and ^{13}C NMR data.

A molecular formula of N1999A2 was not determined by mass spectrum, because there was no parent ion peek on the measurement using EI, CI, FD, positive-FAB or negative-FAB ionization method. In fact, the only other ion consistently observed under any ionization technique was at m/z 281, corresponding to a naphtoate fragment. Detailed 1D- and 2D-NMR studies of N1999A2 including ^1H-^1H COSY, NOESY, DEPT, HMQC and HMBC (J=5.0 Hz, 10.0 Hz, 12.5 Hz) experiments, were performed in mixed solvent of DMSO-d_6 and acetonitrile-d_3 (1:1) at 15°C. Although all NMR experiments were taken for 7 days, N1999A2 was stable under this condition. The half-life time in DMSO:acetonitrile (1:1) solution was 459 hours at room temperature under dark condition. On the ^1H NMR spectrum, 21 proton signals were observed (Table 1). When the solvent for ^1H NMR measurements was changed to acetonitrile-d_3:deuterium oxide (4:1), 4 signals (δ 10.82 ppm, δ 5.36 ppm, δ 5.81 ppm, δ 4.67 ppm) disappeared. Observations by HMBC from the 4 proton signals to C-13, C-14, C-2' and C-10' indicated that these proton signals are assigned to hydroxy group. On the ^{13}C NMR spectrum, 27 carbons were observed (Table 1). The DEPT and HMQC spectra revealed that the N1999A2 molecule contains one carbonyl group, fifteen tertiary carbons, eight methines, three methylenes and one methyl group. The carbon signals of δ 84.0 ppm, δ 89.1 ppm, δ 98.0 ppm and δ 100.3 ppm were characteristic to alkyne carbon, and N1999A2 contained the naphtoate moiety, A2dec1, which is similar to the component of neocarzinostatin chromophore. Therefore, N1999A2 was considered to resemble to neocarzinostatin chromophore except for

Table 1. ^{13}C and ^1H NMR data of N1999A2 in DMSO-d6:acetonitrile-d3 (1:1)

Carbon		^{13}C (ppm)	^1H (ppm)	J (Hz)	Carbon		^{13}C (ppm)	^1H (ppm)	J (Hz)
1	C	129.8			1'	C	110.4		
2	C	84.0			2'	C	157.1		
3	C	100.3			3'	CH	116.7	7.13 (d)	9.5
4	C	64.4			4'	CH	130.6	8.22 (d)	9.5
5	CH	53.0	3.94 (d)	1.3	4a'	C	122.3		
6	C	98.0			5'	C	136.5		
7	C	89.1			6'	C	120.9		
8	CH	100.9	5.59 (brs)		7'	C	153.6		
9	C	161.4			8'	CH	103.7	7.41 (s)	
10	CH_2	37.2	3.29 (ddd)	18.6, 7.1, 2.2	8a'	C	131.4		
			2.89 (d)	18.6	9'	C=O	168.3		
11	CH	77.0	6.12 (m)		10'	CH_2	57.6	5.05 (d)	5.4
12	CH	140.9	6.82 (t)	2.1	11'	CH_3	56.0	3.91 (s)	
13	CH	70.8	3.63 (m)		2'-OH			10.82 (s)	
14	CH_2	63.4	3.63 (m)		10'-OH			5.18 (t)	5.4
			3.55 (m)						
13-OH			5.36 (d)	4.7					
14-OH			4.67 (t)	5.5					

lack of the NMR signals corresponding to carbohydrate and carbonate moieties. Detailed analysis of the ^{1}H and ^{13}C NMR spectra, a suite of 2D correlation experiments, and comparison with neocarzinostatin chromophore[3] allowed complete assignment of N1999A2 structure.

The HMBC experiment performed by the condition of J = 10 Hz showed the correlations of enediyne core moiety except for C-3 carbon. Then, the HMBC correlation was measured by another J value (5.0 Hz). In this HMBC spectrum, the ^{1}H-^{13}C long-range coupling between H-13 and C-3 could be observed. The result of HMBC experiments is summarized in Figure 2. Finally, two valence bonds remaining on each C-4 and C-5 participated in the epoxide formation. The chemical shifts of C-4 (δ 64.4 ppm) and C-5 (δ 53.0 ppm) were close to the corresponding signals of neocarzinostatin chromophore (δ 63.8 and δ 55.2 ppm, respectively), and no more protons and carbons attached to C-4 or C-5 were observed on NMR spectrum. Thus, the new 9-membered ring enediyne structure of N1999A2 was established except for the stereochemistry. Studies on the stereochemistry of N1999A2 are now underway.

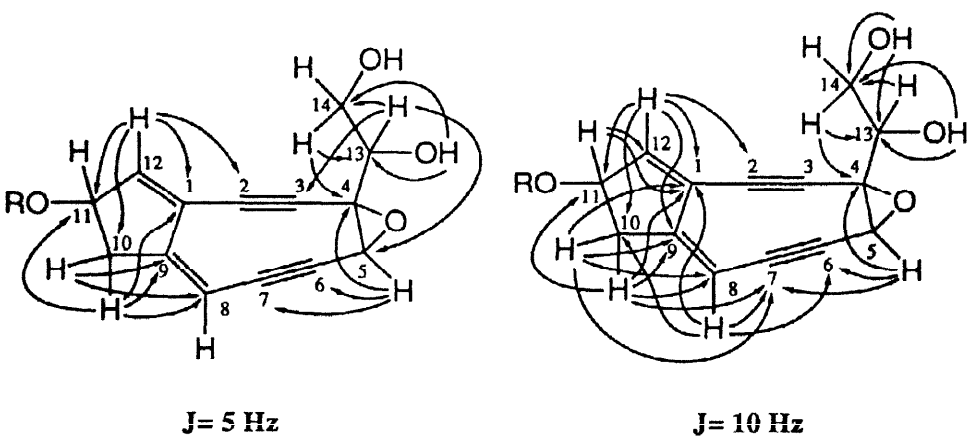

Figure 2. HMBC experiments (J= 5 Hz and J= 10 Hz) of N1999A2 (**1**) in DMSO-d6:Acetonitrile-d3 (1:1).

Figure 3 summarizes the histograms of the DNA cleavage sites (pBR322 SalI-NruI fragment) of N1999A2 in comparison with those of neocarzinostatin chromophore.[5] The result clearly shows that N1999A2 has more random DNA cutting profile than neocarzinostatin chromophore, although its base specificity (T»A>C»G) is similar to that of neocarzinostatin chromophore. Similar phenomenon was also detected in the BamHI-SphI fragment of pBR322 DNA. Goldberg *et al.* have suggested from the NMR experiments that the DNA binding specificity of neocarzinostatin chromophore is determined by the 2'-methyl sugar moiety which recognizes the T·A base pair.[6] By comparison of DNA cleavage by neocarzinostatin chromophore and the corresponding aglycone, whereas, Myers *et al.* indicated that the aminoglycoside dose not appear to be a major determinant of the base specificity.[7] Further, recent comparative study of DNA cleavage between namenamicin and calichemicin suggested that the lowered cleavage efficiency and the altered selectivity of namenamicin are probably attributed to several structure features of the carbohydrate moiety.[8] The present antibiotic N1999A2 may also help to elucidate the role of the carbohydrate residue of neocarzinostatin chromophore in DNA binding and cleavage. Of special interest is the fact the new antibiotic N1999A2 possesses a unique enediyne chromophore similar to neocarzinostatin but is not chromoprotein.

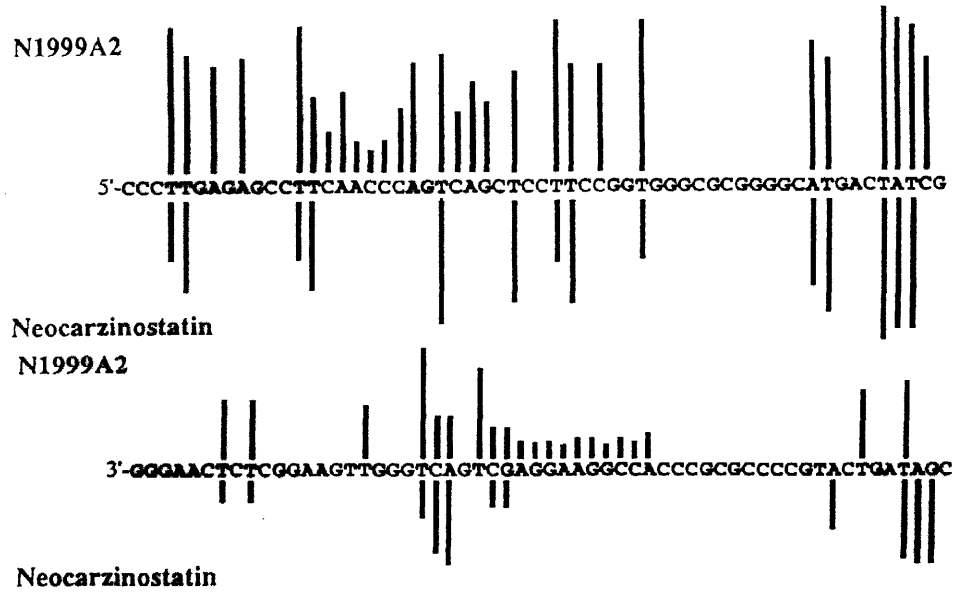

Figure 3. Histograms of DNA cleavage by N1999A2 and neocarzinostatin chromophore. Relative DNA cutting frequencies were obtained from densitometric scans of the gel autoradiograms of 5'- and 3'-end-labeled DNAs. The heights of the bars represent the relative cleavage intensities at the indicated bases.

Acknowledgments

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4) The supernatant obtained by ammonium sulfate precipitation of the culture broth of *Streptomyces* sp. AJ9493 evidently showed strong cytotoxicity, but the precipitate presented only remarkably weak activity. In addition, this chromophore was not incorporated to the apoprotein of neocarzinostatin.
5) Parallel incubations of 5'- or 3'-^{32}P-labeled 323-base pair restriction fragment (SalI/NruI) from plasmid pBR322 were conducted with N1999A2 (12.5-50 μM) and, separately, neocarzinostatin chromophore (10-40 μM) in the presence of dithiothreitol (20 mM) and calf thymus DNA (5 μg/ml) at 37°C and pH 7.0. Quantitative analysis of the DNA cleavage products was achieved by gel electrophoresis using a denaturing 10% polyacrylamide gel.
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