Paramagnetic Enediyne Antibiotic C-1027: Spin Identification and Characterization of Radical Species

Masahiro Hirama,*,† Kimio Akiyama,‡ Toshiyuki Tanaka,§ Takeshi Noda,† Kyo-ichiro Iida,† Itaru Sato,† Ryuji Hanaishi,† Sumiko Fukuda-Ishisaka,† Masaji Ishiguro,⊥ Toshio Otani,|| and John E. Leet#

Department of Chemistry, Graduate School of Science Tohoku University, and CREST, Japan Science and Technology Corporation (JST), Sendai 980-8578, Japan Institute for Chemical Reactions, Tohoku University Sendai 980-8577, Japan, Center for Tsukuba Advanced Research Alliance, University of Tsukuba Tsukuba 305-8577, Japan, Suntory Institute for Bioorganic Research, Osaka 618-0024, Japan, Tokushima Research Center, Taiho Pharmaceutical Co. Ltd., Tokushima 771-0132, Japan, and Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492

Received September 8, 1999
Revised Manuscript Received November 18, 1999

A number of extremely potent enediyne antitumor antibiotics have been isolated from culture filtrates of *Streptomyces* species.[1] Although these antibiotics are believed to generate *p*-benzyne-type biradicals through cycloaromatization under certain conditions, no direct EPR measurements of carbon radical species generated from enediyne antibiotics have been reported.[2] A chromoprotein antibiotic, C-1027, which is a 1:1 complex composed of a carrier apoprotein and a DNA-cleaving enediyne chromophore (**1**),[3] is found to show an EPR spectrum.[4] The naked chromophore **1** is highly labile and undergoes spontaneous cycloaromatization at ambient temperature.[3b−d,5] We have postulated that **1** is protected by the apoprotein through kinetic stabilization and is in equilibrium with the *p*-benzyne form (**2**), although the equilibrium concentration of **2** is extremely small (Figure 1).[4] Here, we characterize the radical species in C-1027 antibiotic by EPR spectroscopy, which provides strong evidence for equilibration between **1** and **2**.

Powdered C-1027 antibiotic always exhibits a steady EPR spectrum (X-band, 9.4 GHz) at room temperature (Figure 2a). As long as the antibiotic is sealed in a vial and refrigerated, the broad powder-pattern EPR spectrum can be observed for at least

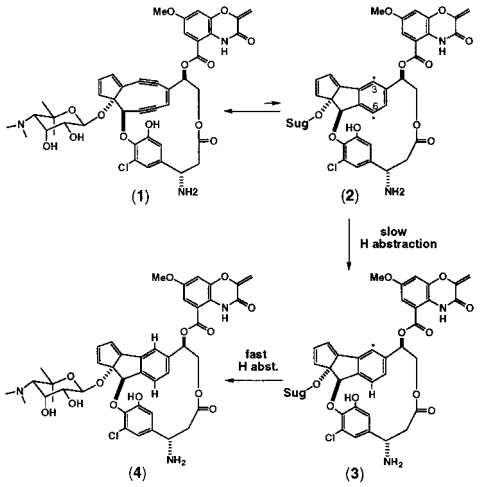

Figure 1.

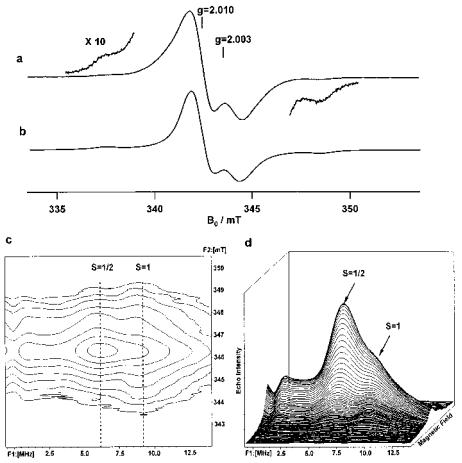

Figure 2. Continuous wave (a, X-band) and pulsed two-dimensional nutation EPR spectra (c, contour plot; d, staked plot) of C-1027 antibiotic powder observed at room temperature. Simulation spectra for the radicals were calculated assuming a homogeneous line width of 1.5 mT with *g* values of 2.010 and 2.003, respectively. The zero-field splitting parameters for the radical pair in the text were obtained by computer simulation. To reproduce the observed spectrum, three components were added in the appropriate ratio.

4 years. The chromophore **1** must be responsible for these signals, because the apoprotein itself does not yield an EPR spectrum. At least two signals with different *g* values, approximately 2.01 and 2.003, appeared to overlap. Neither signal shows large hyperfine couplings. The *g* values, 2.01 and 2.003, indicate that these components should be assigned as a peroxy radical and a carbon-centered radical, respectively.[6] The latter signal became stronger when the antibiotic was kept in a vacuum.

We searched for triplet species related to the *p*-benzyne-type biradical **2** in the EPR spectra, since there remains a possibility that **2** in equilibrium is detectable due to a small singlet−triplet energy gap.[7] Careful analysis of the X-band EPR spectrum indicated a very weak pair of signals at 3373.8 and 3484.3 G (Figure 2a), which could be the outermost signals of randomly oriented triplet species. If this is the case, the zero-field splitting parameter, *D*, is easily read as $2|D| = 11.05$ mT, and the inner signals may overlap with the signals of peroxy and carbon-centered radicals. This EPR spectrum was successfully simulated

* Corresponding author: (e-mail) hirama@ykbsc.chem.tohoku.ac.jp; (tel) 81-22-217-6563; (fax) 81-22-217-6566.
† Department of Chemistry, Tohoku University.
‡ Institute for Chemical Reactions, Tohoku University.
§ University of Tsukuba.
⊥ Suntory Institute for Bioorganic Research.
|| Taiho Pharmaceutical Co. Ltd.
Bristol-Myers Squibb Pharmaceutical Research Institute.

(1) For reviews, see: Nicolaou, K. C.; Dai, W.-M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1387. Grissom, J. W.; Gunawardena, G. U.; Klingberg, D.; Huang, D. *Tetrahedron* **1996**, *52*, 6453.

(2) Some EPR studies on neocarzinostatin were reported, see: Edo, K.; Iseki, S.; Ishida, N.; Horie, T.; Kusano, G.; Nozoe, S. *J. Antibiot.* **1980**, 1586. Sheridan, R. P.; Gupta, R. K. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 213. Edo, K.; Koide, Y. In *Neocarzinostatin*; Maeda, H., Edo, K., Ishida, N., Eds.; Springer: Tokyo, 1997.

(3) (a) Zhen, Y.; Ming, X.; Yu, B.; Otani, T.; Saito, H.; Yamada, Y. *J. Antibiot.* **1989**, *42*, 1294. (b) Minami, Y.; Yoshida, K.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2633. (c) Yoshida, K.; Minami, Y.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2637. (d) Yoshida, K.; Minami, Y.; Otani, T.; Tada, Y.; Hirama, M. *Tetrahedron Lett.* **1994**, *35*, 5253. (e) Okuno Y.; Otsuka, M.; Sugiura, Y. *J. Med. Chem.* **1994**, *37*, 2266. (f) Cobuzzi, R. J.; Kotsopoulos, S. K.; Otani, T.; Beerman, T. A. *Biochemistry* **1995**, *34*, 583. (g) Iida, K.; Fukuda, S.; Tanaka, T.; Hirama, M.; Imajo, S.; Ishiguro, M.; Yoshida, K.; Otani, T. *Tetrahedron Lett.* **1996**, *37*, 4997.

(4) (a) Iida, K., Hirama, M. *J. Am. Chem. Soc.* **1995**, *117*, 8875. (b) Hirama, M. *Pure Appl. Chem.* **1997**, *69*, 525−530.

(5) Mita, T.; Kawata, S.; Hirama, M. *Chem. Lett.* **1998**, 959.

(6) McCain, D.; Plke, W. E. *J. Magn. Reson.* **1975**, *20*, 52.

10.1021/ja993256h CCC: $19.00
Published on Web 01/19/2000

by superimposing the two signals centered at $g = 2.010$ and 2.003 and the triplet signal with $|D/hc| = 0.0052$ cm^{-1} and $|E/hc| = 0.0017$ cm^{-1} (Figure 2b). A forbidden $\Delta m_S = \pm 2$ transition spectrum in the $g = 4$ region, which is often an indicator of a triplet state, was not observed, because the $\Delta m_S = \pm 2$ transition is known to be extremely weak or completely absent in systems with very small D values.[8] However, the existence of a triplet component has been unambiguously demonstrated by the two-dimensional electron spin transient nutation pulsed EPR measurement.[9] The contour plot (Figure 2c) and the staked plot (Figure 2d) clearly revealed the $S = 1$ species ($\omega_n = 8.9$ MHz) in addition to $S = {}^1/_2$ ($\omega_n = 6.3$ MHz). Thus, at least three paramagnetic species are contained within the C-1027 antibiotic.

The D value corresponding to spin−spin dipolar interaction is very small in the present system. The average distance (r) between the two unpaired electrons of the observed triplet species was calculated to be 8 Å by point dipole approximation [$D = -({}^3/_2)g^2\beta^2 r^{-3}$]. The distance between the unpaired electrons in *p*-benzyne σ-biradical is estimated to be less than 5 Å, because the distance between the radical carbon sites is ca. 2.7 Å.[7i] Therefore, we conclude that the triplet species is not the biradical **2** but instead is a possible radical pair derived from **2**.

Our NMR structural study[10] on the complex between the C-1027 apoprotein and the stable aromatized chromophore (**4**)[3b] suggests that the α-proton of Gly96 residue can be abstracted by the nearby C6 radical of **2** in equilibrium with **1**. Thus, we propose a mechanism of radical generation in C-1027 antibiotic (Figure 3). The possible slow hydrogen atom abstraction from the apoprotein by **2** within the binding pocket and the subsequent addition of O_2 to the carbon-centered peptide radical (**5**) would form a peroxy radical (**6**).[11] The triplet species detected by EPR is most likely a transient radical pair between a glycinyl radical (**5**) and an aryl radical (**3**) immediately after hydrogen abstraction. The NMR-derived binding structure[10] indicates that the distance between C3 of **4** and the Cα atom of Gly96 is 7.3 ± 0.4 Å, which agrees with the distance between members of the radical pair calculated from EPR data (vide supra). The peroxy radical detected by EPR is very likely an alkyl-O_2 adduct (**6**), based on the lower reactivity between phenyl radical[12] and O_2. The carbon-centered radical lacking large hyperfine coupling would be the aryl radical **3** with no strongly interacting *o*-hydrogen.[13] Although aryl radicals are known to abstract hydrogen at the rate of diffusion control in solution[14] and cannot be detected by EPR at ambient temperature,[13] **3** is likely to have a sufficient lifetime to be detected within the binding pocket before hydrogen abstraction. However, **3** should possess some freedom of motion to break the radical pair and further, at a certain period due to the inherent high reactivity,[14] abstract hydrogen from the apoprotein to form the stable product **4**. Trapping the alkyl radical **5** by O_2, on the other hand, is likely to occur more rapidly[15] during the period of hydrogen abstraction by **3**.

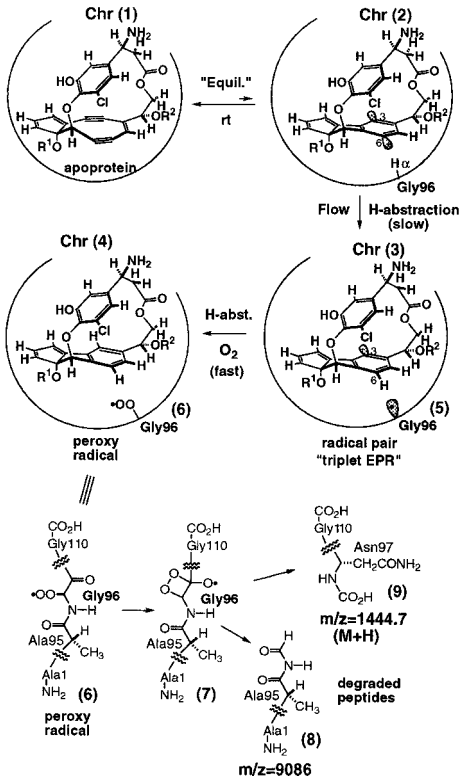

Figure 3. Proposed mechanism for generating paramagnetic species in C-1027 antibiotic.

Evidence for degradation of the apoprotein through **6** was obtained by MALDI-TOF-MS. An antibiotic aged for 4 years showed strong new peaks at 9086 and 1444.7 Da, which correspond to apoprotein fragments **8** and **9**, respectively, in addition to the original peak at 10 498 Da corresponding to the intact apoprotein. Formation of dioxetane (**7**) from **6** and subsequent peptide cleavage would be a rational explanation for the generation of these two fragments. MALDI-TOF-MS also showed appreciable peaks corresponding to oxygenated aromatized chromophores at 862.2 and 878.2 Da, besides the significant peak at 846.2 Da [M+1] corresponding to **4**, which suggests that **2** and/or **3** were trapped by O_2 to some extent.

The apparent rate of bimolecular hydrogen abstraction by the C6-σ radical of **2** is very slow due to the extremely low equilibrium concentration and the inherent slow rate[4,7d−f] in addition to the near orthogonal C6−Hα−Cα orientation,[7e] as suggested in the binding structure of **4** (∼91°).[10] Therefore, the C-1027 antibiotic can exhibit a steady EPR spectrum because of a constant flow of radicals generated by dynamic processes (Figure 3). A similar EPR spectrum was also observed for kedarcidin antibiotic powder (Supporting Information).[16]

The present study shows strong evidence for equilibrium between **1** and **2** in the apoprotein and a chemical mechanism of self-inactivation of the C-1027 antibiotic. These observations may also strengthen our understanding of the molecular basis for mode of action of antitumor chromoprotein antibiotics in cleaving DNA and aid in the design of new anti-cancer drugs with specific delivery systems.

Supporting Information Available: EPR and MALDI-TOF-MS of C-1027, and EPR of kedarcidin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA993256H

(7) (a) Schreiner, P. R. *J. Am. Chem. Soc.* **1998**, *120*, 4184. (b) Bergman, R. G. *Acc. Chem. Res.* **1973**, *6*, 25. (c) Chapman, O. L.; Chang, C.-C.; Kolc, J. *J. Am. Chem. Soc.* **1976**, *98*, 5703. (d) Schottelius, M. J.; Chen, P. *J. Am. Chem. Soc.* **1996**, *118*, 4896. (e) Logan C. F.; Chen, P. *J. Am. Chem. Soc.* **1996**, *118*, 2113. (f) Chen, P. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1478. (g) Marquardt, R.; Balster, A.; Sander, W.; Kraka, E.; Cremer, D.; Radziszewski, J. G. *Angew. Chem., Int. Ed.* **1998**, *37*, 955. (h) Wenthold, P. G.; Squires, R. R.; Lineberger, W. C. *J. Am. Chem. Soc.* **1998**, *120*, 5279. (i) Cramer, C. J. *J. Am. Chem. Soc.* **1998**, *120*, 6261. (j) Wenk, H. H.; Sander, W. *Eur. J. Org. Chem.* **1992**, *57*, 7.

(8) Dougherty, D. A. In *Kinetics and Spectroscopy of Carbenes and Biradicals*; Platz, M. S., Ed.; Plenum: New York, 1990; Chapter 5.

(9) Isoya, J.; Kanda, H.; Norris, J. R.; Tang, J.; Bowman, M. K. *Phys. Rev.* **1990**, *B41*, 3905.

(10) Details will be reported separately.

(11) Roberfroid, M.; Calderon, P. B. *Free Radicals and Oxidation Phenomena in Biological Systems;* Marcel Dekker: New York, 1995.

(12) Preidel, M.; Zellner R. *Ber. Bunsen-Ges. Phys. Chem.* **1989**, *93*, 1417.

(13) Kasai, P. H.; Clark, P. A.; Whipple, E. B. *J. Am. Chem. Soc.* **1970**, *92*, 2640.

(14) Scaiano, J. C.; Stewart, L. C. *J. Am. Chem. Soc.* **1983**, *105*, 3609.

(15) Fossey, J.; Lefort, D.; Sorba, J. *Free Radicals in Organic Chemistry*; John Wiley & Sons: Chichester, 1995.

(16) 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. Kawata, S.; Ashizawa, S.; Hirama, M. *J. Am. Chem. Soc.* **1997**, *119*, 12012.