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Rational Design of a Supra C-1027: Kinetically Stabilized Analogue of the Antitumor Enediyne Chromoprotein

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The chromoprotein natural product C-1027[1] is composed of an 11-kDa apoprotein[2] and a highly reactive chromophore (**1**, Figure 1)[3,4] and displays potent antitumor activity. C-1027 chromophore **1** is bound noncovalently in a cleft of the apoprotein and is dissociable. When not bound to the apoprotein, **1** is the most reactive compound among enediyne natural products[5] and quickly aromatizes via a Masamune−Bergman rearrangement at room temperature without any external activator (**1** → **3**, $t_{1/2} = 0.8$ h in ethanol). *p*-Benzyne biradical **2**, generated through this rearrangement, exerts its potent biological activity by abstracting hydrogens from the sugar portions of double-stranded DNA, which ultimately leads to oxidative cleavage.[6]

Our previous studies indicated that the hydrogen abstraction by **2** is a rate-limiting step and **2** is in equilibrium with **1**.[7] Consequently, chromophore **1** constantly produces **2**, even in its apoprotein.[8] Based on the three-dimensional structure of the complex formed between **3** and the apoprotein, as determined by solution NMR,[9] low accessibility of **2** to hydrogen sources kinetically prevents facile decomposition of C-1027. In addition, the unstable **1** is likely to be escorted by the apoprotein through the cells until it reaches its target, double-stranded DNA.[10] Thus, the apoprotein functions not only as a simple stabilizer but also as an effective drug delivery system (DDS).

Despite these ideal properties as a DDS for the reactive antitumor agent, the apoprotein is not able to completely inhibit the radical-mediated reaction of **2**, and C-1027 slowly decomposes upon aging. Our mass spectrometry (MS) analyses of the aged C-1027 revealed that Gly96 of the apoprotein appears to be responsible for the self-decomposition pathway of C-1027 (Figure 2).[8,11,12]

These investigations prompted us to prepare a more stable analogue of C-1027 by engineering an alternative apoprotein. The challenge was to make a new vessel that increases the stability of **1**, while retaining the binding affinity. To meet these requirements, we planned to utilize kinetic isotope effects to decelerate the radical reaction.[12,13] A supra C-1027 apoprotein was designed to have deuterium instead of protium at the α-hydrogen position of glycine (Figure 2). Here, we report the preparation of the D-Gly apoprotein and its significant stabilization effects on chromophore **1**.

To express the D-Gly apoprotein, *Escherichia coli* containing a vector with the C-1027 apoprotein coding sequence[14] was cultured in a medium containing glycine-d_5 (98% D). In this way, D-Gly apoprotein containing four extra vector-derived amino acids at the N-terminus was isolated in a pure form (16 mg from a 10 L culture). Isotopic incorporation in glycine was calculated to be 78.4 ± 2.8% from the relative integral volume of the cross-peaks of the glycine α-protons on 2D NMR spectra. Through the biosynthetic pathway, the protons of serine and cysteine were labeled with deuterium to some extent. Importantly, all deuterated amino acids, except Gly96, in the C-1027 apoprotein were distant from the reaction site[9] and were considered to be inconsequential to subsequent stability experiments. We also prepared the nonenriched recombinant H-Gly apoprotein as a control.

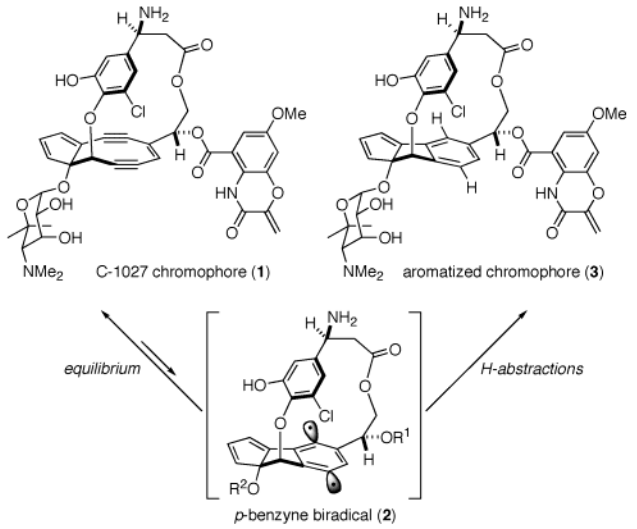

Figure 1. Structures of the C-1027 chromophore (**1**), *p*-benzyne biradical (**2**), and aromatized chromophore (**3**).

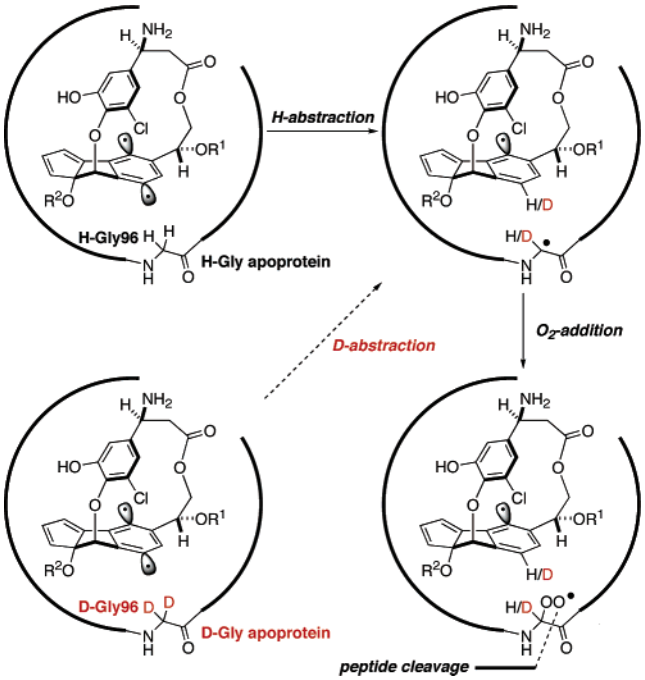

Figure 2. Proposed mechanism of self-degradation of C-1027 and design of the D-Gly C-1027 apoprotein. The apoprotein is represented as a circle.

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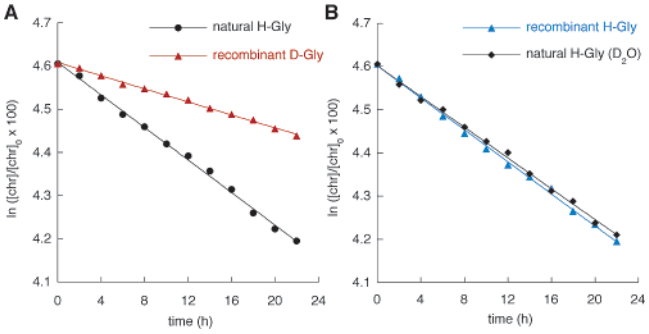

Figure 3. Time course of the C-1027 chromophore content when the H/D-Gly C-1027 holoprotein was incubated at 37 °C in phosphate buffer (pH 6.8). Plots of time against the chromophore content of natural H-Gly (A, black ●), recombinant D-Gly (A, red ▲), recombinant H-Gly (B, blue ▲) holoproteins in H_2O buffer, and natural H-Gly holoprotein in D_2O buffer (B, black ◆).

Having successfully obtained the D-Gly apoprotein, the next task was to form the complex with unstable **1**. After careful experimentation, a reliable method to incorporate **1** into the apoprotein was established. First, the natural C-1027 was injected into an HPLC system [ODS column; eluent, 50% acetonitrile in phosphate buffer], where **1** was extracted from the complex under the column conditions. Eluted **1** was collected in a vial containing a solution of excess D-Gly apoprotein, and immediate complexation occurred in the vial. The obtained solution was then further purified by hydrophobic chromatography to separate the D-Gly holoprotein and the remaining D-Gly apoprotein. Through the same procedure, we prepared the nonenriched holoproteins from both natural and recombinant H-Gly apoproteins.

The chromophore-stabilizing ability of each apoprotein (natural, recombinant H-Gly, and D-Gly apoproteins) was evaluated. The holoproteins were incubated in neutral buffer at 37 °C, and the same quantity of solution was analyzed by HPLC every 2 h. The amount of chromophore decreased in a time-dependent manner, and the chromophore content over time was found to obey first-order kinetics (Figure 3). As expected, natural and recombinant H-Gly apoproteins stabilized the chromophore to the same extent, indicating that the four additional amino acids of the recombinant protein do not affect the functional properties. To exclude the possibility that water acts as a hydrogen-donor, the natural H-Gly apoprotein was incubated in D_2O buffer solution. The slope of the graph is indistinguishable from that with H_2O buffer, thus demonstrating that solvent water had no effect on the experiments.

Most importantly, the D-Gly apoprotein exhibited a better chromophore-stabilizing ability (Figure 3A). Because the deuteration ratio of the D-Gly apoprotein is 78.4 ± 2.8%, the rate constant of the reaction of hypothetical 100% D-Gly holoprotein (k_D) was calculated by fitting the data to the equation given by $\ln([\mathrm{chr}]/[\mathrm{chr}]_0 \times 100) = \ln[21.6\exp(-k_H t) + 78.4\exp(-k_D t)]$, where the chromophore in each protein molecule decays independently with first-order kinetics. In this way, the kinetic isotope effect (k_H/k_D) is determined to be 4.1, and the chromophore in the D-Gly apoprotein is 4-fold longer-lived ($t_{1/2}$ = 151 h) than that in the H-Gly apoproteins ($t_{1/2}$ = 37 h). The observed kinetic isotope effect (4.1) is even larger than those seen in organic solvents (2.8−3.8),[7a] which confirmed the high site-specificity and the kinetic significance of the radical abstraction of **1** from D-Gly96.

In summary, a supra C-1027 was created by reducing the self-decomposition pathway by means of the kinetic isotope effect. This work successfully demonstrated the novel modification of a natural product to acquire superior properties by integrating the physicochemical properties of the small molecule and the 3D-structure of the protein, and the present findings are likely to be applicable to other biologically important natural products and proteins.

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Supporting Information Available: Materials and methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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