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Award Accounts

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Exploring the Chemistry and Biology of Antitumor Enediyne Chromoprotein C-1027

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C-1027 is an extremely potent antitumor agent, and belongs to a family of chromoprotein antitumor antibiotics, which contain a carrier apoprotein and highly unstable enediyne chromophore. The enediyne spontaneously aromatizes to generate *p*-benzyne biradical, and subsequently abstracts hydrogens from the DNA sugar backbone, resulting in the sequence-selective double-strand cleavage. On the other hand, the C-1027 apoprotein functions as a carrier, and is likely to escort the reactive chromophore through the cells until reaching the target DNA. The excitement surrounding this molecule lies in the complex molecular architecture of the chromophore, the important biological activity, and the fascinating mode of action. In this account, our studies in the chemistry and biology of C-1027 are presented in three parts: (1) synthetic study of the C-1027 chromophore, (2) spin-trapping study of the C-1027-induced DNA cleavage, and (3) rational design of a kinetically stabilized analog of C-1027.

In 1989, Otani and co-workers isolated the macromolecular antitumor antibiotic C-1027 from the culture broth of *Streptomyces globisporus C-1027*.[1] This compound shows extreme potency against cultured cancer cells as well as marked growth inhibition of transplantable tumors in mice and human cancers inoculated into nude mice. Like other macromolecular antibiotics, such as neocarzinostatin,[2] kedarcidin,[3] and maduropeptin,[4] C-1027 is composed of an apoprotein complexed with a highly reactive chromophore (**1**, Fig. 1).[5,6] The apoprotein (**6**, Fig. 2) is a single polypeptide chain of 110 amino acid residues.[7] C-1027 chromophore **1** is bound noncovalently in a cleft of the apoprotein and is a member of the enediyne family, which is characterized by two acetylenic groups conjugated to a double bond within a nine- or ten-membered ring.[8] When not bound to the apoprotein, **1** is the most reactive of the enediyne natural products, and the enediyne moiety of **1** quickly aromatizes via a Masamune–Bergman rearrangement[9] at room temperature without an external activator (**1** → **2** → **3**/**4** → **5**, $t_{1/2} = 0.8$ h in ethanol, Fig. 1). Upon sequence-selective binding of **1** to the minor groove of double-strand DNA, *p*-benzyne (*p*-didehydrobenzene) biradical **2**,[10] generated from **1**, exerts its potent biological activity by abstracting hydrogens from

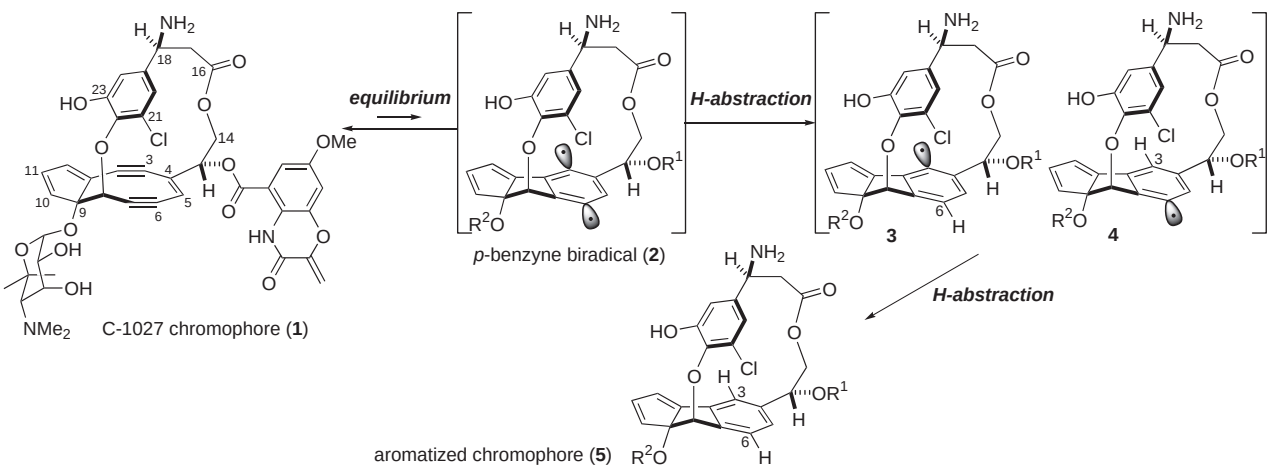

Fig. 1. Structure of the C-1027 chromophore and its Masamune–Bergman rearrangement.

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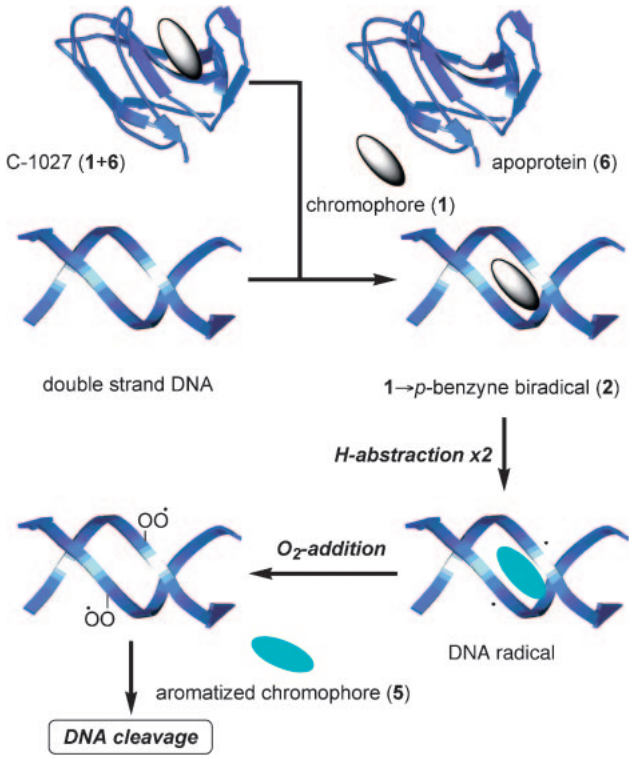

Fig. 2. Overview of the C-1027-mediated DNA cleavage reaction.

the sugar portions of the DNA, which ultimately leads to oxidative cleavage (Fig. 2).[11,12] In addition, the unstable **1** is likely to be escorted by the apoprotein through the cells before reaching its target DNA.[13] Therefore, while the chromophore is responsible for the sequence-selective DNA recognition and damage, the apoprotein functions as an effective drug-delivery system (DDS).

The excitement surrounding this molecule lies in the complex molecular architecture of **1**, the important biological activity, and the fascinating mode of action. In this account, our studies into the chemistry and biology of C-1027 are presented. These can be divided into three categories: (1) synthetic study of the C-1027 chromophore, (2) spin-trapping study of the C-1027-induced DNA cleavage, and (3) rational design of a kinetically stabilized analog of C-1027.

1. Studies Directed toward Total Synthesis of the C-1027 Chromophore

1.1 Synthetic Strategy. The chemical instability and the complex architecture distinguish chromophore **1** as a challenging target for total synthesis.[14–16] The structure of **1** is highly unusual, characterized by a chlorocatechol-containing ansa-bridge, a strained bicyclo[7.3.0]dodecatrienediyne, an appended benzoxazine[17] and an amino sugar.[18] The challenge is further heightened by the presence of non-biaryl atropisomerism arising from hindered rotation of the chlorocatechol ring in the ansa-bridge.[19]

Our synthetic strategy is outlined in Fig. 3. The total synthesis of chromophore **1** would be attained from the framework **7** by attaching the amino sugar[20] and the benzoxazine, and subsequently introducing two olefins (C4–C5 and C10–C11). In

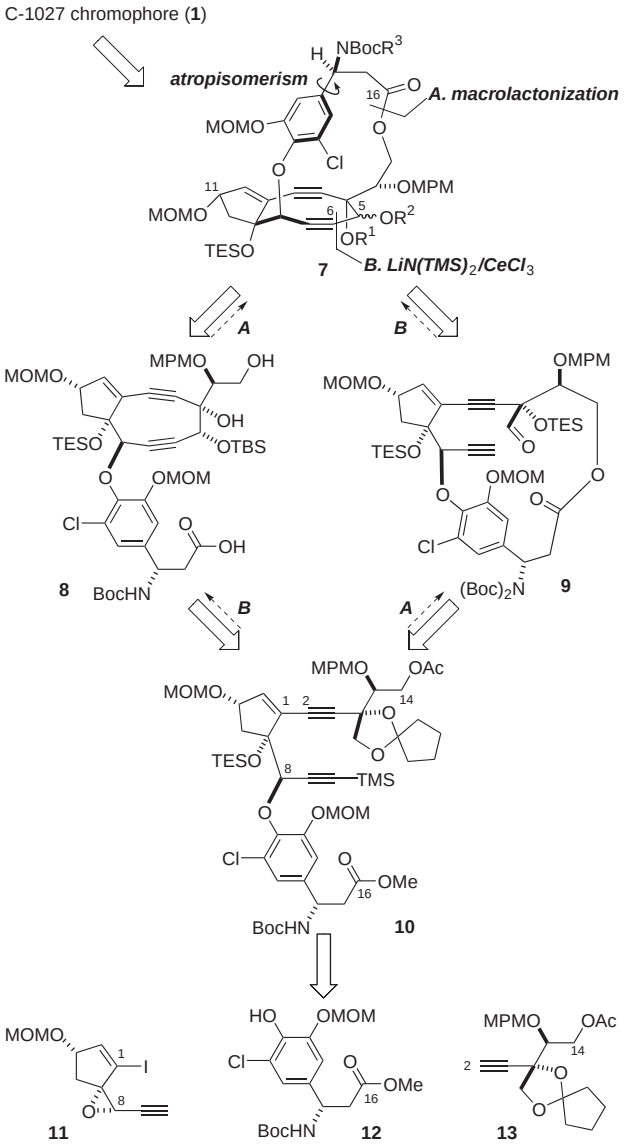

Fig. 3. Retrosynthesis of the C-1027 chromophore.

turn, we planned to construct **7** through atropselective macrolactonization at C16 (step **A**) and formation of the nine-membered ring by linking C5 and C6 (step **B**) using a $LiN(TMS)_2/CeCl_3$-promoted cyclization reaction.[21,22] Two key intermediates, **8** and **9**, were designed depending on the order of steps **A** and **B**, and both were traced back to compound **10**. Finally, the highly unsaturated substrate **10** was to be synthesized through the coupling of three fragments in a convergent manner (**11**, **12**, and **13**).

1.2 Fragment Assembly. The synthesis of the key intermediate **10** started with the known enone **14**[23] (Fig. 4).[24] Nucleophilic addition of vinylmagnesium bromide to **14** occurred from the opposite side of the bulky TBS ether to afford tertiary alcohol **15** as the sole isomer. Selective ozonolysis of the terminal olefin of **15** and subsequent reductive workup generated aldehyde **16**. Treatment of **16** with ethynylmagnesium bromide exclusively produced diol **18** bearing a β-hydroxy group at C8. The stereochemical outcome of the reaction was presumably governed by magnesium chelation and the presence of

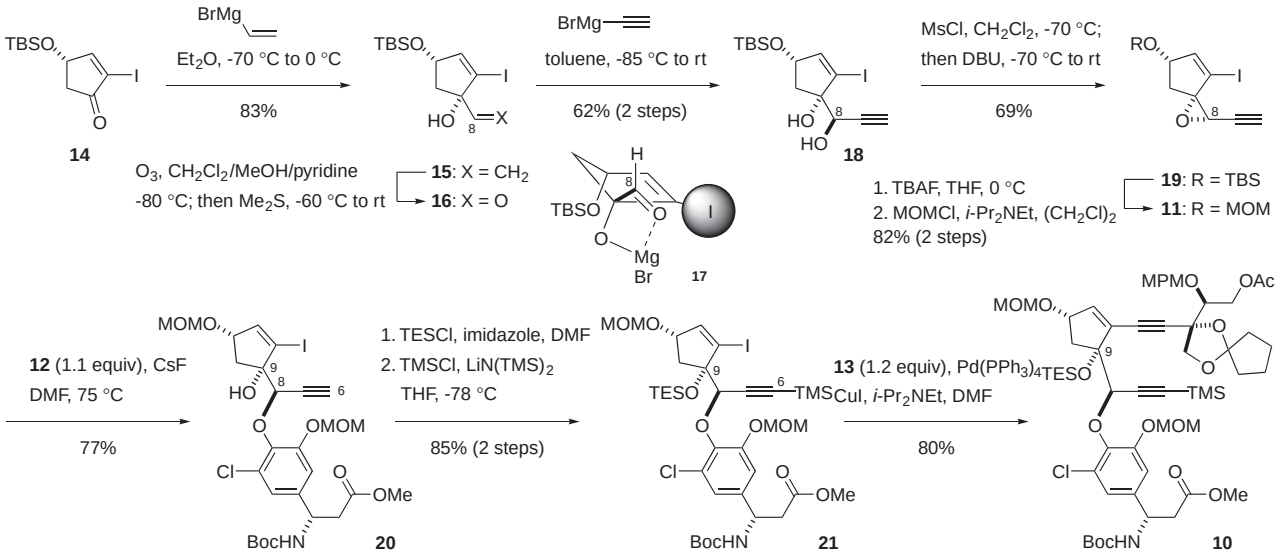

Fig. 4. Coupling of the three fragments.

the bulky iodine, forcing the nucleophile to attack from the less hindered side of the five-membered chelate **17**. The secondary alcohol of **18** thus obtained was converted to epoxide **19** by selective monomesylation and subsequent DBU-treatment. Next, the TBS group of **19** was replaced by the MOM group to give **11** in a two-step sequence.

Aryl ether formation between **11** and β-tyrosine moiety **12** was realized by the action of CsF in DMF,[25] leading to **20**. The tertiary alcohol of **20** was converted to TES ether, and the TMS group was introduced to the terminal acetylene to afford **21**. Then, Sonogashira coupling[26] of **21** with acetylene moiety **13**[27] in the presence of catalytic $Pd(PPh_3)_4$ and CuI delivered the key intermediate **10**.

1.3 Synthesis of the Fully Functionalized Nine-Membered Diyne Core. First, construction of the nine-membered ring of **8** (Fig. 3, step **B**) was performed prior to the macrocyclization (step **A**).[28] To assess the feasibility of the diyne formation in the complex setting, we screened the variously functionalized precursor aldehydes, two of which are shown in Fig. 5. The difference in the cyclization yields of monocarbamate **22** and biscarbamate **23** clearly demonstrated that masking the acidic C18–NH was crucial for this base-promoted condensation. Treatment of **23** with a 1:1 mixture of $LiN(TMS)_2$ and $CeCl_3$ in THF gave rise to the nine-membered diyne **25** as the sole isomer in 82% yield. Interestingly, upon formation of the nine-membered ring, the TES group at the C4–OH of **23** was intramolecularly transposed to the C5–hydroxy group of **25**.

As shown in Fig. 6, the same substrate design for the cyclization was successfully applied to the differentially protected biscarbamate **26**. When **26** was subjected to the $LiN(TMS)_2$/$CeCl_3$-mediated cyclization conditions, the bicyclo[7.3.0]dodecenediyne core **27** was isolated as a single isomer in 78% yield. Subsequent DIBAL-H reduction of **27** simultaneously removed both Piv and Boc groups to generate mono-Boc alcohol **28**. The primary alcohol was then oxidized to the corresponding carboxylic acid **29** via a two-step protocol. Finally, the primary TBS group at C14 of **29** was selectively removed using PPTS in MeOH, leading to the fully functionalized seco-acid **8**

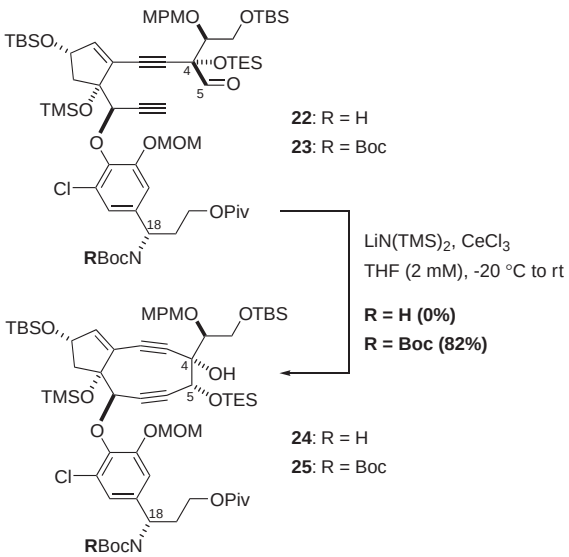

Fig. 5. Protective group effect in the nine-membered ring formation.

(45% yield). Diyne **8** was chemically unstable upon heating ($t_{1/2} = 3$ h in C_6D_6 at 50 °C).[29]

Unfortunately, macrolactonization of **8** under Yamaguchi conditions[30] at room temperature afforded **30** only in 5% yield. The instability of **30** impeded greater forcing and/or higher temperature conditions; thus further optimization of the reaction was abandoned.

Despite the unsuccessful macrocyclization, the chemistry developed here taught us some valuable lessons. In particular, the exploitation of the bis-Boc protection of C18–NH, which enabled the effective nine-membered ring formation of highly functionalized substrates (**23** and **26**), is the key development in this synthesis.

1.4 Synthesis of the Chromophore Framework through Atropselective Macrolactonization. Because of the chemical instability of the nine-membered diyne **8** and its low-yield-

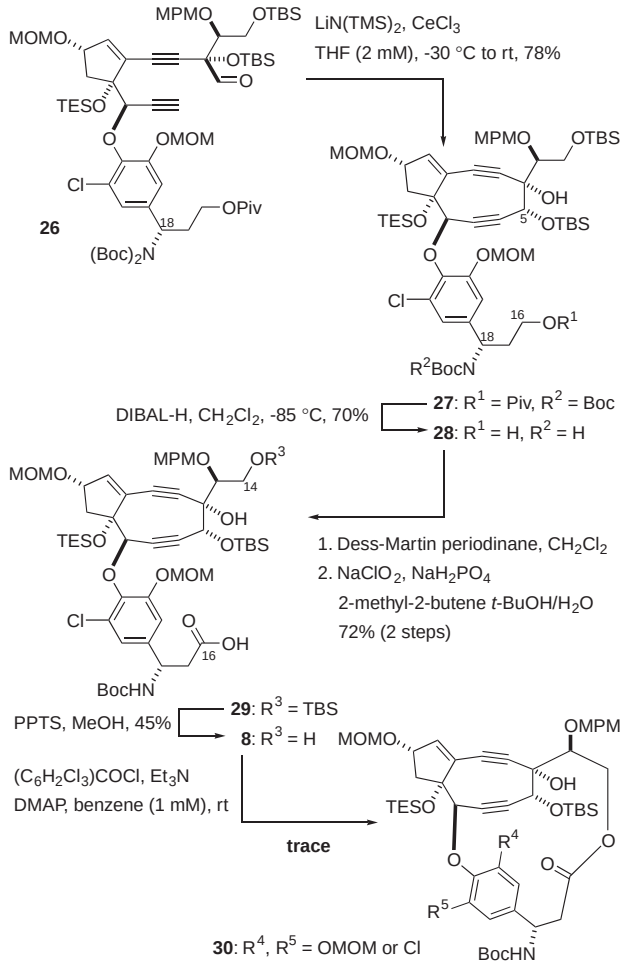

Fig. 6. Formation of the nine-membered diyne and attempted macrocyclization.

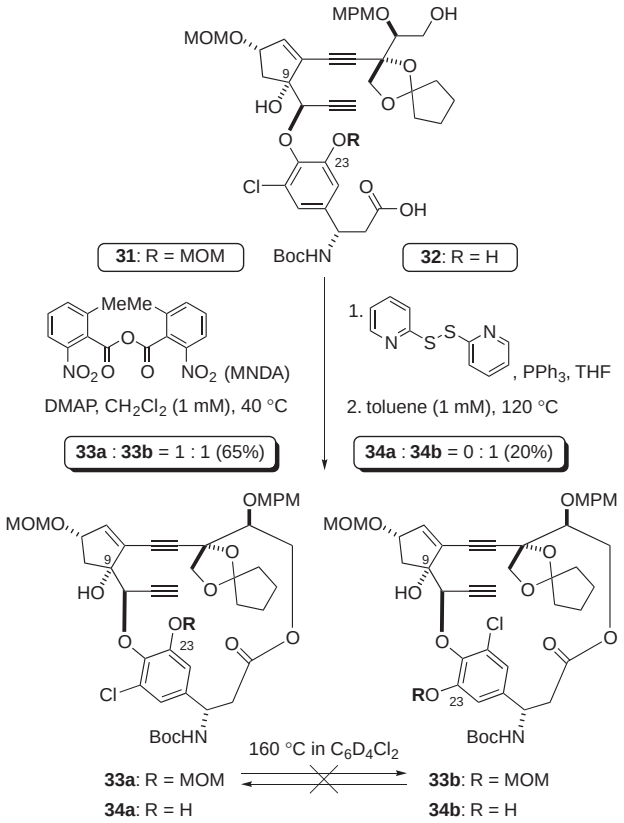

Fig. 7. Attempted atropselective macrolactonization.

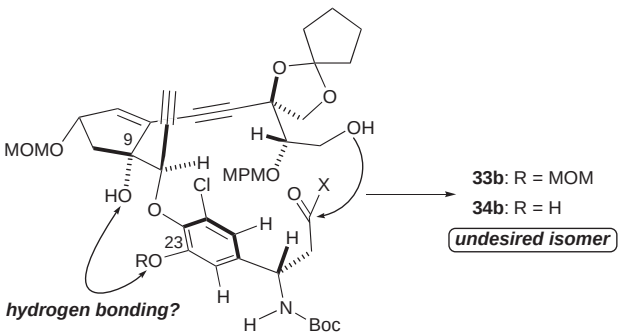

Fig. 8. Potential hydrogen bonding in the transition state of the macrolactonization reaction.

ing macrolactonization, we modified our synthetic route with a change in the order of steps **A** and **B** (from **B** → **A** to **A** → **B** starting from **10**, Fig. 3).[24,27]

To examine the effect of the substitution pattern on yield and atropselectivity, several seco-acids bearing different protective groups were subjected to macrolactonization (Fig. 7). Cyclization of **31** (C9–OH, C23–OMOM) was achieved by the powerful method recently developed by Shiina:[31] treatment of **31** with 2-methyl-6-nitrobenzoic anhydride (MNBA) and DMAP at 40 °C produced macrolactones in high yield, but with a non-atropselective outcome (**33a**:**33b** = 1:1). On the other hand, Corey–Nicolaou macrolactonization[32] of **32**, in which the MOM group at the C23–phenol of **31** was replaced with hydrogen, generated only the undesired atropisomer **34b** in modest yield.

Isomerization to enrich the desired atropisomer **33a** was unsuccessful. Upon separately heating atropisomers **33a** and **33b** to 160 °C, no isomerization occurred, suggesting that these macrocycles are highly rigid. Thus, selective formation of the desired atropisomer would be possible only by controlling the transition state of the macrolactonization using an appropriately functionalized substrate.

From the NOESY data and the molecular modeling, the undesired macrolactones **33b** and **34b** were found to have similar conformations, in which the substituents at C9 and C23 are in spatial proximity, suggesting hydrogen bonding. This hydrogen bonding could also fix the conformation of the transition state as illustrated in Fig. 8, which explains the sole formation of **34b** from **32**. In the cyclization of **31**, the larger unfavorable steric interaction between the C23–OMOM group and the C9–tertiary alcohol could counteract the attractive hydrogen bond, which could be the reason for the non-stereoselective outcome observed. Hence, the atropselectivity of the macrolactonization appears to be controlled by the balance between steric interaction and hydrogen bonding of the C9– and C23–substituents.

From these considerations, placement of a bulky protective group on the C9–tertiary alcohol could enforce an atropselective macrolactonization leading to the desired isomer, because both increased steric interaction with the C23–OMOM group

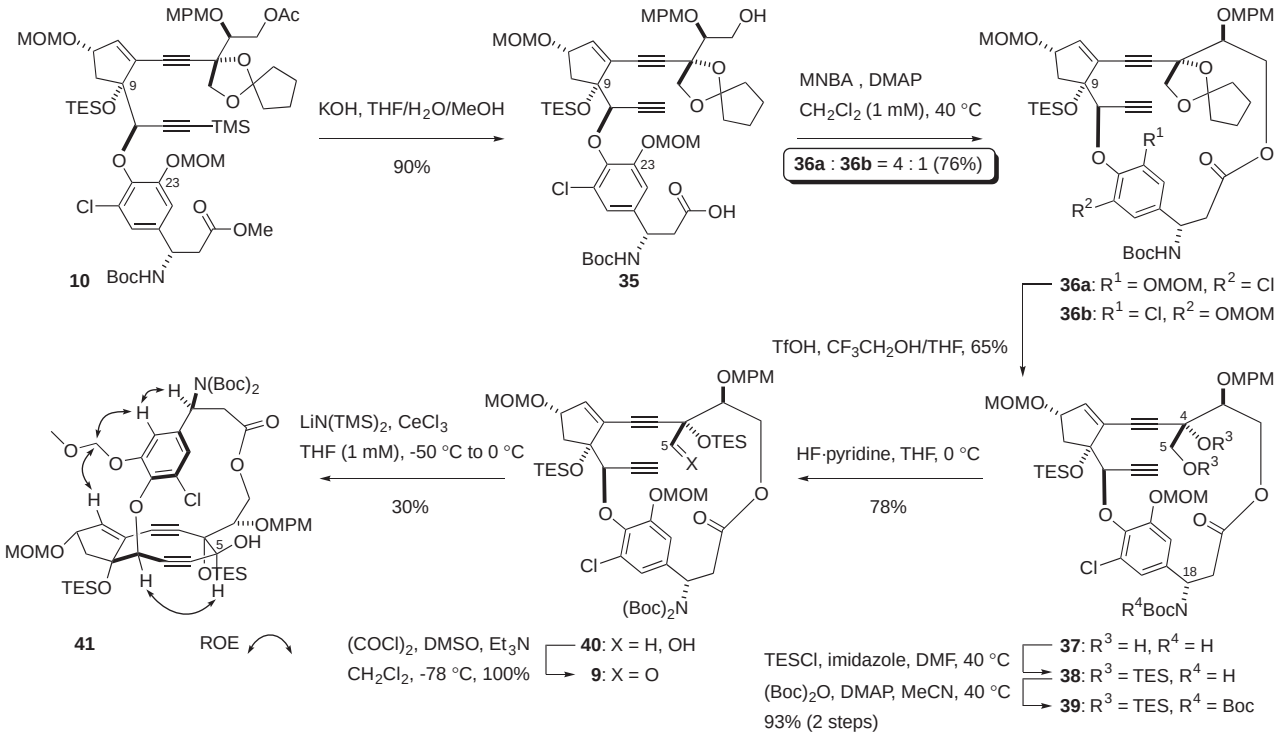

Fig. 9. Synthesis of the C-1027 chromophore framework through atropselective macrolactonization.

and elimination of the hydrogen bond would impose an energetic penalty on the transition state of the unwanted atropisomer. In support of this hypothesis (Fig. 9), macrolactonization of seco-acid **35**, having C23–OMOM and C9–OTES groups, indeed delivered the desired isomer **36a** in 61% yield with 4:1 atropselectivity under Shiina conditions. Remarkably, the yield of the desired atropisomer (**33a** vs **36a**) was doubled by strategic protection of the C9–alcohol.

With the desired ansamacrolide **36a** in hand, our next focus was to construct the nine-membered diyne ring. Before doing so, the reaction conditions of protective and functional group manipulations were carefully tuned. First, the cyclopentylidene ketal of **36a** was selectively removed using TfOH in CF_3CH_2OH/THF[33] without affecting other acid labile protective groups (MOM, TES, MPM, and Boc) to afford 1,2-diol **37**. After conversion of **37** to tris-TES ether **38**, another Boc group was introduced using $(Boc)_2O$ and DMAP to mask the acidic C18–NH proton of **38**, leading to biscarbamate **39**. Then, the primary alcohol at C5 was selectively liberated from **39** using HF·pyridine to produce **40**, which was oxidized to aldehyde **9** with $(COCl)_2$ and DMSO.

Cyclization of **9** was promoted by $LiN(TMS)_2/CeCl_3$ in THF, giving rise to the strained nine-membered diyne **41** as the sole isomer in 30% yield. Thus, this $LiN(TMS)_2/CeCl_3$ reagent combination proved to be applicable to this highly complex and conformationally rigid system (**9**). The correct atropisomerism of **41** was again confirmed by ROESY experiments at this stage, indicating the high energy barrier to rotation of the chlorocatechol in all of the synthetic intermediates. Diyne **41** slowly decomposed at room temperature as in the case of **8**.

The key features in the first successful synthesis of the C-1027 chromophore framework were (i) strategic utilization of protective groups of the 1,5-diol to attain atropselective macrocyclization, and (ii) $LiN(TMS)_2/CeCl_3$-promoted acetylide–aldehyde condensation to build the nine-membered diyne ring in a highly unsaturated and heavily substituted macrocycle.

1.5 Synthesis of the Bicyclo[7.3.0]dodecatrienediyne Core. The most formidable problem for the total synthesis of **1** from the C-1027 chromophore framework **41** is apparently the formation of the reactive bicyclo[7.3.0]dodecatrienediyne (Fig. 10).[34] We therefore undertook, as a model study, the synthesis of trienediyne **44** from diyne **42**, which possesses the same structure as **41** except for the β-tyrosine moiety.

Due to the general instability of strained nine-membered diynes, a synthetic scheme en route to **44** should constitute a series of mild reaction conditions. Moreover, introduction of the C4–C5 olefin should be at the last stage in order to avoid the consumption of synthetic intermediates via Bergman rearrangement. Hence, the plan was to construct the cyclopentadiene structure prior to the enediyne through dehydration of the C11–alcohol (**42** → **43**). The C4–C5 olefin was then to be directly installed via deoxygenation of epoxide **43**,[35] which in turn would be formed from the protected C4,C5-*trans*-diol **42**.

As shown in Fig. 11, synthesis of diyne **42** was realized by $LiN(TMS)_2/CeCl_3$-promoted cyclization of aldehyde **47**, which was synthesized by assembly of the three fragments (**11**, **45**, and **46**). After derivatization of **42** to its mesylate **48**, the MOM group of **48** was selectively removed using Me_2BBr[36] in the presence of other potentially reactive protective groups (MPM and TES), leading to alcohol **49**. TBAF treatment of bis-TES ether **49** under carefully controlled temperature conditions led to chemoselective deprotection of the C4–OTES and concomitant epoxide formation to afford **50**.

Dehydration of allylic alcohol **50** turned out to be no easy task. Introduction of the leaving group to **50** did not induce *anti*-elimination, but typically resulted in decomposition. We

therefore decided to utilize an arylselenoxide derivative that could produce the olefin through *syn*-elimination in neutral conditions. Treatment of α-alcohol **50** with *o*-nitrophenyl selenocyanate and tributylphosphine in THF[37] produced β-selenide **51** through S_N2-displacement. The resultant **51** was oxidized with hydrogen peroxide at room temperature to afford the desired cyclopentadiene **43** in high yield.[38]

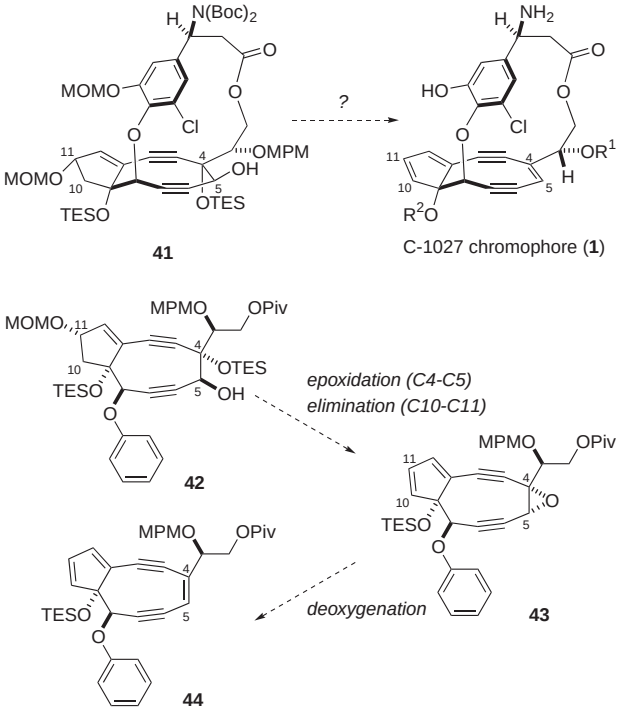

Fig. 10. Synthetic plan of the bicyclo[7.3.0]dodecatrienediyne structure.

The last reaction of this model study was deoxygenation from epoxide **43**. To isolate the target product **44**, deoxygenation of **43** should be faster than the Masamune–Bergman reaction of the resultant enediyne. For this purpose, two powerful but mild metal reductants (SmI_2[39] and WCl_6/n-BuLi[40]), which are known to reduce epoxides to olefins, were applied to **43**. While SmI_2-mediated reduction of **43** proceeded smoothly, the undesired exo-olefin **54** was isolated. In this reaction, elimination of the C13–OMPM in organosamarium intermediate **53** preceded elimination of the samarium alkoxide at C5 that would lead to the desired product **44**.

Gratifyingly, the Sharpless conditions (WCl_6/n-BuLi) were found to generate the targeted bicyclo[7.3.0]dodecatrienediyne **44**. Upon exposure of **43** to low-valent tungsten in deuterated THF, epoxide deoxygenation took place at room temperature, and the exceedingly labile enediyne **44** was isolated in 11% yield. In ethanol, **44** was quickly transformed to aromatized compound **55**. The half-life of **44** in deuterated dichloromethane was 35 min at 25 °C, which is even shorter than that of the natural product **1**.[41]

This study has resulted in the first synthesis of the highly reactive bicyclo[7.3.0]dodecatrienediyne core of the C-1027 chromophore **1**. By virtue of its neutral nature and high chemoselectivity, the synthetic route described here should be applicable for the total synthesis of **1** from its framework **41**.

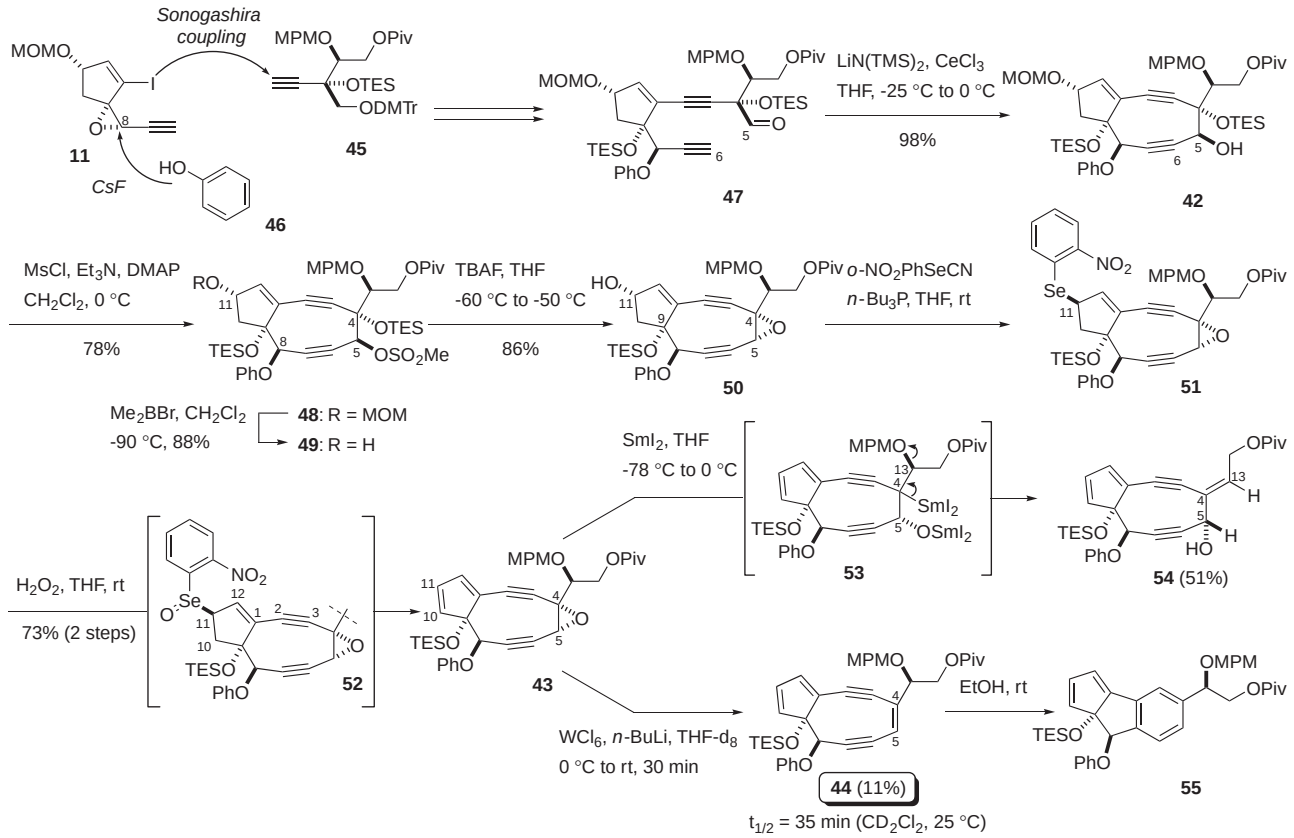

Fig. 11. Synthesis of bicyclo[7.3.0]dodecatrienediyne.

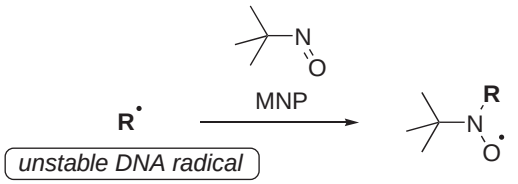

Fig. 12. Spin-trapping reagents used in this study.

2. Spin-Trapping Study of DNA Cleavage Induced by C-1027

It was demonstrated that the C-1027 chromophore **1** delivered the enediyne portion within the minor groove of DNA, and then initiated *p*-benzyne biradical formation (Fig. 2). The highly reactive biradical would then be perfectly positioned to strip hydrogen atoms from the sugar phosphate backbone of adjacent strands of DNA, and would initiate a series of reactions leading to double-strand scission. Goldberg reported that the DNA lesions showed a five-nucleotide sequence specificity and that 5′-GTTAT/5′-ATAAC was the most preferred sequence.[11d,e] Although the outcome of C-1027-mediated DNA damage has been studied in considerable detail, no direct observation of radical intermediates has been reported. As part of our continuing interest in the chemical behavior of C-1027, we conducted an ESR study on the DNA-cleaving reaction.[42,43]

The difficulty associated with the direct ESR measurement arises from the extremely short life-time of the DNA carbon radicals under physiologically relevant conditions. We therefore applied a spin-trapping method using the nitroso reagent MNP (2-methyl-2-nitrosopropane, Fig. 12).[44] Spin-trapping allows the capture of a reactive radical by converting it to a more stable radical adduct detectable by ESR at room temperature. Importantly, hyperfine-coupling (hfc) parameters of the adducts permit identification of the initial radical trapped.

As a DNA substrate, we selected the dodecamer 5′-GCC**GTTA$_1$T**GCCG/5′-CGGC**ATA$_2$A$_3$C**GGC (**56**), because Goldberg showed from extensive gel electrophoresis studies that **1** abstracts hydrogen atoms of the C4′, C1′, and C5′ positions from the A_1, A_2, and A_3 nucleotide sugars of **56**, respectively, through the sequence selective binding indicated in the bold face.[11d,e]

Interestingly, a mixture of C-1027 and MNP in aqueous buffer did not show any signal, indicating that MNP did not trap the radicals **2**–**4** (Fig. 1). No spin-adduct was observed either, when the single-strand DNA (5′-GCCGTTATGCCG) and C-1027 were added to MNP. In sharp contrast to these results, a combination of double-strand DNA **56**, MNP, and C-1027 in the buffer provided the highly anisotropic spectrum shown in Fig. 13 ($g = 2.006$, $A_N = 1.5$ mT). These results indicate that **1** was bound to **56** and consequently abstracted hydrogen from **56** to generate DNA carbon radicals trapped by MNP. An identical ESR spectrum was also observed under anaerobic conditions (data not shown), from which it is speculated that the initially-formed DNA radicals were trapped before reacting with molecular oxygen. Thus, the radical intermediates formed during C-1027-induced DNA cleavage have been observed using spin-trapping methods under physiologically relevant conditions.

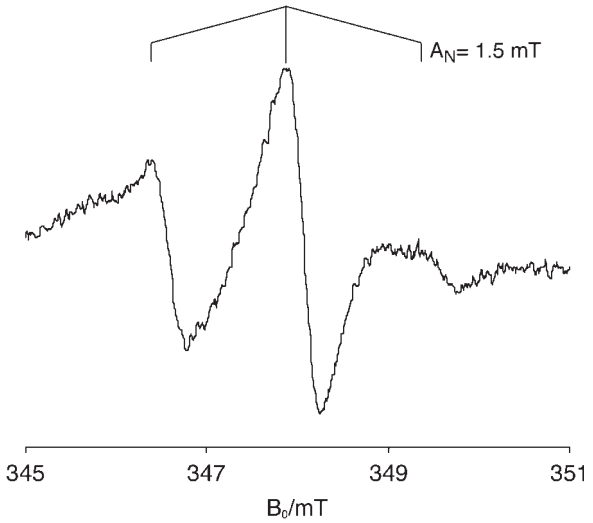

Fig. 13. X-band ESR spectra of MNP spin adducts: (A) C-1027 with double-stranded DNA **56** and MNP in NTE buffer (100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, and pH 7.5) under aerobic conditions.

3. Design and Preparation of a Kinetically Stabilized Analog of C-1027

p-Benzyne formation (**2**, Fig. 1) from the C-1027 chromophore **1** is central to its biological activity. The previous studies of **1** and related model compounds indicated that hydrogen abstraction by the cyclized *p*-benzyne intermediate (**2** → **3**/**4**) is a rate-limiting step and that **2** is in equilibrium with **1**.[41,45] Consequently, the chromophore **1** constantly produces **2**, even when bound with the apoprotein.[46]

Based on the three-dimensional structure of the complex formed between **3** and the apoprotein, as determined by solution NMR,[47] low accessibility of **2** to hydrogen sources kinetically prevents facile decomposition of C-1027. Thus, the apoprotein functions both as a stabilizer and as an effective DDS for **1**.

Despite these remarkable 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 (Fig. 14).[46,48] The previous mass spectrometry (MS) analyses of aged C-1027 showed mass peaks that correspond to apoprotein fragments **57** and **58**. Formation of the Gly96 radical and subsequent oxidative peptide cleavage would be a probable explanation for the generation of these two fragments. Therefore, Gly96 of the apoprotein appears to be responsible for the self-decomposition pathway of C-1027.

These investigations prompted us to prepare a more stable analog of C-1027 by engineering an alternative apoprotein. The challenge was to make a new vessel that increased the stability of **1**, while retaining the binding affinity. To meet these requirements, we planned to utilize kinetic isotope effects in order to decelerate the radical reaction.[49] A supra C-1027 apoprotein was thus designed to have deuterium instead of protium at the α-hydrogen position of glycine (Fig. 15).[50]

Recombinant D-Gly apoprotein was isolated from a culture of *Escherichia coli* containing a vector with the C-1027 apoprotein coding sequence in a medium containing glycine-d_5

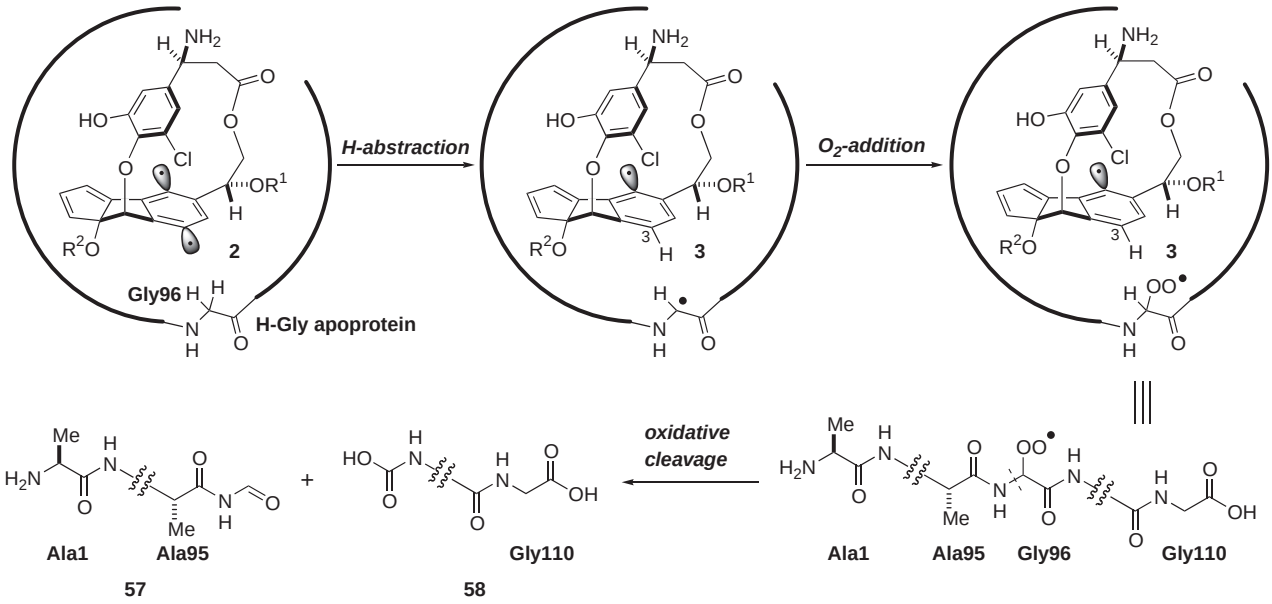

Fig. 14. Proposed mechanism of the self-degradation of C-1027. The apoprotein is represented as a circle.

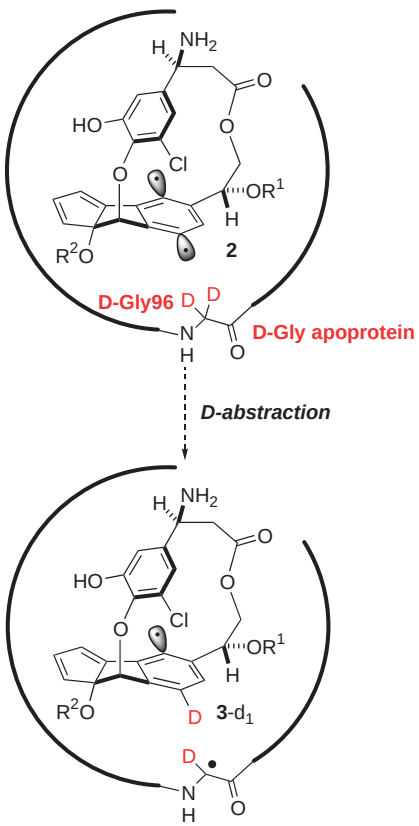

Fig. 15. Design of D-Gly C-1027 apoprotein.

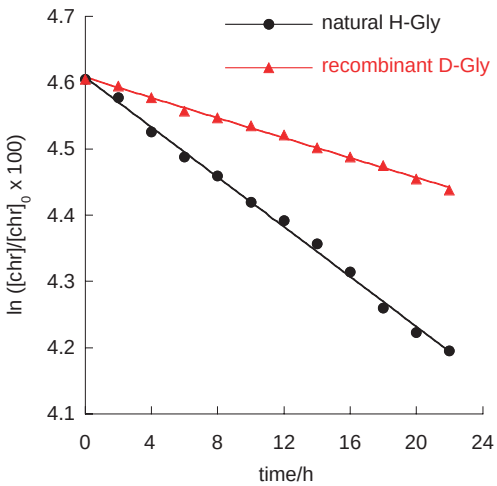

Fig. 16. Time course of C-1027 chromophore content when H/D-Gly C-1027 holoprotein was incubated at 37 °C in phosphate buffer (pH 6.8). Plots of time against chromophore content of natural H-Gly (black circles), and recombinant D-Gly (red triangles) holoproteins.

(98%D).[51] Isotopic incorporation in glycine of the apoprotein thus obtained was calculated to be 78.4 ± 2.8% from 2D NMR spectra. Complexation between the unstable chromophore **1** and the recombinant D-Gly apoprotein was achieved using an HPLC technique, yielding the D-Gly holoprotein.

The chromophore-stabilizing ability of natural and D-Gly apoproteins was then evaluated. The holoproteins were incubated in a neutral buffer at 37 °C, and the same quantity of solution was analyzed by HPLC. The amount of chromophore decreased in a time-dependent manner, and the chromophore content over time was found to obey first-order kinetics (Fig. 16). Most importantly, the D-Gly apoprotein exhibited a better chromophore-stabilizing ability. The kinetic isotope effect (k_H/k_D) was determined to be 4.1, and the chromophore in the D-Gly apoprotein has a lifetime four times longer ($t_{1/2}$ = 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),[41] 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 modifi-

cation of a natural product to acquire superior properties by integrating the physicochemical properties of the small molecule and the 3D-structure of the protein.

4. Conclusion

All of the studies described above have originated from the fascinating chemistry and biology of the cytotoxic natural product C-1027, have been rich in intellectual excitement, and have been valuable learning experiences. Major progress toward the total synthesis of the C-1027 chromophore **1** has already been achieved, which will provide a framework for its end game. The present findings in the spin-trapping study and the preparation of supra C-1027 are likely to be applicable to other radical-mediated processes of biologically important natural products and proteins. It is my hope and dream that the molecules prepared here and in the future will find applications in the new anticancer drug designs.

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