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Design, Synthesis and Sequence Selective DNA Cleavage of Functional Models of Bleomycin—II. 1,2-*trans*-Disubstituted Cyclopropane Units as Novel Linkers[1]

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Abstract—The design and syntheses of functional models for bleomycin in which AMPHIS, a simplified model of the metal-chelating subunit of bleomycin is connected to distamycin analogs with a series of linkers, are described. Kinetic studies and DNA cleavage assay show that 1,2-*trans*-disubstituted cyclopropane units are the best linkers within this series. Study of selective DNA cleavage on high resolution polyacrylamide sequencing gels indicates that the linker modified hybrids generally cleave selectively at the 5' end of poly T sites and at the 3' end of poly A sites. Cleavage activity is enhanced for most of the compounds related to those with shorter linkers, previously reported, (Huang, L.; Quada, Jr J. C.; Lown, J. W. *Bioconjugate Chem.* **1995**, *6*, 21, Ref. 1) probably as a result of the linker allowing the active complex to approach the target deoxyribose more closely and efficiently. Certain of the compounds, ones containing a (*S*)-methyl in the linker and the (*S,S*)-cyclopropyl linker, exhibit unique cleavage sites, indicating that these linkers allow the hybrids to locate novel, individual DNA binding sites.

Introduction

An important objective in modern bioorganic and medicinal chemistry concerns the design of synthetic models that mimic various aspects of biologically active molecules. Detailed study of such models could lead to development of better chemotherapeutic agents, novel artificial restriction enzymes and molecular biological and diagnostic tools. There is currently considerable interest in the design and synthesis of artificial sequence-specific DNA-cleaving molecules which are bifunctional conjugates of DNA cleaving groups and DNA recognition units.[2] It has been demonstrated that the linker moiety between the reactive group and the carrier is an important factor affecting the efficiency of DNA cleavage.[2b,3] A recent study of ours found that bis-netropsins in which two DNA binding netropsin motifs are connected by enantiomerically pure C_2-symmetric linkers, especially 1,2-*trans*-cyclopropanedicarboxamide, show efficient DNA binding via isohelical and strand selective recognition.[4] Further studies including footprinting and 2-D NMR methods revealed that the cyclopropane linkers with the appropriate absolute configuration permit the two netropsin units to match the natural right-handed twist of the base pairs along the minor groove of DNA.[5]

Bleomycins (BLM), including the semisynthetic derivatives peplomycin and liblomycin, are a family of glycopeptide antitumor antibiotics which are clinically used in combination chemotherapy against several types of cancer (Fig. 1).[6] The therapeutic effect of BLM is believed to arise from its ability to cause DNA degradation. This process involves a redox-active metal ion such as Fe^{2+} and a source of oxygen and causes predominantly the cleavage of double-stranded DNA at '5-GT-3' and '5-GC-3' sites by the initial selective abstraction of the H-4' atom of the deoxyribose moiety in pyrimidine residues.[7] Extensive investigation indicated that the BLM glycopeptide can be structurally divided into several parts according to their roles in DNA scission. The pyrimidoblamic acid-*erythro*-β-hydroxy-L-histidine moiety forms the subunit responsible for iron chelation and dioxygen activation; the bithiazole and positively charged side-chain provide affinity for double-helical DNA and may provide sequence selectivity; the carbamoyl disaccharide moiety increases the efficiency of DNA cleavage through stabilization of the BLM–Fe(II)–O_2 complex and the peptide linker integrates each part of BLM at an appropriate distance and in a suitable orientation. Since the total synthesis and the intriguing mechanism of BLM action was established,[8,9] there has been considerable interest in the design, synthesis and biological evaluation of bleomycin model compounds.[9–11] Of particular interest has been the role of each moiety in the sequence selectivity of BLM, which has not been conclusively established. Studies by the groups of Umezawa, Hecht and Ohno indicated that the length and the chirality of the linker in bleomycin or analogs are important factors in the design of bleomycin analogs.[11,12] Ohno's work on PYML(6)[10b] (a slightly simplified model of the BLM metal binding moiety, see Fig. 2) bleomycin models indicated that the DNA binding moiety also controlled sequence selectivity. PYML(6)-bleomycin was shown to have the BLM-like selectivity,[11a] and PYML(6)-distamycin[11b,c] had the AT selectivity characteristic of distamycin. On the other hand, a series of analogs of deglyco-BLM

demethyl A_2 prepared by Hecht *et al.* containing oligoglycine spacers of varying length[12a,b] were all shown to retain the DNA cleavage selectivity of the natural product, indicating that the metal-binding moiety selected the cleavage sequence. Our efforts in this area have been aimed at directing the DNA cleavage activity to novel sequences. We have reported the first part of our studies on design, synthesis and DNA sequence selective cleavage of a simplified synthetic functional model of bleomycin **1(f–h)** in which methyl 2-(2-aminoethyl)-aminomethyl-pyridine-6-carboxyl-histidinate (AMPHIS) served as metal-chelating subunit and poly-*N*-methylpyrrole moieties were used as the DNA binding moiety (see Fig. 2).[1,13] Ethidium bromide assay and electrophoretic mobility experiments revealed that the relative rate of DNA cleavage increased with the number of *N*-methylpyrrole units in the DNA recognition moiety. Polyacrylamide sequencing gel electrophoresis demonstrated that the poly-*N*-methylpyrrole moiety directs the hybrids to AT-rich sequences of DNA. We report herein our further studies on hybrids in which AMPHIS-distamycin (DST) was connected through various linker moieties designed to further refine the DNA sequence recognition properties.

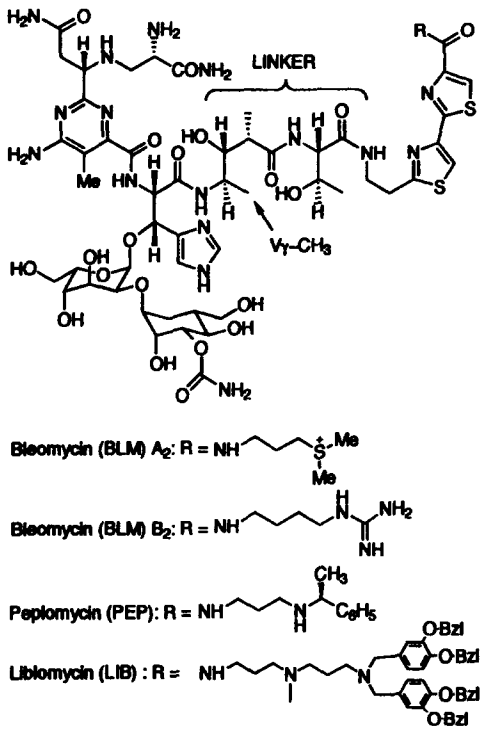


Figure 1. Natural bleomycin A_2, B_2, and semisynthetic analogs peplomycin and liblomycin.

Results and Discussion

If we consider bleomycin models as bifunctional molecules, a linker with C_2-symmetry (which permits the bidentate bis-netropsin to match the natural right-handed twist of double stranded DNA)[5] should also increase the DNA binding affinities of the bifunctional hybrids. Thus, we designed **1a** and **b** (Fig. 2), in which a pair of enantiomers of 1,2-*trans*-cyclopropane-dicarboxylic acid derivatives (CPA) were used as linkers. For comparison, we designed **1d** and **e**, in which (*R*)- and (*S*)-alanine were incorporated to replace the Vγ-CH_3 that has been reported to be important for the orientation of the metal-complexing moiety (Fig. 1),[11c] and **1c**, containing an achiral linker with the same length as **1d** and **e**.

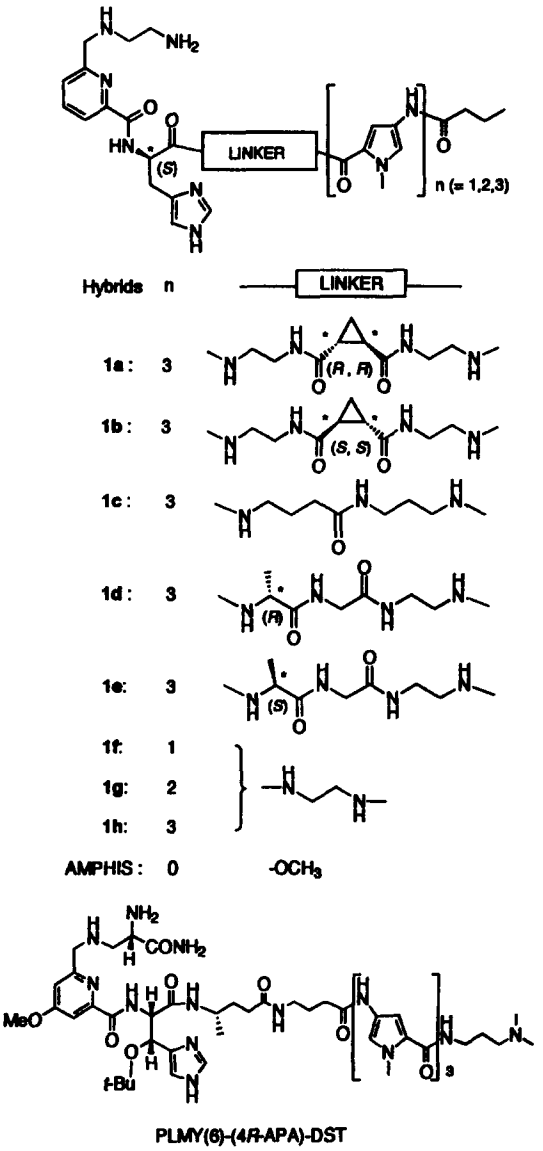


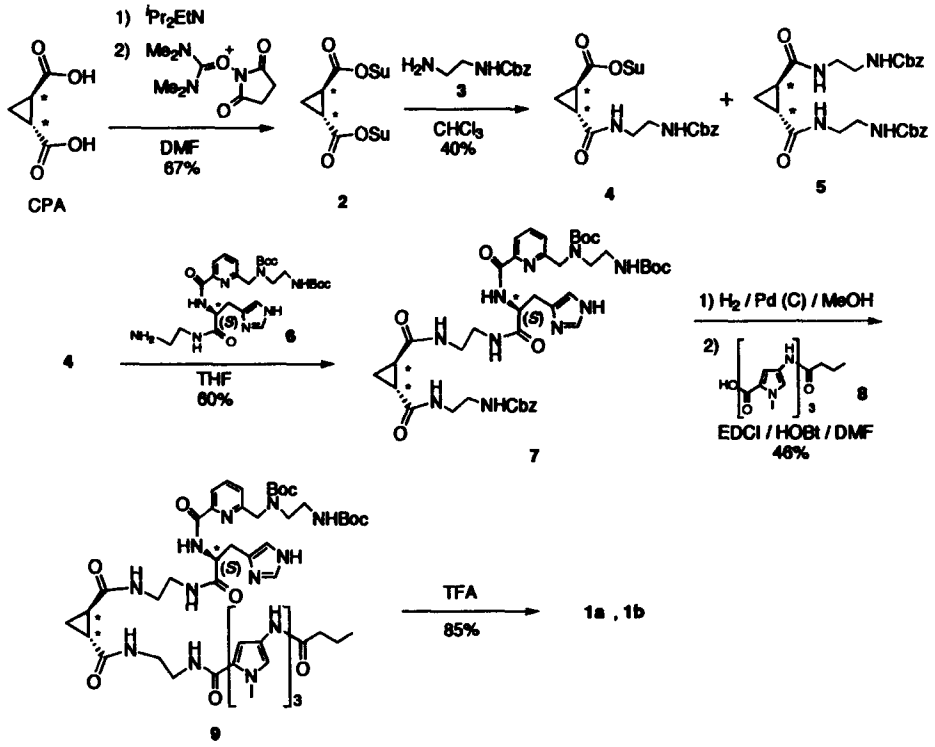
Figure 2. Compounds **1a–e**: designed functional models of bleomycin with different linkers. Compounds **1f–h**: basic models previously reported.

Synthesis

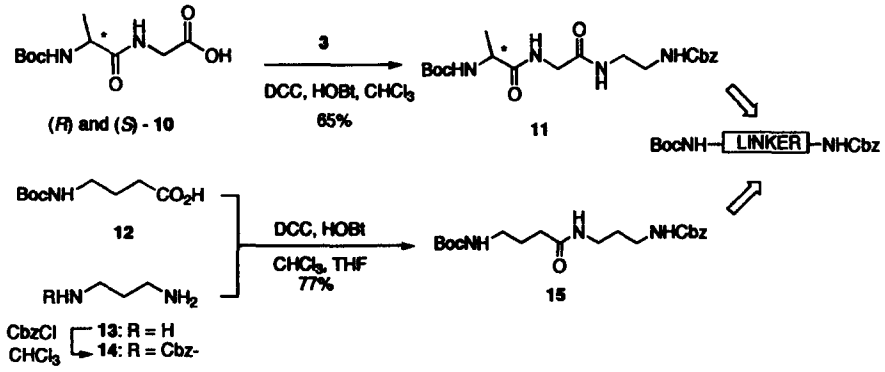
Condensation of amine **3**[13] with (1*S*,2*S*)- or (1*R*,2*R*)-**2**[5,14] (Scheme 1), derived from the reaction of 1,2-*trans*-cyclopropanedicarboxylic acid with *O*-(succinimidyl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate in the presence of di-*iso*-propylethylamine in chloroform provided a pair of enantiomers of the active monoester **4** in a yield of 40% with diamide **5** as a byproduct.

Coupling of **4** with the metal-chelating moiety **6**[1,13] in tetrahydrofuran afforded **7** in 60% yield. Removal of the benzyloxy carbonyl group of **7** by hydrogenation in the presence of 10% Pd(C) in methanol, followed by coupling with the distamycin unit **8**[1,13] in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and HOBt in dimethylformamide gave a pair of diastereomers **9**. Deprotection of **9** in trifluoroacetic acid at 0 °C, followed by purification on Amberlite XAD-2 resin resulted in AMPHIS-(1*S*,2*S*) and (1*R*,2*R*)-CPA-DST **1a** and **b**, respectively, in approximately 85% yield.

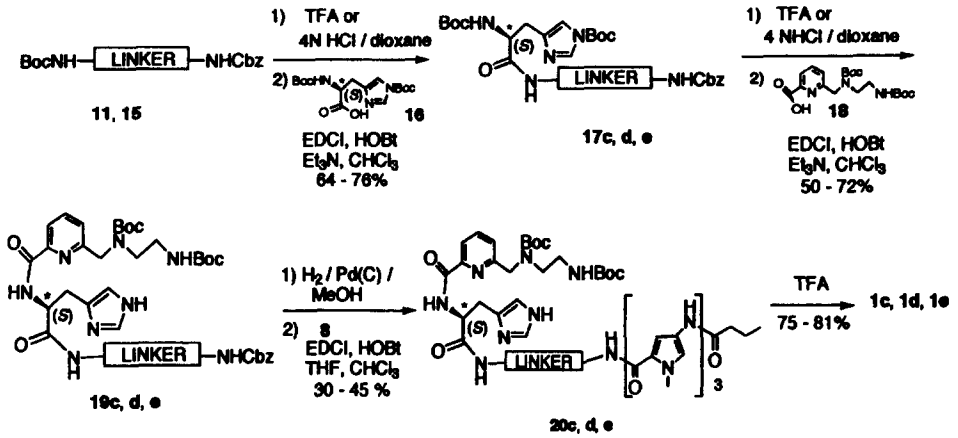
Compounds **1c–e** were synthesized by a different approach. Coupling of peptides (*R*)- and (*S*)-Boc-Ala-Gly-CO_2H (**9**) (Scheme 2) with **3** in the presence of dicyclohexylcarbodiimide (DCC) and HOBt in chloroform afforded (*R*)- and (*S*)-Boc-Ala-Gly-Cbz (**11**) in 65% yield. Similar coupling of commercially available **12** with **14**, derived from the condensation of 1,3-diaminopropane (**13**) with benzyl chloroformate in chloroform, gave linker (**15**) in 77% yield. The protected linkers **11** and **15** (Scheme 3) were then stirred in trifluoroacetic acid or 4 N HCl dioxane solution at 0 °C to remove the Boc group and the amine salts were coupled with protected histidine **16** to afford the intermediates **17c–e** in 64–76% yield. Deprotection of **17** with trifluoroacetic acid or 4 N HCl in dioxane and coupling of the amine salts with known compound **18** under the same coupling conditions as for **17** provided the protected hybrids **19c–e** in 50–72% yield. Removal of Cbz groups on **19** by hydrogenation and coupling of the free amine with **8** in the presence of EDCI and HOBt in THF and chloroform gave **20c–e** in 30–45% yield. Finally, deprotection and purification by the same procedure as for **1a** and **b** afforded (*S*)-AMPHIS-ABA-DAP-DST (**1c**) and AMPHIS-(*R*) and (*S*)-Ala-Gly-DST (**1d** and **e**), respectively, in 75–81% yield.



Scheme 1. Synthesis of **1a** and **b**, hybrids with 1,2-*trans*-disubstituted cyclopropane linkers.



Scheme 2. Syntheses of linkers, (*R*) and (*S*)-Boc-Ala-Gly-Cbz (**11**) and Boc-ABA-DAP-Cbz (**15**).



Scheme 3. Syntheses of the hybrids, **1c–e**.

DNA cleavage studies

Studies of the kinetics and sequence preference of drug–DNA reactions of the Fe(II) complexes of **1a–e** and their cleavage of supercoiled covalently closed circular DNA in the presence of biologically compatible reductants such as 1,4-dithiothreitol (DTT) were conducted by ethidium binding assay, agarose gel electrophoresis, and polyacrylamide sequencing gel electrophoresis.

The ethidium bromide binding assay is convenient for studies of the kinetics of reactions of supercoiled DNA with drugs.[15] Supercoiled covalently closed circular (CCC) and nicked open circular (OC) DNAs permit intercalation of ethidium to different extents, which is revealed by the characteristic fluorescence intensity of bound ethidium. The difference of the fluorescence between undenatured and denatured assay solutions provides information about extent of cleavage via binding ethidium because the heat denatures cleaved DNA, which results in a decrease in fluorescence. The results are shown in Figure 3. The relative rates of CCC-PM2 DNA cleavage by the Fe(II)-hybrid complexes are **1a, 1b, 1c > 1d, 1e, 1h**. The results are consistent with current understanding about the relevance of linker length to cleavage efficiency. Ohno reported[11c] a 10-fold difference in the efficiency of DNA cleavage between PYML(6)-(4*R*)- and (4*S*)-APA-distamycin, which differ only in the configuration of the methyl group in the linker. In the case of chiral methyl containing models **1d** and **e**, however, there is no significant difference in DNA cleavage activity. Independent evidence for the DNA cleavage was obtained by agarose gel electrophoresis experiments. After incubation of the complexes with PM2 DNA and DTT for 25 min, the reaction mixtures were loaded on the gel. The results are shown in Figure 4. Under the experimental conditions, all of the complexes converted CCC DNA (form I) to OC DNA (form II) and **1a** and **b** were evidently more efficient than the others.

Linker modified bleomycin models **1a–e** were prepared to investigate how the linker influences DNA cleavage in this system. Figure 5 displays a comparison of the

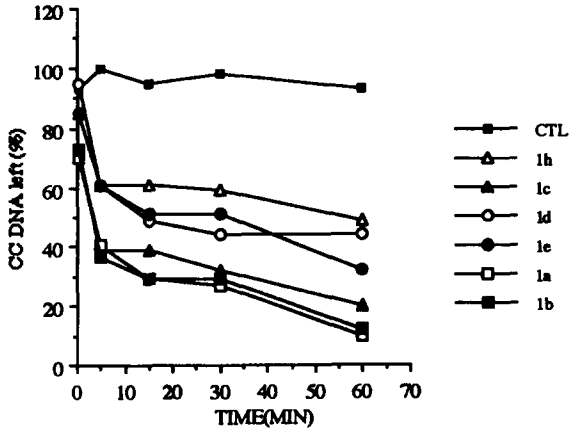


Figure 3. Plot of the percentage of the closed circular DNA left measured by ethidium fluorescence assay against reaction time. The reactions were run at 24 °C under aerobic conditions. Reaction mixture (53 μL) contained Fe(II)–1 (1:1) in 8×10^{-5} M, 25 μg mL^{-1} of PM-2 supercoiled DNA (CC) in 25 mM Tris buffer pH 7.3, 1 mM of 1,4-dithiothreitol (DTT). Reaction mixture (10 μL) was pipetted for each point into 2 mL of F_{12} assay solution and denatured at 95 °C for 3 min. The fluorescence measured was divided by that of a control reaction treated identically.

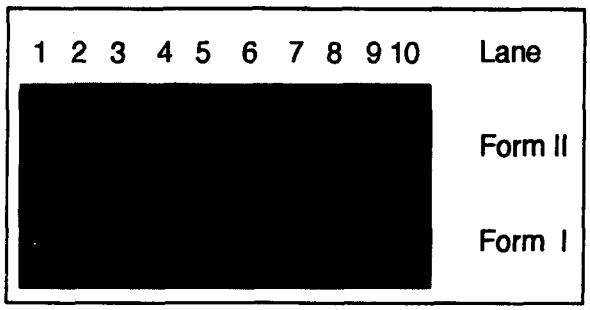


Figure 4. Supercoiled DNA cleavage by Fe(II)–1 (1:1) complexes. The reactions were run under the same conditions as shown in Figure 3. Lane 1: DNA alone; Lane 2: 5 μM of Fe(II); Lane 3: 80 μM of Fe(II)–**1h**; Lane 4: 80 μM of Fe(II)–**1c**; Lane 5: 80 μM of Fe(II)–**1d**; Lane 6: 80 μM of Fe(II)–**1e**; Lane 7: 80 μM of Fe(II)–**1a**; Lane 8: 40 μM of Fe(II)–**1a**; Lane 9: 80 μM of Fe(II)–**1b**; Lane 10: 40 μM of Fe(II)–**1b**. Form I: closed circular DNA; Form II: open circular DNA.

DNA sequence selective cleavage of the linker modified compounds **1a–e** with that of **1h**, which contains a short linker, and bleomycin A_2 on a 158 bp fragment of pBR322 (bases 30–187).[16] The hybrids are

all less active than BLM (2 μM versus 10 μM of hybrids), and all had very different (and a reduced level of) selectivity compared to BLM. All compounds cleave DNA selectively within or near the AT-rich sequences (bases 56–62 and 89–100), but there are significant differences in selectivity within the series. Most of the linker modified compounds have cleavage sites farther in the 5' direction along the upper binding site, especially **1e** and **b** (position A90). Cleavage by **1e** and **d** in the centre of the lower binding site (A58 and A59) is also enhanced relative to **1h** (which contains the short linker). It appears that the linker modifications in this series of compounds enhances cleavage at some sites but has no effect or even reduces activity at other sites. Owing to the heterogeneous nature of the binding sequences on this DNA detailed interpretation of the variation in cleavage between these compounds is not possible, but it is apparent that linker structure can have an impact on position of cleavage.

In order to understand the relationship between linker structure and cleavage more fully a similar DNA cleavage experiment utilizing an AT rich 241 bp fragment of SV40 (bases 347–588)[17] was performed. Figure 6 shows the results of this experiment which are more easily interpreted because of the homogenous binding sequences. The apparent inactivity of BLM compared to hybrids **1** in the figure is actually due to overcleavage of the DNA substrate in the BLM lane. Cleavage of the linker modified compounds generally extends farther in the 3' direction at poly A binding sites and farther in the 5' direction at poly T binding sites than cleavage of the short linker compound **1h**. Assuming that the pyrrole ends of all compounds bind to DNA at the same position, extension of the cleavage position probably arises directly from increased linker length allowing the active complex to reach farther along the minor groove. Some of the compounds also have unique cleavage sites, some of which are quite isolated and therefore may be due to binding at a site accessible only to that structure. For example, **1e**, which carries a (*S*)-methyl group, has new cleavage sites at A399 and A400, at A487, and at A509, all of which are within the poly A binding sites (rather than at the 3' end, where **1h** cleaves) and which are not cleaved by any of the other compounds. Hybrid **1b**, with the (*S,S*)-cyclopropyl linker, has a new cleavage band (A393) 12 bases away from the nearest cleavage site and five bases away from the 5' end of the A_7 binding site. Compound **1b** also has cleavage sites at A463, A465, and G470, which are in a region without any AT stretch longer than three bases. Possibly, therefore, **1b** is capable of efficient binding to a three base site.

In addition to the extension of cleavage and unique sites of cleavage, some of the linker modified compounds have improved efficiencies of cleavage relative to **1h** at the 'original' sites. Hybrid **1c** has greatly enhanced cleavage at T417, and both **1c** and **e** have enhanced cleavage at A447 and A448. This may be due to the increased linker length allowing a

Figure 5. Sequencing gel autoradiogram of cleavage of a 5'-^{32}P labeled 158-bp restriction fragment by bleomycin and linker modified compounds **1**. DNA lane contains untreated DNA; control lane contains 10 μM Fe(II) + 250 μM DTT. BLM lane contains 2 μM Fe(II)–BLM A_2. Central lanes contain 10 μM Fe(II)–**1** as indicated; each of these lanes also contained 250 μM DTT. Lanes marked G, GA, C, and CT were treated by the Maxam–Gilbert sequence reactions for those bases.

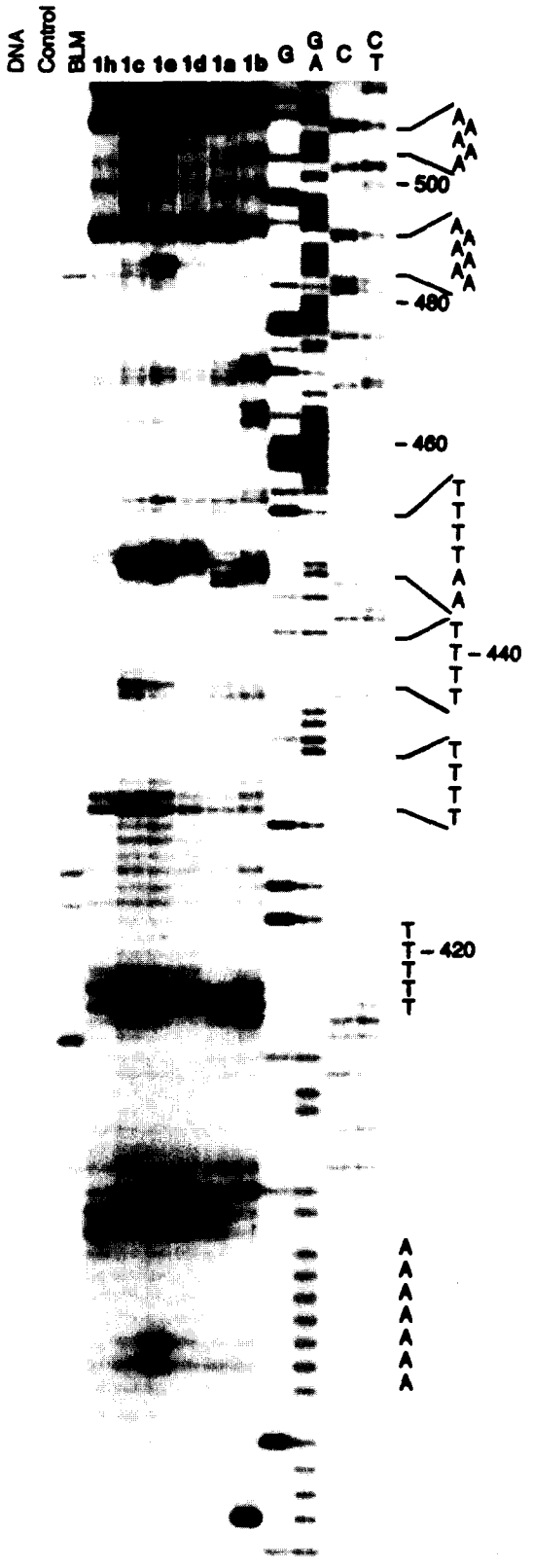


Figure 6. Sequencing gel autoradiogram of cleavage of a 5'-^{32}P labeled 241-bp restriction fragment by bleomycin and linker modified compounds **1**. DNA lane contains untreated DNA; control lane contains 10 μM Fe(II) + 250 μM DTT. BLM lane contains 2 μM Fe(II)–BLM A_2. As for Figure 5, central lanes contain 10 μM Fe(II)–**1** and 250 μM DTT. G, GA, C, and CT lanes were produced by the appropriate Maxam–Gilbert sequence reactions.

conformation in which the active complex is closer to the site of hydrogen abstraction on a particular deoxyribose residue.

As is the case with most model studies, our AMPHIS-DST series of BLM models has yet to approach the level of cleavage efficiency and selectivity of the natural product. The linker-modified compounds reported herein have, however, improved upon earlier models we reported,[1] and some, notably (*S*)-methyl carrying compound **1e** and in **1b**, bearing the (*S,S*)-cyclopropyl linker, have unique single-base cleavage sites. These will serve as the basis for further refinement and development of DNA cleavage activity and selectivity. Structural studies are being performed and should also provide detailed information about mode of binding and how to improve it. These studies should assist in designing the next generation of hybrids and will be reported in due course.

Experimental

Chemistry

Melting points were determined with an electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at ambient temperature on a Bruker WH-300 spectrometer. Fast atom bombardment high-resolution mass spectra (FABHRMS) were recorded on a modified Kratos MS-50 mass spectrometer equipped with a VG 11 250J data system. Accurate masses were calculated interactively with the data system using a reference (such as CsI in glycerol) peaks. FT-IR spectra were recorded on a Perkin Elmer 1760 spectrophotometer interfaced to a PE 7700 microcomputer. Optical rotations were measured on PE 241 polarimeter at sodium D line (589 nanometers) at ambient temperature. Analytical thin-layer chromatography was performed on silica-coated plastic plates (silica gel 60 F-254, Merck) and visualized under UV light. Preparative separations were performed by flash chromatography on silica gel (Merck, 70–230 or 230–400 mesh). Tetrahydrofuran was dried by distillation from sodium benzophenone ketyl. Anhydrous dimethylformamide was purchased from Aldrich. All other solvents were used as received and were reagent grade where available.

O-(N-Succinimidyl)-2-{[2-(benzyloxycarbonyl)aminoethyl]-aminocarbonyl}cyclopropanecarboxylate (4). To a stirred solution of **2** (324 mg, 1 mmol) in $CHCl_3$ (5 mL) was added dropwise amine **3** (194 mg, 1 mmol) in $CHCl_3$ (5 mL) and stirring was continued for 1 h. The mixture was filtered and evaporated. The residue was subjected to flash chromatography on silica column, eluted with CH_2Cl_2:MeOH (9:1). The product **4** (161 mg) was obtained as white crystals in 40% yield. For (1*R*,2*R*)-**4**, mp: 106–108 °C; $[\alpha]^{20}_D$ –89 (*c* 1.14; $CHCl_3$); IR ($CHCl_3$ cast) υ_{max} 3317 (br), 2944 (w), 1737 (s), 1713 (s), 1645 (m), 1560 (m), 1210 (m) cm^{-1}; 1H NMR ($CDCl_3$): δ 7.34 (*s*, 5H), 6.68 (*br*, 1H, -CONH-), 5.30

(*br*, 1H, -CONH-), 5.10 (*s*, 2H), 3.50–3.25 (*m*, 4H), 2.85 (*s*, 4H), 2.45 (*m*, 1H), 2.12 (*m*, 1H), 1.65 (*m*, 1H), 1.50 (*m*, 1H); HRMS (CI) m/z calcd for $C_{19}H_{21}N_3O_7$ (M^+): 403.1379, found: 403.1380.

(S)-Bis(Boc)-AMPHIS-CPA-Cbz (7). A solution of active ester **4** (120 mg, 0.30 mmol) and amine **6** (173 mg, 0.30 mmol) in $CHCl_3$ (20 mL) was stirred at room temp. overnight. The reaction mixture was then concentrated and the residue was purified on silica column, eluted with CH_2Cl_2:MeOH (9:1). The coupling product **7** (150 mg, 58%) was obtained as white foam. (1*R*,2*R*)-**7**: R_f (CH_2Cl_2:MeOH = 8.5:1.5) 0.33; $[\alpha]^{20}_D$ –23 (*c* 1.10; $CHCl_3$); IR ($CHCl_3$ cast) υ_{max} 3300 (br), 2977 (w), 2934 (w), 1652 (s), 1593 (s), 1453 (m), 1366 (m), 1249 (m), 1167 (m), 755 (m); ^{1}H NMR ($CDCl_3$:DMSO-d_6 = 5:1): δ 8.54 (*s*, 1H, -CONH-), 8.27 (*br s*, 1H, -CONH-), 8.05 (*br*, 1H, -CONH-), 7.93 (*br m*, 2H), 7.87 (*m*, 1H), 7.78 (*m*, 1H), 7.30 (*m*, 1H, -CONH-), 7.22 (*s*, 5H), 7.18 (*br m*, 1H, -CONH-), 7.07 (*m*, 1H), 6.74 (*s*, 1H), 6.05 (*br m*, 1H, -CONH-), 4.98 (*s*, 2H), 4.87 (*m*, 1H), 4.50 (*m*, 2H), 3.40–2.90 (*m*, 14H), 1.95 (*m*, 2H), 1.40 (*s*, 4.5H), 1.30 (*s*, 9H), 1.25 (*s*, 4.5H), 1.07 (*m*, 2H); FABHRMS m/z calcd for $C_{42}H_{58}N_{10}O_{10}H$ (M^+ + H): 863.4415, found: 863.4438.

(S)-Bis(Boc)-AMPHIS-CPA-DST (9). A suspension of **7** (138 mg, 0.161 mmol) and 10% Pd(C) (40 mg) in MeOH (2 mL) was hydrogenated at atmospheric pressure (H_2-balloon) for 5 h and then filtered. The filtrate was concentrated and dissolved in $CHCl_3$ (1 mL). In another flask, a mixture of **8** (110 mg, 0.242 mmol), HOBt (35 mg, 0.242 mmol) and DCC (53 mg, 0.242 mmol) in THF (1 mL) was stirred at room temp. for 30 min and poured into the above amine solution. The reaction mixture was stirred at room temp. for 48 h. After the solvent was removed under reduced pressure, the residue was chromatographed on silica gel column, eluted with gradient solvent (CH_2Cl_2:MeOH = 9:1–4:1). The product **9** (58 mg) was obtained as an amorphous foam in a yield of 46%. (1*R*,2*R*)-**9**: R_f (CH_2Cl_2:MeOH = 4:1) 0.41; $[\alpha]^{20}_D$ –15 (*c* 0.73; MeOH); IR (CH_2Cl_2, cast) υ_{max} 3300 (br), 2955 (m), 2925 (m), 1655 (s), 1651 (s), 1578 (s), 1524 (s), 1464 (m), 1434 (m), 1406 (m), 1251 (w), 1214 (m), 1192 (s), 1159 (w), 775 (w) cm^{-1}; ^{1}H NMR ($CDCl_3$:DMSO-d_6 = 5:1): δ 9.20 (*s*, 1H, -CONH-), 9.16 (*s*, 1H, -CONH-), 9.07 (*s*, 1H, -CONH-), 8.50 (*br*, 1H, -CONH-), 7.88 (*br*, 1H, -CONH-), 7.80 (*br m*, 1H, -CONH-), 7.55 (*m*, 1H), 7.44 (*m*, 1H), 7.24 (*s*, 1H), 6.97 (*m*, 1H), 6.88 (*m*, 2H), 6.80 (*s*, 1H), 6.58 (*s*, 1H), 6.50 (*m*, 2H), 6.47 (*s*, 1H), 5.58 (*br*, 1H, -CONH-), 4.43 (*m*, 1H), 4.15 (*m*, 2H), 3.53 (*s*, 3H), 3.52 (*s*, 3H), 3.49 (*s*, 3H), 3.17–2.50 (*m*, 14H), 1.90 (*t*, *J* = 7.5 Hz, 2H), 1.70 (*m*, 2H), 1.30 (*sex*, *J* = 7.5 Hz, 2H), 1.12 (*s*, 4.5 H, *t*-Bu), 1.02 (*s*, 9H, *t*-Bu), 0.97 (*s*, 4.5H, *t*-Bu), 0.87 (*m*, 2H), 0.58 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z calcd for $C_{56}H_{76}N_{16}O_{12}H$ (M^+ + H): 1165.5906, found: 1165.5867. For (1*S*,2*S*)-**9**: R_f (CH_2Cl_2:MeOH = 4:1) 0.41; $[\alpha]^{20}_D$ +37 (*c* 0.80; MeOH); IR ($CHCl_3$ cast) υ_{max} 3302 (br), 2965 (w), 2932 (w), 1649 (s), 1574 (s), 1524 (s), 1434 (s), 1405 (m), 1366 (m), 1251 (w), 1165 (w), 1105 (m), 756 (w) cm^{-1}; ^{1}H NMR ($CDCl_3$:DMSO-d_6 = 5:1): δ 9.22 (*s*, 1H, -CONH-), 9.19 (*s*, 1H, -CONH-), 9.08 (*s*, 1H, -CONH-), 8.43 (*br*, 1H, -CONH-), 7.93 (*br*, 1H, -CONH-), 7.80 (*br*, 1H, -CONH-), 7.52 (*d*, *J* = 7.0 Hz, 1H), 7.40 (*t*, *J* = 7.0 Hz, 1H), 7.23 (*s*, 1H), 6.93 (*t*, *J* = 7.0 Hz, 1H), 6.82 (*d*, *J* = 2.0 Hz, 1H), 6.77 (*d*, *J* = 2.0 Hz, 1H), 6.60 (*d*, *J* = 2.0 Hz, 1H), 6.49 (*d*, *J* = 2.0 Hz, 1H), 6.48 (*s*, 1H), 6.45 (*d*, *J* = 2.0 Hz, 1H), 6.42 (*d*, *J* = 2.0 Hz, 1H), 5.70 (*br m*, 1H, -CONH-), 4.37 (*m*, 1H), 4.10 (*m*, 2H), 3.50 (*s*, 3H), 3.48 (*s*, 3H), 3.43 (*s*, 3H), 3.10–2.50 (*m*, 14H), 1.85 (*t*, *J* = 7.5 Hz, 2H), 1.62 (*m*, 2H), 1.26 (*sex*, *J* = 7.5 Hz, 2H), 1.08 (*s*, 4.5 H, *t*-Bu), 0.98 (*s*, 9H, *t*-Bu), 0.92 (*s*, 4.5H, *t*-Bu), 0.80 (*m*, 2H), 0.54 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z found: 1165.5857.

(S)-AMPHIS-CPA-DST (1a, b). (1*R*, 2*R*)-**9** (27 mg, 0.023 mmol) in trifluoroacetic acid (1 mL) was stirred at 0 °C for 30 min and the mixture was then evaporated under reduced pressure to remove the solvent. The residue was made basic with dilute aqueous ammonia (1 mL) to pH 9 and loaded on a column of Amberlite XAD-2 resin. After washing with H_2O until the eluate was neutral, the compound was washed out with MeOH. Evaporation of the methanolic solution provided **1a** (18 mg, 81%) as white amorphous foam. **1a**: R_f (AcOEt:*n*-BuOH:H_2O = 1:1:1) 0.38; $[\alpha]^{20}_D$ –14 (*c* 1.45; MeOH); IR ($CHCl_3$ cast) υ_{max} 3284 (br), 2961 (m), 2935 (m), 1652 (s), 1576 (s), 1533 (s), 1457 (m), 1436 (m), 1349 (w), 1262 (w), 1205 (w), 766 (w) cm^{-1}; ^{1}H NMR (DMSO-d_6:CD_3OD = 5:1): δ 7.92 (*t*, *J* = 7.0 Hz, 1H), 7.86 (*d*, *J* = 7.0 Hz, 1H), 7.60 (*d*, *J* = 7.0 Hz, 1H), 7.55 (*s*, 1H), 7.22 (*s*, 1H), 7.19 (*s*, 1H), 7.15 (*s*, 1H), 7.03 (*s*, 1H), 6.86 (*s*, 2H), 6.81 (*s*, 1H), 4.58 (*m*, 1H), 3.88 (*s*, 2H), 3.84 (*s*, 3H), 3.82 (*s*, 3H), 3.78 (*s*, 3H), 3.23–2.98 (*m*, 10H), 2.77 (*m*, 2H), 2.66 (*m*, 2H), 2.20 (*t*, *J* = 7.5 Hz, 2H), 1.94 (*m*, 2H), 1.58 (*sex*, *J* = 7.5 Hz, 2H), 1.04 (*m*, 2H), 0.87 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z calcd for $C_{46}H_{60}N_{16}O_8H$ (M^+ + H): 965.4858, found: 965.4824.

Compound **1b** was obtained by the similar procedure. (1*S*, 2*S*)-**9** (20 mg, 0.0172 mmol) afforded **1b** (14 mg) in 85% yield. **1b**: R_f (AcOEt:*n*-BuOH:H_2O = 1:1:1) 0.38; $[\alpha]^{20}_D$ +33 (*c* 1.58; MeOH); IR ($CHCl_3$ cast) υ_{max} 3286 (br), 3099 (m), 2934 (m), 1649 (s), 1576 (s), 1465 (w), 1406 (m), 1348 (w), 1262 (w), 1120 (w), 770 (w) cm^{-1}; ^{1}H NMR (DMSO-d_6:CD_3OD = 5:1): δ 7.92 (*d*, *J* = 7.0 Hz, 1H), 7.84 (*d*, *J* = 7.0 Hz, 1H), 7.61 (*d*, *J* = 7.0 Hz, 1H), 7.55 (*s*, 1H), 7.22 (*d*, *J* = 2.0 Hz, 1H), 7.18 (*d*, *J* = 2.0 Hz, 1H), 7.14 (*d*, *J* = 2.0 Hz, 1H), 7.04 (*d*, *J* = 2.0 Hz, 1H), 6.96 (*d*, *J* = 2.0 Hz, 2H), 6.81 (*s*, 1H), 4.60 (*m*, 1H), 3.88 (*s*, 2H), 3.84 (*s*, 3H), 3.81 (*s*, 3H), 3.79 (*s*, 3H), 3.22–2.95 (*m*, 10H), 2.76 (*m*, 2H), 2.65 (*m*, 2H), 2.20 (*t*, *J* = 7.5 Hz, 2H), 1.92 (*t*, *J* = 6.0 Hz, 2H), 1.58 (*sex*, *J* = 7.5 Hz, 2H), 1.04 (*m*, 2H), 0.88 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z found: 965.4830.

(S) and (R)-Boc-Ala-Gly-Cbz (11). To a solution of peptide **10** (248 mg, 1 mmol) and amine **3** (213 mg, 1.1 mmol) in chloroform (20 mL) was added DCC (227 mg, 1.1 mmol) and the mixture was stirred overnight.

After diluting with EtOAc (50 mL), the reaction mixture was filtered and concentrated. The residue was purified by flash chromatography on silica column, eluted with mixed solvent of CH_2Cl_2:MeOH (20:1–10:1). The coupling product **11** (280 mg, 65% yield) was obtained as white foam. IR ($CHCl_3$ cast) υ_{max} 3317 (br), 2979 (w), 2935 (w), 1685 (s), 1662 (s), 1531 (s), 1252 (m), 1166 (w) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.34 (*s*, 5H), 7.18 (*br*, 1H, -CONH-), 7.00 (*br*, 1H, -CONH-), 5.70 (*br*, 1H, -CONH-), 5.28 (*br*, 1H, -CONH-), 5.08 (*s*, 2H), 4.10 (*m*, 1H), 3.88 (*m*, 2H), 3.50–3.25 (*m*, 4H), 1.45 (*s*, 9H), 1.34 (*d*, *J* = 7.0 Hz, 3H); HRMS (CI) m/z calcd for $C_{20}H_{30}O_6N_4H$ (M^+ + H): 423.2244, found: 423.2224.

(S)-Bis(Boc)-His-ABA-DAP-Cbz (17c). A mixture of **15** (786 mg, 2 mmol) and trifluoroacetic acid (5 mL) was stirred at 0 °C for 30 min and subsequently evaporated *in vacuo* to remove the solvent completely. After the residue was washed with ether and dried *in vacuo*, the amine–TFA salt was obtained as a white powder. In another flask, a mixture of the acid **16** (783 mg, 2.2 mmol), EDCI (422 mg, 2.2 mmol), HOBt (297 mg, 2.2 mmol) and triethylamine (0.40 mL) in $CHCl_3$ (20 mL) was stirred for 30 min and poured into the above amine-TFA salt. After being stirred overnight, the reaction mixture was diluted with $CHCl_3$ (30 mL), washed with H_2O, saturated aqueous $NaHCO_3$ solution and dried on anhydrous Na_2SO_4. Flash chromatography on silica column, eluted with CH_2Cl_2:MeOH (9:1) provided **17c** (847 mg, 67% yield) as a white foam. R_f (CH_2Cl_2:MeOH = 9:1) 0.38; $[\alpha]^{20}_D$ +11 (*c* 0.53; $CHCl_3$); IR (CH_2Cl_2 cast) υ_{max} 3307 (br), 2979 (w), 2936 (w), 1800 (w), 1757 (s), 1709 (s), 1658 (s), 1530 (s), 1455 (w), 1440 (w), 1391 (m), 1371 (m), 1328 (w), 1255 (s), 1156 (m), 1012 (w), 773 (w) cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.20 (*s*, 1H), 7.36 (*s*, 5H), 7.22 (*s*, 1H), 7.13 (*br*, 1H, -CONH-), 7.00 (*br m*, 1H, -CONH-), 6.05 (*br m*, 1H, -CONH-), 5.65 (*br m*, 1H, -CONH-), 5.09 (*s*, 2H), 4.46 (*m*, 1H), 3.37–3.15 (*m*, 6H), 3.03 (*m*, 2H), 2.15 (*m*, 2H), 1.79 (*m*, 2H), 1.69 (*m*, 2H), 1.61 (*s*, 9H), 1.41 (*s*, 9H); FABHRMS m/z calcd for $C_{31}H_{46}O_8N_6H$ (M^+ + H): 631.3455, found: 631.3433.

Compounds **17d** and **e** were synthesized from **11d** and **e** in the same way. Compound **11d** and **e** (434 mg, 1 mmol) provided **17d** (475 mg, 72% yield) and **17e** (523 mg, 76% yield), respectively. (*S*)-Bis(Boc)-His-(*R*)-Ala-Gly-Cbz (**17d**): R_f (CH_2Cl_2:MeOH = 9:1) 0.40; $[\alpha]^{20}_D$ –8.8 (*c* 1.00; $CHCl_3$); IR ($CHCl_3$ cast) υ_{max} 3308 (br), 2980 (w), 2936 (w), 1758 (m), 1712 (s), 1660 (s), 1530 (s), 1391 (m), 1254 (s), 1156 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.38 (*br*, 1H, -CONH-), 8.16 (*s*, 1H), 8.10 (*br*, 1H, -CONH-), 7.69 (*br*, 1H, -CONH-), 7.35 (*s*, 5H), 7.10 (*s*, 1H), 6.72 (*br*, 1H, -CONH-), 6.45 (*br*, 1H, -CONH-), 5.48 (*br*, 1H, -CONH-), 5.10 (*s*, 2H), 4.40 (*m*, 1H), 4.27–4.13 (*m*, 2H), 3.56 (*m*, 1H), 3.50–3.20 (*m*, 6H), 1.65 (*s*, 9H), 1.42 (*s*, 9H), 1.40 (*d*, *J* = 5.0 Hz, 3H); FABHRMS m/z calcd for $C_{31}H_{45}N_7O_9H$ (M^+ + H): 660.3357, found: 660.3334. (*S*)-Bis-(Boc)-His-(*S*)-Ala-Gly-Cbz (**17e**): R_f (CH_2Cl_2:MeOH = 9:1) 0.40; $[\alpha]^{20}_D$ –1.8 (*c* 0.57; $CHCl_3$); IR ($CHCl_3$ cast) υ_{max} 3310 (br), 2976 (w), 2933 (w), 1760 (m), 1710 (s), 1658 (s), 1525 (s), 1388 (m), 1252 (m), 1159 (m) cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.47 (*br*, 1H, -CONH-), 7.94 (*s*, 1H), 7.33 (*s*, 5H), 7.11 (*s*, 1H), 7.05 (*br*, 1H, -CONH-), 6.77 (*br*, 1H, -CONH-), 6.27 (*br*, 1H, -CONH-), 5.94 (*br*, 1H, -CONH-), 5.08 (*s*, 2H), 4.28–4.10 (*m*, 3H), 3.70 (*m*, 1H), 3.46–3.20 (*m*, 6H), 1.65 (*s*, 9H), 1.43 (*s*, 9H), 1.41 (*d*, *J* = 5.0 Hz, 3H); FABHRMS m/z found: 660.3321.

(S)-Bis(Boc)-AMPHIS-ABA-DAP-Cbz (19c). A solution of **17c** (63 mg, 0.10 mmol) in trifluoroacetic acid (1 mL) was stirred at 0 °C for 30 min and subsequently evaporated *in vacuo* to remove the solvent. The amine–TFA salt was obtained as a powder after the residue was washed with Et_2O and dried *in vacuo*. In another flask, acid **18** (40 mg, 0.1 mmol), EDCI (21 mg, 0.11 mol), HOBt (15 mg, 0.11 mmol) and triethylamine (50 μL) in $CHCl_3$ (1 mL) was stirred for 30 min and poured into the amine–TFA salt. After being stirred overnight, the mixture was diluted with $CHCl_3$ (10 mL), washed with H_2O, saturated aqueous $NaHCO_3$ solution, and dried on anhydrous Na_2SO_4. Purification on silica column, eluted with CH_2Cl_2:MeOH (9:1) provided **19c** (40 mg, 50% yield) as white amorphous foam. R_f (CH_2Cl_2:MeOH = 9:1) 0.43; IR (CH_2Cl_2 cast) υ_{max} 3320 (br), 2975 (w), 2933 (w), 1700 (s), 1527 (s), 1478 (w), 1453 (m), 1409 (w), 1366 (m), 1266 (m), 1250 (m), 1168 (m), 1081 (w), 735 (w) cm^{-1}; ^1H NMR ($CDCl_3$:DMSO-d_6 = 5:1): δ 8.73 (*br*, 1H, -CONH-), 8.00 (*s*, 1H), 7.87 (*br*, 1H, -CONH-), 7.80 (*br m*, 1H, -CONH-), 7.67 (*m*, 1H), 7.42 (*m*, 1H), 7.20 (*s*, 5H), 7.18 (*s*, 1H), 6.86 (*m*, 1H), 6.10 (*br*, 1H, -CONH-), 5.35 (*br*, 1H, -CONH-), 4.92 (*s*, 2H), 4.88 (*m*, 1H), 4.44 (*d*, *J* = 7.0 Hz, 2H), 3.35 (*m*, 2H), 3.28–2.98 (*m*, 10H), 2.03 (*m*, 2H), 1.65 (*m*, 2H), 1.50 (*m*, 2H), 1.35 (*s*, 4.5H, *t*-Bu), 1.25 (*s*, 9H), 1.22 (*s*, 4.5H, *t*-Bu); FABHRMS m/z calcd for $C_{40}H_{57}O_9N_9H$ (M^+ + H): 808.4357, found: 808.4358.

Compounds **19d** and **e** were obtained from **17d** and **e** by the similar procedure. Compounds **17d** and **e** (195 mg, 0.30 mmol) afforded **19d** (149 mg, 59% yield) and **19e** (180 mg, 72% yield), respectively. (*S*)-Bis(Boc)-AMPHIS-(*R*)-Ala-Gly-Cbz **19d**: R_f (CH_2Cl_2:MeOH = 9:1) 0.28; $[\alpha]^{20}_D$ +1.9 (*c* 0.57; $CHCl_3$); IR ($CHCl_3$ cast) υ_{max} 3317 (br), 2977 (w), 2933 (w), 1662 (s), 1522 (s), 1453 (w), 1366 (m), 1249 (m), 1167 (m), 755 (m) cm^{-1}. ^1H NMR (DMSO-d_6): δ 8.80 (*br*, 1H, -CONH-), 8.45 (*br*, 1H, -CONH-), 7.93 (*m*, 1H), 7.87 (*m*, 1H), 7.55 (*br*, 1H, -CONH-), 7.60 (*s*, 1H), 7.33 (*m*, 1H), 7.32 (*s*, 5H), 7.20 (*br*, 1H, -CONH-), 6.90 (*s*, 1H), 6.83 (*br*, 1H, -CONH-), 5.00 (*s*, 2H), 4.65 (*q*, *J* = 7.0 Hz, 1H), 4.47 (*m*, 2H), 4.18 (*t*, *J* = 6.5 Hz, 1H), 3.67 (*d*, *J* = 6.5 Hz, 2H), 3.31 (*m*, 2H), 3.17–2.88 (*m*, 8H), 1.42 (*s*, 4.5H, *t*-Bu), 1.37 (*s*, 9H), 1.22 (*s*, 4.5H, *t*-Bu), 1.18 (*d*, *J* = 7.0 Hz, 3H), FABHRMS m/z calcd for $C_{40}H_{56}O_{10}N_{10}H$ (M^+ + H): 837.4259, found: 837.4224. (*S*)-Bis(Boc)-AMPHIS-(*S*)-Ala-Gly-Cbz **19e**: R_f (CH_2Cl_2:MeOH = 9:1) 0.29; $[\alpha]^{20}_D$ +2.3 (*c* 1.22; $CHCl_3$); IR ($CHCl_3$ cast) υ_{max} 3316 (br), 2977 (w), 2934 (w), 1662 (s), 1522 (s),

1250 (m), 1167 (m) cm^{-1}. ^1H NMR ($CDCl_3$:DMSO-d_6 = 5:1): δ 8.58 (*br*, 1H, -CONH-), 8.43 (*br*, 1H, -CONH-), 7.85 (*br*, 1H, -CONH-), 7.70 (*d*, *J* = 7.5 Hz, 1H), 7.55 (*t*, *J* = 7.5 Hz, 1H), 7.27 (*s*, 1H), 7.06 (*d*, *J* = 7.5 Hz, 1H), 7.00 (*s*, 5H), 6.70 (*br*, 1H, -CONH-), 6.43 (*s*, 1H), 5.45 (*br*, 1H, -CONH-), 4.70 (*s*, 2H), 4.25 (*s*, 2H), 4.05 (*t*, *J* = 6.5 Hz, 1H), 3.71 (*dd*, *J* = 18.0 Hz, *J* = 6.5 Hz, 1H), 3.58 (*dd*, *J* = 18.0 Hz, *J* = 6.5 Hz, 1H), 3.25–2.85 (*m*, 11H), 1.22 (*s*, 4.5H, *t*-Bu), 1.15 (*d*, *J* = 6.5 Hz, 3H), 1.12 (*s*, 9H), 1.08 (*s*, 4.5H, *t*-Bu); FABHRMS m/z found: 837.4269.

(S)-Bis(Boc)-AMPHIS-ABA-DAP-DST (20c). A suspension of **19c** (68 mg, 0.084 mmol) and 10% Pd(C) (30 mg) in MeOH (1 mL) was stirred under an atmosphere of hydrogen (H_2-balloon) for 4 h. Filtration and evaporation of the mixture provided the free amine. In another flask acid **8** (42 mg, 0.093 mmol), HOBt (13 mg, 0.096 mmol), and DCC (19 mg, 0.092 mmol) in DMF (0.75 mL) was stirred at room temp. for 30 min and subsequently transferred into the above free amine. After stirring for 2 days, the mixture was evaporated *in vacuo* to remove the solvent. Separation of the residue by flash chromatography on silica column with CH_2Cl_2:MeOH (8.5:1.5) as eluent afforded the coupling product **20c** (28 mg, 30% yield) as a white foam. R_f (CH_2Cl_2:MeOH = 4:1) 0.46; $[\alpha]^{20}_D$ +8.3 (*c* 0.64; MeOH); IR ($CHCl_3$ cast) υ_{max} 3298 (br), 2964 (w), 2931 (w), 1650 (s), 1574 (s), 1523 (s), 1463 (m), 1434 (s), 1405 (s), 1366 (m), 1252 (m), 1208 (w), 1163 (m), 1105 (w), 1062 (w), 755 (w) cm^{-1}; ^1H NMR (DMSO-d_6:$CDCl_3$ = 1:1): δ 9.46 (*s*, 1H, -CONH-), 9.43 (*s*, 1H, -CONH-), 9.32 (*s*, 1H, -CONH-), 8.90 (*br*, 1H, -CONH-), 7.81 (*br*, 1H, -CONH-), 7.70 (*m*, 1H), 7.61 (*s*, 1H), 7.56 (*m*, 1H), 7.25 (*m*, 2H), 7.21 (*s*, 1H), 7.19 (*s*, 1H), 7.10 (*s*, 1H), 6.85 (*s*, 1H), 6.84 (*s*, 1H), 6.79 (*s*, 1H), 6.72 (*s*, 1H), 5.80 (*br*, 1H, -CONH-), 4.78 (*m*, 1H), 4.48 (*d*, *J* = 7.0 Hz, 2H), 3.83 (*s*, 3H), 3.81 (*s*, 3H), 3.78 (*s*, 3H), 3.50–2.90 (*m*, 12H), 2.30 (*m*, 2H), 2.18 (*t*, *J* = 7.5 Hz, 2H), 1.60 (*m*, 6H), 1.41 (*s*, 4.5H, *t*-Bu), 1.28 (*s*, 9H), 1.25 (*s*, 4.5H, *t*-Bu), 0.87 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z calcd for $C_{54}H_{75}O_{11}N_{15}H$ (M^+ + H): 1110.5848, found: 1110.5822.

Compound **19d** (129 mg, 0.154 mmol) afforded **20d** (70 mg) in 70% yield and **19e** (85 mg, 0.102 mmol) resulted in **20e** (53 mg) in 45% yield. (*S*)-Bis(Boc)-AMPHIS-(*R*)-Ala-Gly-DST (**20d**): R_f (CH_2Cl_2:MeOH = 4:1) 0.35; $[\alpha]^{20}_D$ +25 (*c* 0.71; MeOH); IR (CH_2Cl_2 cast) υ_{max} 3307 (br), 2975 (w), 2935 (w), 1657 (s), 1525 (s), 1434 (m), 1406 (m), 1252 (m), 1166 (m), 755 (m) cm^{-1}. ^1H NMR ($CDCl_3$:DMSO-d_6:CD_3OD = 5:1:1): δ 7.46 (*m*, 1H), 7.38 (*m*, 1H), 7.27 (*m*, 1H), 6.92 (*s*, 1H), 6.88 (*s*, 1H), 6.79 (*s*, 1H), 6.62 (*s*, 1H), 6.57 (*s*, 1H), 6.48 (*s*, 1H), 6.46 (*s*, 1H), 6.37 (*s*, 1H), 4.41 (*m*, 1H), 4.12 (*s*, 2H), 3.95 (*q*, *J* = 7.0 Hz, 1H), 3.53 (*s*, 3H), 3.51 (*s*, 3H), 3.46 (*s*, 3H), 3.57–3.30 (*m*, 2H), 3.10–2.74 (*m*, 10H), 1.89 (*t*, *J* = 7.5 Hz, 2H), 1.31 (*sex*, *J* = 7.5 Hz, 2H), 1.11 (*s*, 4.5H, *t*-Bu), 1.02 (*s*, 9H), 0.99 (*d*, *J* = 7.0 Hz, 3H), 0.95 (*s*, 4.5H, *t*-Bu), 0.59 (*t*, *J* = 7.5 Hz, 3H). FABHRMS m/z calcd for $C_{54}H_{74}O_{12}N_{16}H$ (M^+ + H): 1139.5750, found: 1139.5718. (*S*)-Bis(Boc)-AMPHIS-(*S*)-Ala-Gly-DST (**20e**): R_f (CH_2Cl_2:MeOH = 4:1) 0.35; $[\alpha]^{20}_D$ +8.6 (*c* 0.55; MeOH); IR (CH_2Cl_2 cast) υ_{max} 3305 (br), 2975 (w), 2934 (w), 1662 (s), 1519 (s), 1436 (m). 1405 (m), 1366 (w), 1248 (m), 1166 (w), 754 (w) cm^{-1}. ^1H NMR ($CDCl_3$:DMSO-d_6:CD_3OD = 5:1:1): δ 7.58 (*d*, *J* = 6.0 Hz, 1H), 7.46 (*t*, *J* = 6.0 Hz, 1H), 7.40 (*s*, 1H), 7.30 (*m*, 1H), 7.02 (*d*, *J* = 2.0 Hz, 1H), 6.97 (*d*, *J* = 2.0 Hz, 1H), 6.90 (*d*, *J* = 2.0 Hz, 1H), 6.73 (*s*, 1H), 6.65 (*d*, *J* = 2.0 Hz, 1H), 6.60 (*d*, *J* = 2.0 Hz, 1H), 6.39 (*d*, *J* = 2.0 Hz, 1H), 4.61 (*m*, 1H), 4.20 (*s*, 2H), 4.06 (*m*, 1H), 3.63 (*s*, 6H), 3.50 (*s*, 3H), 3.30–2.80 (*m*, 12H), 1.99 (*t*, *J* = 7.5 Hz, 2H), 1.40 (*sex*, *J* = 7.5 Hz, 2H), 1.20 (*s*, 4.5 H, *t*-Bu), 1.13 (*s*, 9H), 1.15 (*d*, *J* = 7.0 Hz, 3H), 1.02 (*s*, 4.5 H, *t*-Bu), 0.68 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z found: 1139.5731.

(*S*)-AMPHIS-ABA-DAP-DST (**1c**). Compound **20c** (18 mg, 0.016 mmol) in trifluoroacetic acid (1 mL) was stirred at 0 °C for 30 min. After the solvent was removed *in vacuo*, the residue was made basic with aqueous NH_3 solution (0.5 mL) to pH 9 and then loaded on Amberlite XAD-2 resin. The column was initially washed with H_2O to neutral and subsequently with MeOH. Concentration of the methanolic fraction provided **1c** (12 mg, 81% yield) as a white foam. R_f (AcOH:*n*-BuOH:H_2O = 1:1:1) 0.39; $[\alpha]^{20}_D$ +2.2 (*c* 1.15; MeOH); IR (MeOH cast) υ_{max} 3281 (br), 2967 (w), 1652 (s), 1644 (s), 1576 (s), 1520 (s), 1433 (s), 1402 (m), 1259 (m), 1120 (w), 1095 (m), 754 (w) cm^{-1}; ^1H NMR (DMSO-d_6:$CDCl_3$:CD_3OD = 1:1:1): δ 7.86 (*d*, *J* = 7.0 Hz, 1H), 7.77 (*t*, *J* = 7.0 Hz, 1H), 7.68 (*s*, 1H), 7.36 (*d*, *J* = 7.0 Hz, 1H), 7.14 (*m*, 2H), 7.06 (*d*, *J* = 2.0 Hz, 1H), 6.88 (*s*, 1H), 6.86 (*d*, *J* = 2.0 Hz, 1H), 6.78 (*d*, *J* = 2.0 Hz, 1H), 6.73 (*d*, *J* = 2.0 Hz, 1H), 4.70 (*m*, 1H), 3.97 (*s*, 2H), 3.82 (*s*, 3H), 3.79 (*s*, 3H), 3.75 (*s*, 3H), 3.25–3.00 (*m*, 8H), 2.97 (*m*, 2H), 2.88 (*m*, 2H), 2.18 (*t*, *J* = 7.5 Hz, 2H), 2.05 (*m*, 2H), 1.77–1.50 (*m*, 6H), 0.87 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z calcd for $C_{44}H_{59}O_7N_{15}H$ (M^+ + H): 910.4800, found: 910.4791.

(S)-AMPHIS-(R) and (S)-Ala-Gly-DST (1d, e). Compound **20d** (40 mg, 0.035 mmol) gave **1d** (28 mg, 85% yield) and **20e** (18 mg, 0.016 mmol) gave **1e** (11.2 mg, 75% yield). Compound **1d**: R_f (*n*-BuOH:AcOH:H_2O = 1:1:1) 0.35; $[\alpha]^{20}_D$ +11 (*c* 0.77; MeOH); IR (MeOH cast) υ_{max} 3313 (br), 2962 (w), 2929 (w), 1653 (s), 1539 (s), 1520 (s), 1451 (m), 1391 (m), 1270 (s), 1175 (w), 1107 (m), 1069 (w), 1026 (w), 730 (m) cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.90 (*s*, 1H, -CONH-), 9.88 (*s*, 1H, -CONH-), 9.75 (*s*, 1H, -CONH-), 8.95 (*br*, 1H, -CONH-), 8.44 (*br*, 1H, -CONH-), 8.00 (*br*, 1H, -CONH-), 7.93 (*m*, 1H), 7.87 (*m*, 1H), 7.62 (*m*, 1H), 7.57 (*s*, 1H), 7.23 (*d*, *J* = 2.0 Hz, 1H), 7.18 (*d*, *J* = 2.0 Hz, 1H), 7.14 (*d*, *J* = 2.0 Hz, 1H), 7.02 (*d*, *J* = 2.0 Hz, 1H), 6.88 (*s*, 1H), 6.87 (*d*, *J* = 2.0 Hz, 1H), 6.83 (*d*, *J* = 2.0 Hz, 1H), 4.67 (*m*, 1H), 4.21 (*m*, 1H), 3.87 (*s*, 2H), 3.83 (*s*, 3H), 3.82 (*s*, 3H), 3.78 (*s*, 3H), 3.68 (*m*, 2H), 3.40–3.00 (*m*, 6H), 2.80 (*t*, *J* = 6.5 Hz, 2H), 2.68 (*t*, *J* = 6.5 Hz, 2H), 2.20 (*t*, *J* = 7.5 Hz, 2H), 1.58 (*sex*, *J* = 7.5 Hz, 2H), 1.21 (*d*, *J* = 7.0 Hz, 3H), 0.89 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z calcd for $C_{44}H_{58}O_8N_{16}H$ (M^+ + H): 939.4701, found: 939.4707.

Compound **1e**: R_f (*n*-BuOH:AcOH:H_2O = 1:1:1) 0.35; $[\alpha]^{20}_D$ +3.5 (*c* 0.86; MeOH); IR (MeOH cast) υ_{max} 3288 (br), 2962 (w), 2931 (w), 1653 (s), 1522 (s), 1436 (m), 1260 (m), 1096 (m), 800 (m) cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.89 (*s*, 2H, -CONH-), 9.76 (*s*, 1H, -CONH-), 9.00 (*br*, 1H,-CONH-), 8.85 (*br*, 1H, -CONH-), 8.52 (*br m*, 1H, -CONH-), 8.02 (*br m*, 1H, -CONH-), 7.92 (*d*, *J* = 7.5 Hz, 1H), 7.89 (*t*, *J* = 7.5 Hz, 1H), 7.78 (*br*, 1H, -CONH-), 7.62 (*d*, *J* = 7.5 Hz, 1H), 7.58 (*s*, 1H), 7.18 (*d*, *J* = 2.0 Hz, 1H), 7.15 (*d*, *J* = 2.0 Hz, 1H), 7.02 (*d*, *J* = 2.0 Hz, 1H), 6.88 (*s*, 1H), 6.87 (*d*, *J* = 2.0 Hz, 1H), 6.83 (*d*, *J* = 2.0 Hz, 1H), 4.67 (*m*, 1H), 4.23 (*m*, 1H), 3.85 (*s*, 2H), 3.83 (*s*, 3H), 3.82 (*s*, 3H), 3.78 (*s*, 3H), 3.74 (*m*, 2H), 3.40–3.00 (*m*, 6H), 2.79 (*t*, *J* = 6.5 Hz, 2H), 2.65 (*t*, *J* = 6.5 Hz, 2H), 2.20 (*t*, *J* = 7.5 Hz, 2H), 1.58 (*sex*, *J* = 7.5 Hz, 2H), 1.25 (*d*, *J* = 7.0 Hz, 3H), 0.88 (*t*, *J* = 7.5 Hz, 3H); FABHRMS m/z found: 939.4685.

Biochemistry

Buffers used and their abbreviations are TE: 25 mM Tris–HCl, pH 7.3; F_{12} (fluorescence assay solution, pH 12): 0.02 M K_3PO_4, 0.5 mM EDTA and 0.5 μg mL^{-1} ethidium bromide; TBE: 0.089M Tris, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0. Fluorescence was measured on a Turner Model 430 spectrofluorometer; PM2 DNA was purchased from Boehringer Mannheim and was used in the experiments without further purification (fluorescence assay showed that it contained more than 80% of covalently closed circular form). Restriction enzymes *Hind* III and *Eco* RV and pBR322 DNA were also from Boehringer Mannheim, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, restriction enzymes *Hpa* II and *Eco*O 109 I, SV40 viral DNA, and sonicated calf thymus DNA were from GIBCO BRL, and [γ-^{32}P]ATP was from New England Nuclear. Blenoxane® was fractionated by a published procedure[7b] to provide bleomycin A_2 and B_2. All other reagents were analytical grade and were used as received.

Kinetic studies

The reactions of the drugs with PM2 DNA were carried out in 53 μL of TE solution which contained 1 A_{260} of DNA, 80 μM drug–Fe(II) complexes, and 1 mM 1,4-dithiothreitol(DTT). Buffered solutions of the drug–Fe(II) complexes were freshly prepared immediately before each experiment. The reactions were run at room temp. and 10 μL of the reaction mixture was pipetted into 2 mL of F_{12} solution at 0, 5, 15, 30, and 60 min. The solution was denatured at 95 °C for 5 min, and cooled to room temp. before measuring the fluorescence. The percentage of CCC DNA remaining was calculated by dividing the fluorescence of drug–DNA reaction mixtures by that of the control mixture.

Electrophoretic mobility shift assays

The reaction of the drugs with PM2 DNA were carried out in 10 μL of TE solution which contained 0.93 A_{260} of DNA and varying concentrations of drugs. The reactions were run at room temp. for 25 min. The resultant reaction mixtures were examined by electrophoresis through 5.6 mm thick 1% agarose gels with TBE running buffer. The gels were run at room temp. at a voltage of 3.33 V cm^{-1} for 17 h. The resulting gels were stained with ethidium bromide in H_2O at a concentration of 0.5 μg mL^{-1}. Bands were visualized by 300 nm UV transillumination and photographed on Polaroid 667 film.

Sequencing gel assays

Plasmid pBR322 was cut with *Hind* III, dephosphorylated with calf intestinal alkaline phosphatase, and labeled at the 5' end using [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled DNA was then cut with *Eco* RV and the desired 158 bp fragment was purified by nondenaturing PAGE and isolated by a crush and soak procedure.[18] SV40 viral DNA was cut with *Hpa* II, dephosphorylated and labeled as for pBR322, cut with *Eco*O 109 I, and purified and isolated as for pBR322. DNA cleavage reactions contained ~50,000 dpm of labeled DNA, 5 μM (nucleotides) sonicated calf thymus DNA, and 5 mM Na cacodylate buffer, pH 7.5, in a 10 μL reaction volume. Fe(II) and DTT solutions were prepared immediately before use. Concentrations of Fe(II), DNA cleaving compound, and DTT are given in the Figures. Reactions were initiated by admixture of all components and were allowed to proceed at 25 °C for 30 min. Reactions were stopped by addition of a loading buffer containing 10 M urea, 1.5 mM EDTA, and 0.05% each of Bromophenol Blue and Xylene Cyanol. The reaction mixtures were then lyophilized and redissolved in 2 μL H_2O, heated to 90 °C for 5 min and chilled on ice before PAGE analysis. Sequence lanes were produced by the method of Maxam and Gilbert.[18,19] Electrophoresis was performed in TBE buffer on 0.4 mm thick, 55 cm long, 8% polyacrylamide gels containing 7 M urea at 55 °C and 2000 V. Gels were autoradiographed at –70 °C using Kodak X-Omat AR film.

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