An Additional Spirocyclization for Duocarmycin SA

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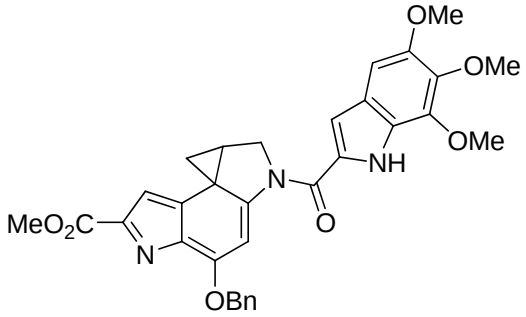


4. A solution of P_4-phosphazene base (1 M in hexanes, 46 μL, 0.046 mmol) was added to a solution of **3** (17 mg, 0.028 mmol) in anhydrous MeCN (0.46 mL) under Ar. The reaction mixture was stirred at 23 °C for 3.5 h, after which an equal volume of toluene was added and the MeCN was removed under a stream of N_2. The toluene solution was subjected to chromatography directly (SiO_2, 0–20% acetone–toluene gradient) to afford **4** (9.0 mg, 56%): ^{1}H NMR (acetone-d_6, 600 MHz) δ 11.39 (br s, 1H), 7.44 (m, 2H), 7.30–7.35 (m, 3H), 6.94 (s, 1H), 6.70 (s, 1H), 6.67 (d, 1H, J = 2.4 Hz), 5.01 (d, 1H, J = 12.0 Hz), 4.95 (d, 1H, J = 12.0 Hz), 4.82 (s, 1H), 3.96 (d, 1H, J = 12.0 Hz), 3.85 (s, 3H), 3.77 (s, 3H), 3.72 (s, 3H), 3.45 (dd, 1H, J = 4.2, 12.0 Hz), 3.33 (s, 3H), 1.82 (t, 1H, J = 7.8 Hz), 1.70 (m, 1H), 0.72 (dd, 1H, J = 5.4, 6.6 Hz); ^{13}C NMR (acetone-d_6, 150 MHz) δ 164.6, 162.6, 153.4, 142.00, 141.97, 138.2, 136.1, 130.9, 130.6, 130.2, 129.7, 125.5, 125.1, 124.3, 111.3, 101.5, 100.3, 99.5, 98.1, 92.2, 71.6, 62.1, 61.8, 57.4, 52.4, 45.3, 36.6, 29.5, 13.7; UV (CH_3CN) λ_{max} 311 nm (ε 23,700), 325 nm (ε 20,100); ESITOF–HRMS *m/z* 568.2077 ($[M + H]^+$, $C_{32}H_{30}N_3O_7$ requires 568.2078).
(–)-**4**: $[\alpha]_D$ –186 (*c* 0.1, THF), natural enantiomer.
(+)-**4**: $[\alpha]_D$ +186 (*c* 0.1, THF), unnatural enantiomer.

NMR analysis of 4. The ^{1}H NMR of **4** displayed three well-defined cyclopropane CH signals at δ 0.72, 1.70, and 1.82, and characteristic diastereotopic signals for C1-H_2 exhibiting a large geminal coupling constant of 12.0 Hz (δ 3.45 and δ 3.96) analogous to those observed with duocarmycin SA itself (Supporting Information Figure S1). The most diagnostic ^{1}H NMR signals proved to be the disappearance of the indole NH (δ 10.30 in acetone-d_6) and a marked shift in the C3-H from δ 8.19 (br s, 1H) in the precursor **3** to δ 4.82 (s, 1H) in **4**. This latter remarkable shift in C3-H is indicative of an aromatic CH in **3** and its conversion to a β-CH of an isolated electron-rich olefin (enamide) in **4**.

The ^{13}C NMR spectrum of **4** also exhibited characteristic distinctions between **4** and the natural product. Although the chemical shift of C-8 in **4** (δ 13.7) is indicative of a cyclopropane, the same carbon in duocarmycin SA is shifted significantly downfield (δ 27.4), reflecting the different electronic environments of the two ring systems. Aside from the C4-carbonyl carbon of **1** (δ 179.0) which is now an olefinic carbon in the extended π-system of **4** (δ 138.2), perhaps the greatest difference in chemical shift is seen with C-2a. This carbon in **1** (δ 163.0) displays the

characteristic chemical shift of a β-carbon of a polarized α,β-unsaturated enone system, whereas the same carbon in **4** shifts dramatically upfield to δ 92.2, once again mirroring the conversion to an electron-rich olefin.

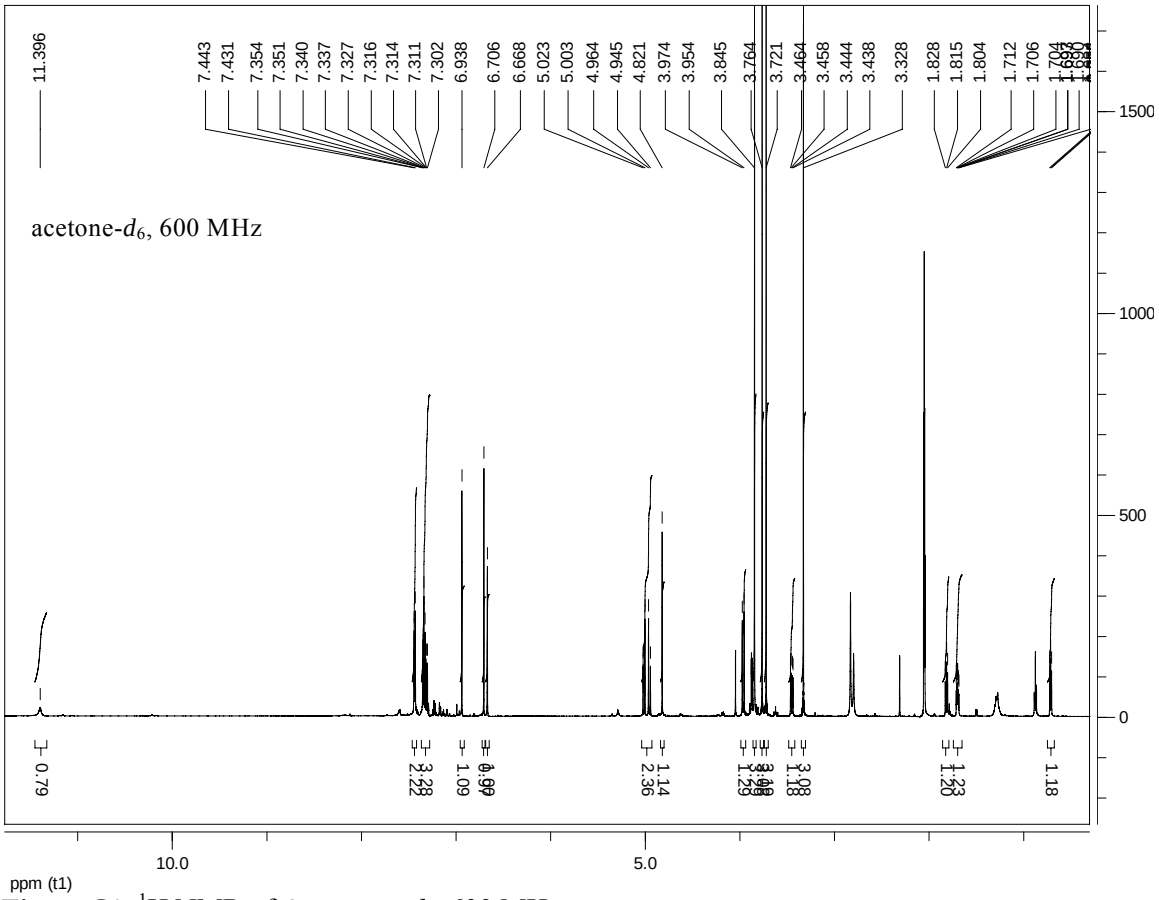


Figure S1. ^{1}H NMR of **4**, acetone-d_6, 600 MHz.

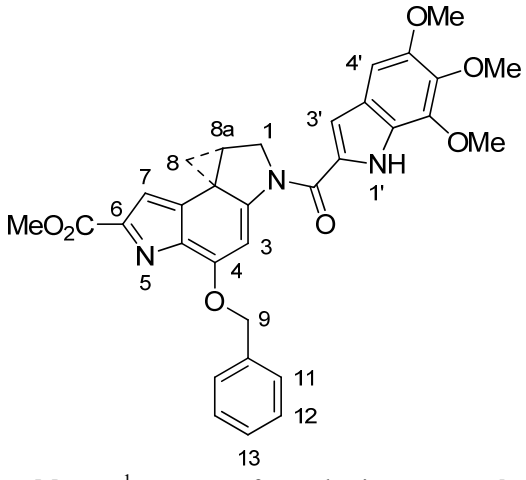


Table S1. ^{1}H NMR of **4** and **1** in acetone-d_6.

Assignment	**4 (acetone-d_6, 600 MHz)**	**Duocarmycin SA (1) (acetone-d_6, 600 MHz)**
1-Ha (1H)	3.96 (d, J = 12.0 Hz)	4.47 (d, J = 10.2 Hz)

1-Hb (1H)	3.45 (dd, J = 4.2, 12.0 Hz)	4.58 (dd, J = 4.8, 10.2 Hz)
3-H (1H)	4.82 (s)	7.11 (s)
5-NH (1H)	---	11.20 (br s)
6-$COOCH_3$ (3H)	3.76 (s)	3.86 (s)
7-H (1H)	6.67 (d, J = 2.4 Hz)	6.75 (s)
8-Ha (1H)	0.72 (t, J = 6.0 Hz)	1.63 (t, J = 4.2 Hz)
8-Hb (1H)	1.82 (dd, J = 6.6, 7.8 Hz)	1.82 (dd, J = 4.2, 7.8 Hz)
8a-H (1H)	1.70 (m)	3.04 (dt, J = 4.8, 7.8 Hz)
9-Ha (1H)	4.95 (d, J = 12.0 Hz)	---
9-Hb (1H)	5.01 (d, J = 12.0 Hz)	---
11-H (2H)	7.44 (m)	---
12-H (2H)	7.35 (m)	---
13-H (1H)	7.31 (m)	---
1'-NH (1H)	11.39 (br s)	10.48 (br s)
3'-H (1H)	6.94 (s)	6.94 (s)
4'-H (1H)	6.71 (s)	6.82 (s)
5'-OCH_3 (3H)	3.72 (s)	3.85 (s)
6'-OCH_3 (3H)	3.33 (s)	3.86 (s)
7'-OCH_3 (3H)	3.85 (s)	4.00 (s)

Table S2. ^{13}C NMR for **4** and duocarmycin SA (**1**) in acetone-d_6.

Assignment	**4 (acetone-d_6, 600 MHz)**	**Duocarmycin SA (1) (acetone-d_6, 600 MHz)**
C1	45.3	56.8
C2a	92.2	163.0
C3	98.1	113.5
C4	*	179.0
C4a	130.5	133.9
C6	124.3	128.6
6-$COOCH_3$	162.6	162.6
6-$COOCH_3$	52.4	53.0
C7	111.3	109.9
C7a	125.5	132.0
C7b	29.4	32.9
C8	13.7	27.4
C8a	36.6	25.5
C9	71.6	---
C10	*	---
C11	129.7	---
C12	130.1	---
C13	129.8	---
C2'	*	131.6
C2'α	164.6	163.4
C3'	100.2	109.4
C3'a	*	125.2
C4'	99.4	99.9
C5'	152.5	152.2

C6’	142.00	142.9
C7’	141.97	141.1
C7’a	125.1	128.2
5’-OCH_3	57.4	57.4
6’-OCH_3	61.8	62.4
7’-OCH_3	62.1	62.5

*Unable to assign using ^{1}H, ^{13}C, HMQC, and HMBC NMR.

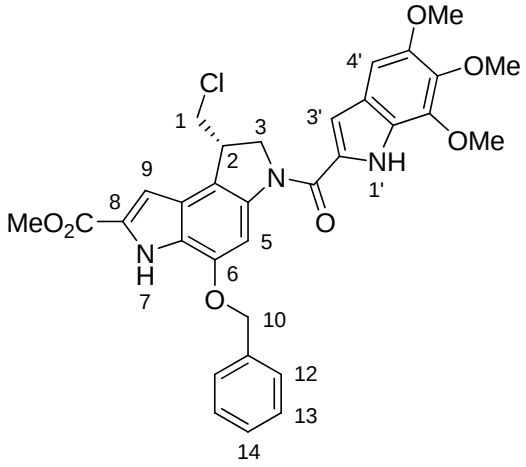


Table S3. ^{1}H NMR of **3** in acetone-d_6.

Assignment	**3 (acetone-d_6, 600 MHz)**
1-Ha (1H)	4.60 (dd, *J* = 3.6, 10.2 Hz)
1-Hb (1H)	4.78 (t, *J* = 10.2 Hz)
2-H (1H)	4.20 (m)
3-Ha (1H)	3.92 (dd, *J* = 7.8, 10.8 Hz)
3-Hb (1H)	4.17 (dd, *J* = 3.6, 10.8 Hz)
5-H (1H)	8.19 (br s)
7-NH (1H)	11.15 (br s)
8-$COOCH_3$ (3H)	3.87 (s)
9-H (1H)	7.30 (d, *J* = 1.8 Hz)
10-H (2H)	5.26 (s)
12-H (2H)	7.57 (d, *J* = 7.2 Hz)
13-H (2H)	7.40 (m)
14-H (1H)	7.34 (m)
1’-NH (1H)	10.30 (br s)
3’-H (1H)	7.08 (d, *J* = 1.8 Hz)
4’-H (1H)	6.98 (s)
5’-OCH_3 (3H)	3.86 (s)
6’-OCH_3 (3H)	3.87 (s)
7’-OCH_3 (3H)	4.03 (s)

Table S4. ^{13}C NMR of **3** (acetone-d_6).

Assignment	**3 (acetone-d_6, 600 MHz)**
C1	56.7
C2	44.7
C3	48.9
C4a	*

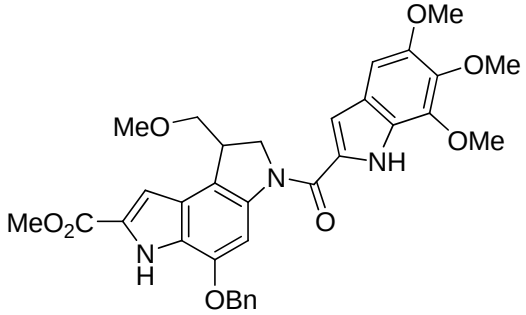
C5	100.3
C6	147.5
C6a	125.8
C8	128.4
8-$COOCH_3$	163.2
8-$COOCH_3$	53.0
C9	107.9
C9a	130.5
C9b	116.4
C10	71.8
C11	138.9
C12	129.74
C13	130.2
C14	129.72
C2’	*
C2’α	161.4
C3’	107.8
C3’a	*
C4’	100.0
C5’	*
C6’	152.0
C7’	*
C7’a	127.3
5’-OCH_3	62.4
6’-OCH_3	57.5
7’-OCH_3	62.5

*Unable to assign using ^{1}H, ^{13}C, HMQC, and HMBC NMR.

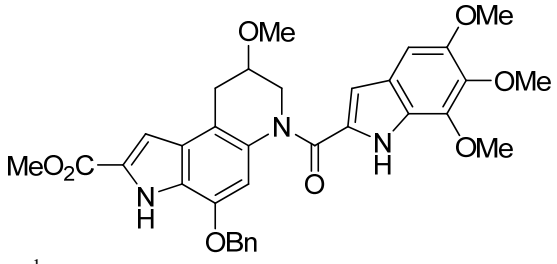
Table S5. UV of **1** and **4**.

Duocarmycin SA (**1**, MeCN)		**4** (MeCN)	
λ_{max}, nm	ε ($cm^{-1}mol^{-1}L$)	λ_{max}, nm	ε ($cm^{-1}mol^{-1}L$)
313	17100	311	23700
355	27800	325	20100

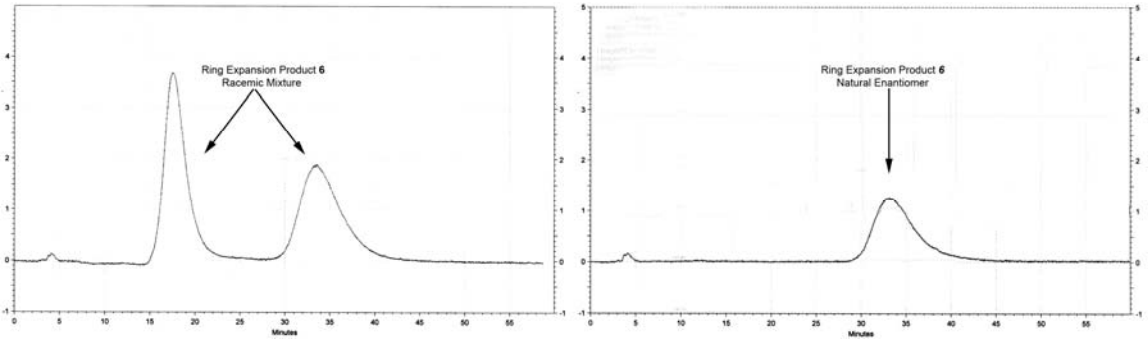
Addition of MeOH to 4. A sample of **4** (3.0 mg, 0.005 mmol) was dissolved in 3 mL of MeOH and stirred for 4 days at ambient temperature. PTLC (SiO_2, 10% acetone–toluene) of the crude reaction mixture afforded **5** (0.8 mg, 25%) and **6** (1.3 mg, 41%) as clear residues.



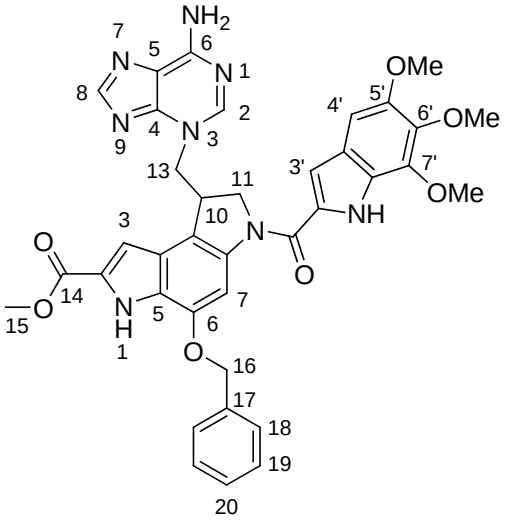
5: ^{1}H NMR (acetone-d_6, 600 MHz) δ 11.06 (br s, 1H), 10.17 (br s, 1H), 8.20 (br s, 1H), 7.60 (d, 2H, J = 7.2 Hz), 7.42 (m, 2H), 7.36 (m, 1H), 7.24 (s, 1H), 7.07 (s, 1H), 7.00 (s, 1H), 5.29 (s, 2H), 4.71 (t, 1H, J = 10.2 Hz), 4.50 (dd, 1H, J = 4.2, 10.2 Hz), 4.05 (s, 3H), 4.00 (m, 1H), 3.879 (s, 3H), 3.877 (s, 3H), 3.87 (s, 3H), 3.85 (t, 1H, J = 4.2 Hz), 3.55 (t, 1H, J = 8.4 Hz), 3.36 (s, 3H); ESIOF–HRMS *m/z* 600.2349 ([M + H]$^+$, $C_{33}H_{34}N_3O_8$ requires 600.2340).



6: ^{1}H NMR (acetone-d_6, 600 MHz) δ 11.11 (br s, 1H), 10.22 (br s, 1H), 7.31 (d, 2H, J = 6.6 Hz), 7.25 (m, 3H), 6.78 (s, 1H), 6.70 (s, 1H), 6.33 (s, 1H), 4.85 (d, 2H, J = 4.2 Hz), 4.27 (dd, 1H, J = 5.4, 13.2 Hz), 3.98 (m, 2H), 3.95 (s, 3H), 3.87 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.36 (s, 3H), 3.33 (dd, 1H, J = 5.4, 16.8 Hz), 3.02 (dd, 1H, J = 4.2, 16.8 Hz); ESITOF–HRMS *m/z* 600.2343 ([M + H]$^+$, $C_{33}H_{34}N_3O_8$ requires 600.2340).



HPLC analysis (ChiralCel OD, 0.46 × 25 cm, 40% *i*-PrOH/hexanes, 1.2 mL/min, 303 nm) of the ring expansion product **6** derived from the addition of MeOH to both racemic and (–)-**4** (natural enantiomer) are illustrated above.



Adenine adduct 7. Calf thymus DNA (Sigma, 303 mg, *ca*. 55 bp equiv) was added to 10 mM sodium phosphate buffer (59 mL, pH 7.1). The mixture was sonicated for 1 h and then stirred vigorously overnight to afford an opaque mixture. A sample of **4** (3.1 mg, 0.006 mmol) was

dissolved in 1.5 mL of DMF, added to the DNA mixture, and the solution was stirred at 23 °C for 24 h. EtOH (118 mL) and 3.0 M NaOAc (5.7 mL) were added and the reaction mixture was cooled to –78 °C for 1.5 h. The mixture was centrifuged for 30 min and the supernatant was removed. The residual pellet was suspended in 73 mL of 10 mM sodium phosphate buffer. *n*-BuOH (76 mL) was added and the mixture was warmed to 100 °C for 30 min. The layers were separated, and the thermal depurination and extraction were repeated. The organic layers were combined and concentrated to provide a white residue. Chromatography (SiO_2, 0–10% MeOH–$CHCl_3$ gradient) afforded **7** as a clear residue (2.5 mg, 68%). The EtOH extract was concentrated and subjected to chromatography (SiO_2, 0–20% acetone–toluene gradient) to afford to afford the mass balance of the reaction (2.0 mg) in two fractions. LC/MS analysis of these fractions indicated a small amount of additional **7** (2%) and a mixture of products consistent with addition of phosphate (12%), water (6%), acetate (5%), and ethanol (6%) to **4**. For **7**: ^{1}H NMR (DMF-d_7, 600 MHz) δ 12.38 (br s, 1H), 11.30 (br s, 1H), 8.40 (s, 1H), 8.17 (br s, 1H), 7.91 (s, 1H), 7.70 (d, 2H, J = 6.6 Hz), 7.45 (m, 2H), 7.38 (m, 1H), 7.21 (s, 1H), 7.09 (d, 1H, J = 1.8 Hz), 6.99 (s, 1H), 5.35 (br s, 2H), 4.91 (dd, 1H, J = 4.2, 13.2 Hz), 4.81 (dd, 1H, J = 10.2 Hz), 4.71–4.66 (m, 2H), 4.63–4.60 (m, 1H), 4.04 (s, 3H), 3.92 (s, 3H), 3.89 (s, 3H), 3.88 (s, 3H); ^{13}C NMR (DMF-d_7, 150 MHz) δ 162.7, 161.1, 156.6, 154.2, 151.6, 150.8, 146.9, 144.5, 141.4, 140.5, 138.3, 132.8, 130.2, 129.5, 128.93, 128.88, 127.8, 126.7, 125.4, 125.0, 115.9, 107.4, 107.1, 99.2, 71.0, 62.0, 61.8, 56.9, 55.8, 53.9, 52.5, 41.3; ESITOF–HRMS *m/z* 703.2626 ($[M + H]^+$, $C_{37}H_{35}N_8O_7$ requires 703.2623).

It is likely that conducting the DNA alkylation reaction at increasing compound–base pair ratios (e.g. 1:5 vs 1:55) would provide detectable and progressively increasing amounts of guanine alkylation and depurination.[7,10] The regioselectivity of the adenine addition was established through use of NMR (^{1}H, ^{13}C, HMQC) and direct comparison with the assigned chemical shifts of the duocarmycin SA–adenine adduct.[S1] The ^{1}H NMR showed a good correlation between the chemical shifts and coupling constants of **7** and the adenine addition product with duocarmycin SA (Table S6). C10-H was found to be a single proton at the characteristic chemical shift of δ 4.61. The C10-H of the alternative addition product resulting in ring expansion would appear as 2H (δ 3.0–4.0) with a very large geminal coupling constant (J = 19.5 Hz) characteristic of duocarmycin B1/C1,[S2] and **6**. The additional chemical shifts of C11-H_2 and C13-H_2, their observed couplings, and the assigned coupling constants were fully consistent with this assignment. Thus, C13-H was found to exist as two signals, one of which (a doublet of doublets) exhibited a large coupling constant of 13.6 Hz. The remaining C13-H was located underneath C11-H, and their coupling constants could not be determined. The ^{13}C NMR of **7** was also found to be in good agreement with those of the duocarmycin SA, duocarmycin A, and yatakemycin adenine adducts, confirming adenine addition to the least substituted cyclopropane carbon. The key signals distinguishing the possible adducts are found in the carbons within or proximal to the fused 5- versus 6-membered ring with **7** exhibiting chemical shifts consistent only with the former. The most distinguishing signal in the ^{13}C NMR (Table S7) was the chemical shift of C10 (δ 41.3), consistent with that observed with the duocarmycin SA–adenine adduct (δ 41.1) and related structures as well as **3** (δ 44.7), and distinct from the shift (δ 33–34) expected of a 6-membered ring adduct. This signal is considerably upfield of both C11 and C13 methylene carbons (δ 53.9, 55.8), consistent with the pattern seen in the analogous adenine adducts with duocarmycin A, duocarmycin SA, and yatakemycin. The similarity of the chemical shifts and coupling constants of the rigorously assigned, closely related agents confirms that the addition of adenine occurs at the least-substituted cyclopropane carbon of **4**.

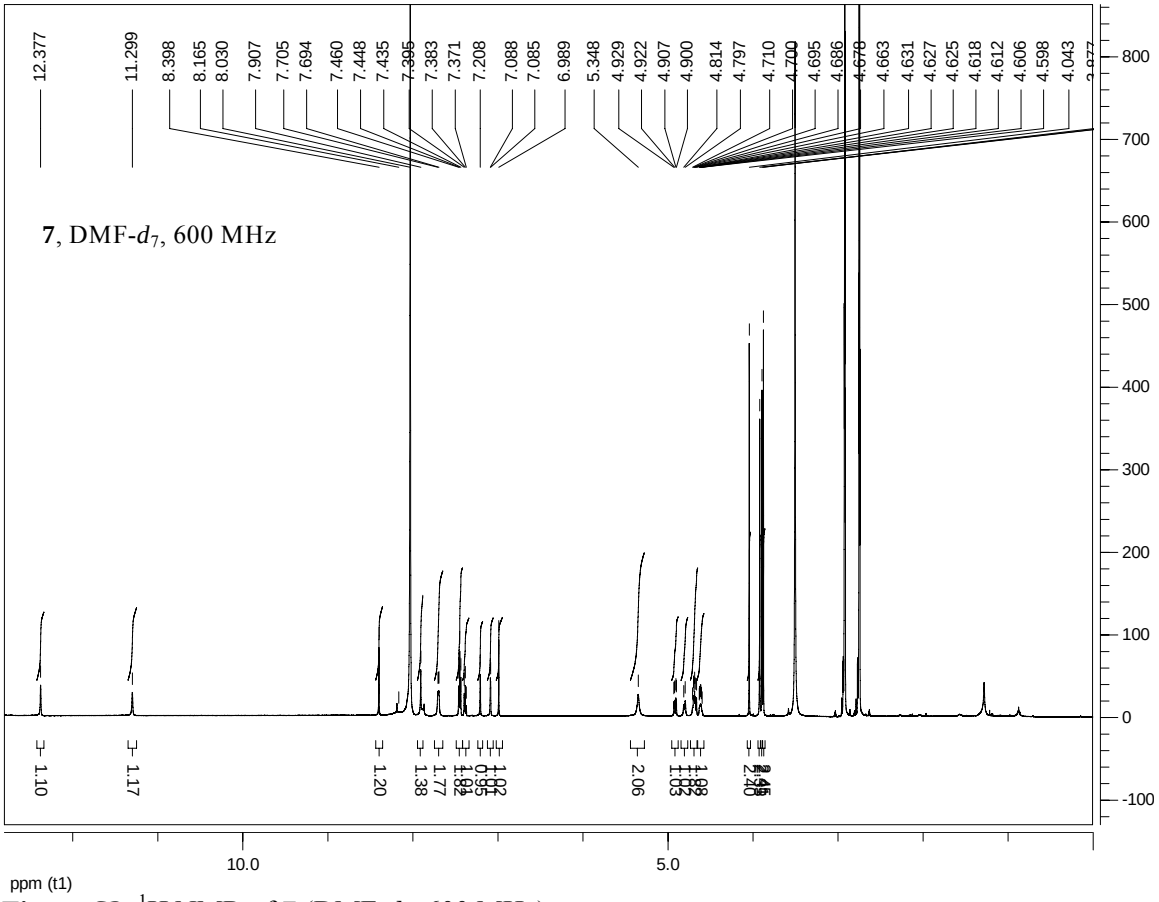


Figure S2. ^{1}H NMR of **7** (DMF-d_7, 600 MHz).

Table S6. ^{1}H NMR comparison between selected signals of **7** and duocarmycin SA–adenine adduct.[S1]

Assignment	**7 (DMF-d_7)**	**Duocarmycin SA–adenine (acetone-d_6)**
NH-1	12.34 (s)	10.73 (s)
C3-H	7.21 (s)	6.97 (s)
C7-H	8.17 (br s, under DMF)	7.92 (br s)
C10-H	4.61 (m)	4.55 (m)
C11-Ha	4.70 (m)[a]	4.61 (dd, J = 11.0, 8.6 Hz)
C11-Hb	4.80 (br d, J = 10.2 Hz)	4.82 (br d, J = 11.0 Hz)
C13-Ha	4.68 (m)[a]	4.67 (dd, J = 13.6, 7.4 Hz)
C13-Hb	4.92 (dd, J = 13.6, 4.2 Hz)	4.89 (dd, J = 13.6, 4.9 Hz)
C15-H	3.92 (s)	3.89 (s)
C16-H	5.35 (s)	---
C18-H	7.70 (d, J = 6.6 Hz)	---
C19-H	7.45 (m)	---
C20-H	7.38 (m)	---
NH-1’	11.30 (br s)	10.16 (s)
C3’-H	7.09 (d, J = 1.8 Hz)	6.89 (s)
C4’-H	6.99 (s)	6.96 (s)
C5’-OCH_3	3.89 (s)	3.85 (s)
C6’-O CH_3	3.88 (s)	3.85 (s)

C7'-O CH_3	4.04 (s)	4.02 (s)
Ade-C2-H	8.40 (s)	7.79 (s)
Ade-C8-H	7.91 (s)	8.22 (s)

[a] C11-H and C13-H obscure each other, resulting in multiplets.

Table S7. ^{13}C NMR comparison between selected signals[a] of **7** and duocarmycin SA–adenine adduct.

Assignment	**7 (DMF-d_7)**	**Duocarmycin SA–adenine (acetone-d_6)**
C3	107.4	106.2
C7	*	113.3
C10	41.3	41.1
C11	55.8	53.9
C13	53.9	58.9
C15	52.5	52.1
Ade-C2	144.5	153.0[b]
Ade-C8	154.2	146.4[b]

***** Correlation between C7-H and C7 not found by HMQC. All carbons present in spectrum.

[a] Signals assigned using ^1H, ^{13}C, and HMQC NMR. Unassigned signals not shown.

[b] Assignment reversed. HMBC of 3-methyl-adenine indicates that δ 146.4 is consistent with C2.

Solvolysis of 4. A sample of **4** (10 μg, 0.018 μmol) was dissolved in 0.1 mL of MeCN and added to a mixture of MeOH (1.5 mL) and universal buffer (pH 2–7; 1.5 mL. 0.2 M boric acid, 0.05 M citric acid, 0.1 M Na_3PO_4, and deionized H_2O). The cuvette was stoppered, inverted 5 times, and UV–Vis spectra was acquired every 1–2 min until the reaction was complete. The increase in absorbance at 240 nm was monitored. The solvolysis rates were calculated from the least-squares treatment of the slope of plots of time versus ln $[(A_f - A_i)/(A_f - A)]$.

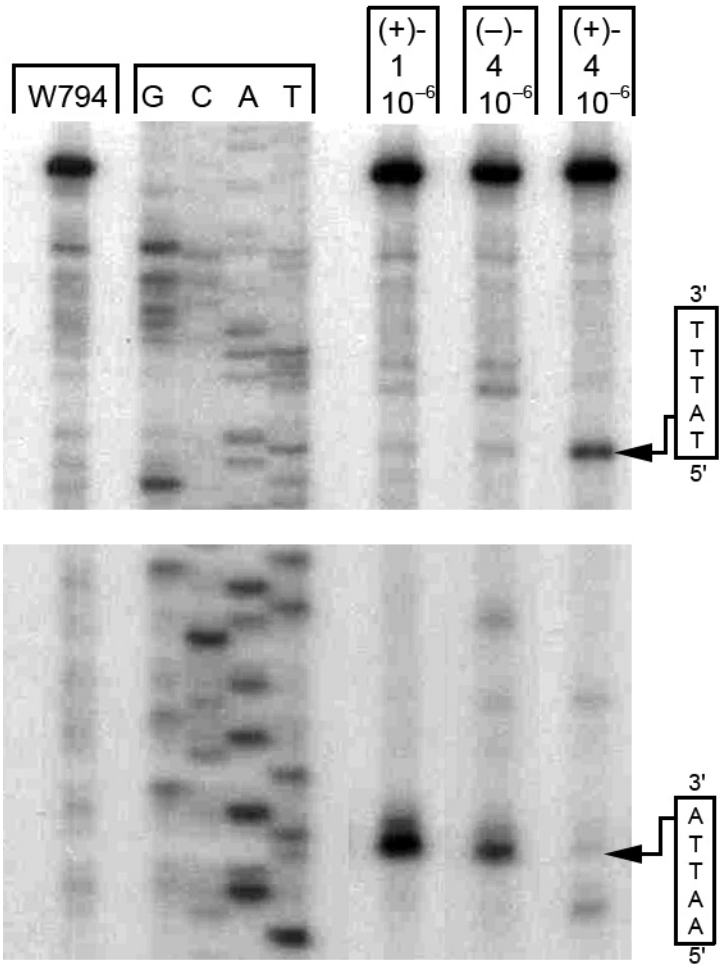


Figure S3. Thermally-induced strand cleavage of w794 DNA (144 bp, nucleotide no. 5238–138) after DNA–agent incubation with (+)-duocarmycin SA (**1**) and **4** (22 h, 23 °C), removal of unbound agent by EtOH precipitation and 30 min thermolysis (100 °C), followed by denaturing 8% PAGE and autoradiography. Lane 1, control DNA; lanes 2–5, Sanger G, C, A, and T sequencing standards; lane 6, (+)-duocarmycin SA (**1**, 1×10^{-6} M); lanes 7 and 8, nat.-(–)-**4** and *ent*-(+)-**4** (1×10^{-6} M).

DNA Alkylation Selectivity. General procedures, the preparation of singly 5' end-labeled double-stranded DNA, gel electrophoresis, and autoradiography were conducted according to procedures described in full details elsewhere.[S3] Eppendorf tubes containing the 5' end-labeled w794 DNA (4.5 μL) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) were treated with the agent in DMSO (0.5 μL at the specified concentration). The solution was vortexed and centrifuged prior to incubation at 23 °C for the specified period of time. Unbound agent was removed by EtOH precipitation. The covalently modified DNA was resuspended in TE buffer (5 μL) and heated at 100 °C (3 × 10 min) to effect thermal depurination at the alkylation sites. Samples were allowed to cool to 25 °C, centrifuged, and 2.5 μL of formamide dye (0.03% xylene cyanol FF, 0.03% bromophenol blue, 8.7% Na_2EDTA 250 mM) was added to each sample. Prior to electrophoresis, each sample was denatured by heating at 100 °C for 5 min, centrifuged and placed in an ice block. The sample was loaded directly onto the gel (3.5 μL) alongside Sanger dideoxynucleotide sequencing reactions run as standards and analyzed by polyacrylamide gel electrophoresis (PAGE, 8% sequencing gel) under denaturing conditions (8 M urea) in TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM Na_2EDTA) followed by autoradiography.

DNA Alkylation Relative Rate. Following the procedure detailed above, an Eppendorf tube containing 5' end-labeled w794 DNA (4.5 μL per time point taken) in TE buffer (pH 7.6) was treated with agent (0.5 μL in DMSO per time point taken, 1×10^{-5} M in DMSO). The solution was mixed, incubated at 23 °C, and a 5 μL aliquot was quenched by EtOH precipitation at the prescribed timepoint. Following EtOH precipitation, samples were resuspended in TE buffer (5 μL, pH 7.6), thermally depurinated (100 °C, 3 × 10 min), formamide dye was added (2.5 μL), and samples were analyzed by PAGE and autoradiography as described above. Relative rates of alkylation for (+)-**1** and (+)- and (–)-**4** were derived from the slopes of the plots of the percent integrated optical density of the alkylation site cleavage bands versus time.

Supporting Information References:

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