Critical Role of the Linking Amide in CC-1065 and the Duocarmycins: Implications on the Source of DNA Alkylation Catalysis

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Abstract: The preparation and evaluation of both enantiomers of **5** are described and it constitutes an analogue of CBI-TMI (**4**), the duocarmycins, and CC-1065 in which the amide linking the alkylation and DNA binding subunits has been replaced by a methylene. The agent proved remarkably stable to acid-catalyzed solvolysis consistent with alkylation subunit stabilization derived from a fully engaged vinylogous amide. It was found to exhibit an acid-catalyzed solvolysis half-life ($t_{1/2}$) of 80 h, 824 h, and ca. 30 500 h (3.3 days, 34 days, and ca. 3.5 years) at pH 1, 2, and 3, respectively, and to be completely stable at pH 7. The removal of the linking amide resulted in a 10^5-fold loss in cytotoxic potency and the complete loss of DNA alkylation capabilities providing an agent that is $>10^6\times$ less effective than **4** and $>10^2\times$ less effective than even *N*-BOC-CBI or *N*-Ac-CBI. These observations highlight the critical importance of the linking amide and implicate a fundamental role in DNA alkylation catalysis. Thus, rather than enhancing DNA alkylation by facilitating C4 carbonyl protonation (acid catalysis), the removal of the linking amide abolished the capabilities for DNA alkylation. This is consistent with the intimate participation of the linking amide in catalysis derived from a DNA binding-induced conformation change that serves to disrupt the alkylation subunit cross-conjugated vinylogous amide stabilization activating the agents for nucleophilic attack.

The duocarmycins (**1**, **2**) and CC-1065 (**3**) are the parent members of a class of exceptionally potent antitumor antibiotics that derive their properties through a sequence selective alkylation of duplex DNA (Figure 1).[1−6] A key structural element of the natural products is the alkylation subunit vinylogous amide and the corresponding N^2 amide linkage that joins the alkylation and DNA binding subunits. In earlier studies,[7−9] we have documented the stabilization derived from the vinylogous amide and quantitated the reactivity consequences of its disruption. These studies along with the demonstration that the DNA alkylation reaction proceeds at a rate independent of pH,[10,11] that the DNA alkylation rates do not correlate with measured rates of acid-catalyzed reactivity (pH 2−3),[3,4,11−15]

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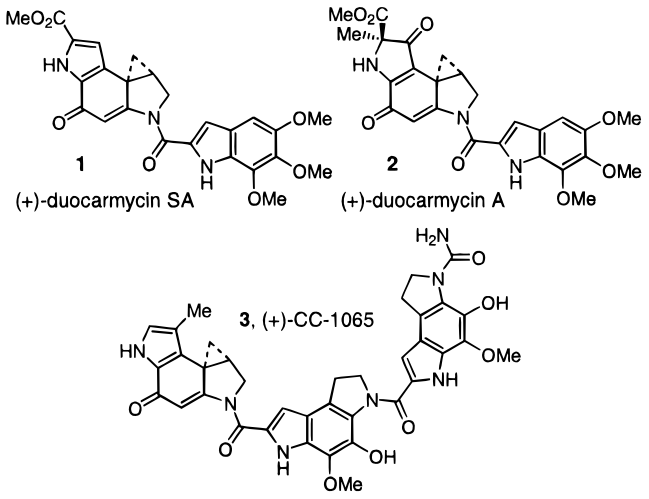

Figure 1.

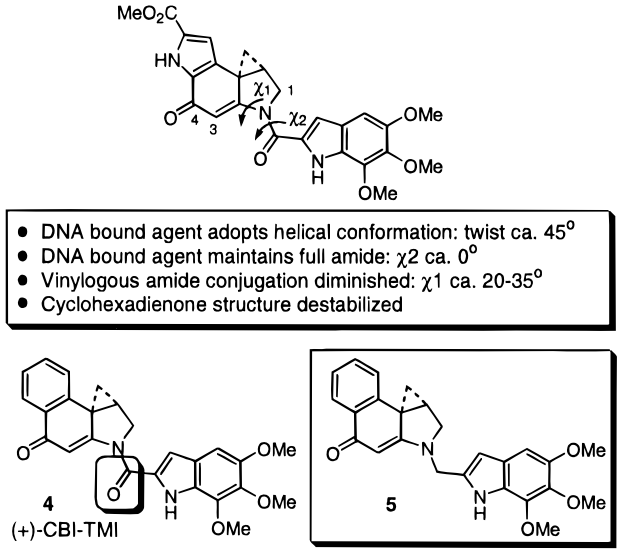

Figure 2.

that DNA alkylation catalysis requires a rigid extended N^2 amide substituent[10] and is independent of the location of the C4 versus C8 alkylation subunit carbonyl[16] suggested that the DNA alkylation catalysis is not derived from a long-postulated C4 carbonyl protonation[2,6,17] (acid catalysis) but rather from a DNA binding-induced conformational change in the agent that disrupts the cross-conjugated vinylogous amide stabilization, activating the agents for nucleophilic attack (Figure 2).[10,18] This conformational change is derived from the adoption of a helical DNA bound conformation with the helical rise (twist) accommodated at the linking amide, the only flexible site in the natural products. Recent high-resolution structural studies of duocarmycin SA bound to a deoxyoligonucleotide at a high affinity alkylation site have defined a twist of 44 ±2° between the planes of the two aromatic subunits and suggested that the bulk of the twist is accommodated in the χ_1 dihedral angle rather than that of the linking amide itself (χ_2).[19] This serves to deconjugate the alkylation subunit vinylogous amide, removing its stabilizing effect, and activates the agents for nucleophilic attack. That this deconjugation could provide sufficient reactivity changes was illustrated in X-ray studies of a series of structural analogues that demonstrated a direct correlation between decreasing vinylogous amide conjugation, increasing reactivity ($10^3-10^4\times$), increasing χ_1 dihedral angle, and concomitant increases in the cyclopropane bond lengths and conjugation.[8,20,21] Moreover, the studies demonstrated that sufficient reactivity changes accompany even partial disruption of the vinylogous amide to account for DNA alkylation catalysis.

Herein, we report the preparation and examination of **5**, which replaces the linking N^2 amide of **4** with a methylene group (Figure 2). Its examination was anticipated to distinguish between catalysis derived from disruption of the vinylogous amide by a DNA binding-induced conformation change (χ_1 versus χ_2 dihedral angle) and acid catalysis involving C4 carbonyl protonation (χ_2 versus χ_1 dihedral angle). The former source of catalysis would not be possible with **5**, thus rendering it ineffective, whereas the latter would be enhanced by the increased basicity of C4 carbonyl resulting from the fully engaged vinylogous amide.

Preparation of 5. The initial synthesis of **5** was accomplished enlisting a set of C2 methylene substituted 5,6,7-trimethoxyindole substrates to generate a reactive alkylating agent in situ (Scheme 1). Introduction of suitable protecting groups for the C2 hydroxymethyl group and the indole nitrogen could be utilized to generate in situ a potentially reactive cyclic carbamate susceptible to nucleophilic displacement providing **5** in a single step.[21] Accordingly, *O*-acylation (Ac_2O-DMAP) of **7** and indole protection (BOC_2O-DMAP) provided **9**, while *O*-silylation (TBDMSCl−imidazole) and BOC protection (BOC_2O-DMAP) furnished **11**. *N*-Alkylation of **12**[22] was achieved using two protocols. The most successful was deprotonation of CBI (**12**, NaH−DMF) and addition of **9** to provide **5** (15%). Somewhat less successful was the coupling of CBI with **11** (Bu_4NF-DMF) which also furnished **5** (5%). Although not extensively investigated, attempts to improve the conversions by rigorous exclusion of water, cooling (−20 °C), or decreased reaction times were not successful, and the initial unoptimized efforts provided sufficient material for our studies.

An alternative and more direct preparation of **5** was accomplished by conversion of *seco*-CBI-TMI (**13**) to the corresponding thioamide with Lawesson's reagent ($(p\text{-MeOPhPS}_2)_2$, C_6H_6, 80 °C, 2.5 h, 57%) followed by catalytic Ra−Ni desulfurization (EtOH, 80 °C, 10 min) and subsequent immediate base-catalyzed spirocyclization of the intermediate **15** (3 equiv of DBU, CH_3CN, 25 °C, 45 min, 33% for two steps), Scheme 1.

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(21) This was first indicated as plausible in efforts to deprotect the TBDMS ether of **11** which provided (2-(((*tert*-butyloxy)carbonyl)oxy)-methyl-5,6,7-trimethoxyindole and 2-hydroxymethyl-5,6,7-trimethoxyindole (Bu_4NF, THF, 20% and 38% respectively) or 2-(acetoxy)methyl-5,6,7-trimethoxyindole (Bu_4NF, 100:1 THF−HOAc, 40%) or from treatment of *N*-(*tert*-butyloxy)carbonyl-2-(((*tert*-butyloxy)carbonyl)oxy)methyl-5,6,7-trimethoxyindole with NaOMe−MeOH which provided 2-(methoxy)methyl-5,6,7-trimethoxyindole (73%).

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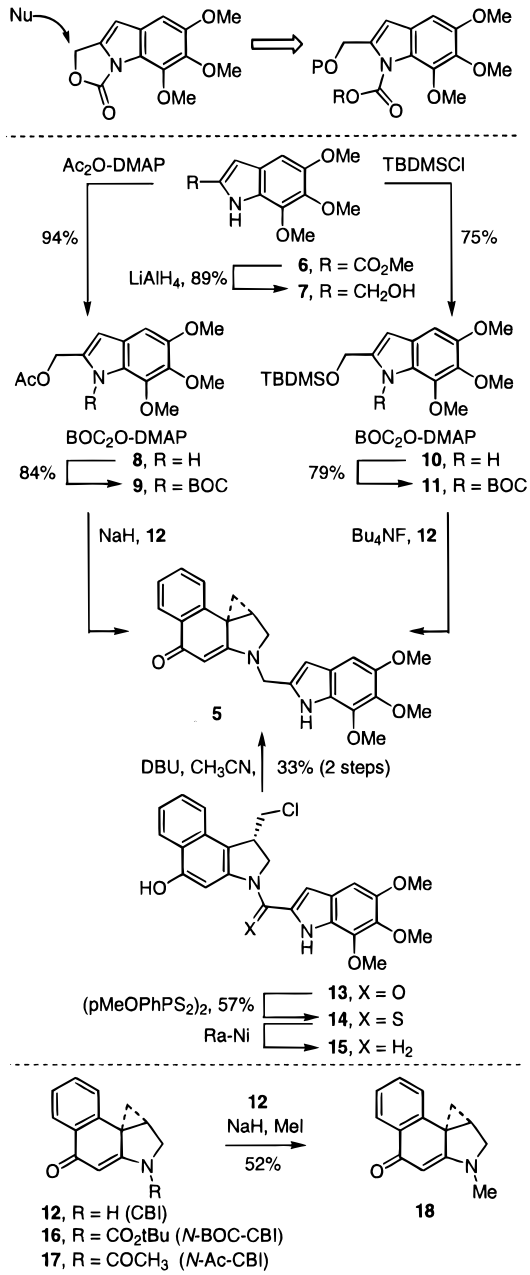
Scheme 1



The current studies also required the preparation of *N*-methyl CBI (**18**) for side-by-side comparison with **5**, **12**, and **16**, **17**, and this was achieved by deprotonation of CBI[22] (**12**, NaH) and *N*-alkylation with iodomethane (Scheme 1).

Acid-Catalyzed Solvolysis Reactivity of 5 and Related Agents. The rates of acid-catalyzed solvolysis of the duocarmycin and CC-1065 alkylating subunits and their analogues have provided insights on the source and extent of their stabilization. The removal of the linking amide and its replacement with a methylene unit (conversion of **4** to **5**) was expected to provide a substrate capable of full engagement of the vinylogous amide. The key question that we wished to address was whether this would lead to increased stabilization or, by virtue of the increased basicity of the C4 carbonyl, render the analogue more susceptible to protonation and acid-catalyzed solvolysis.

The solvolysis of **5** and *N*-methyl CBI (**18**) was followed by UV and conducted at pH 3, 2, and 1. Both **5** and **18** proved remarkably stable exhibiting little reactivity at pH 3 and a very slow solvolysis at pH 2, and only at pH 1 did the rate of

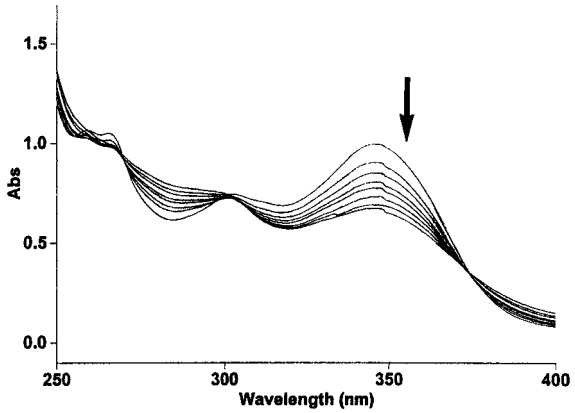


Figure 3. Acid-catalyzed solvolysis of **5** (pH 2). Selected UV scans at 0, 1, 2, 3, 4, 5, 6, and 7 weeks are shown.

Table 1. Solvolysis Studies

agent	pH	$t_{1/2}$ (h)	k (h^{-1})	λ_{max} (nm)
5	3	30528	2.27×10^{-5}	345
	2	824	8.41×10^{-4}	345
	1	80	8.65×10^{-3}	354
N-methyl-CBI (**18**)	3	68135	1.02×10^{-5}	343
	2	1608	4.31×10^{-4}	343
	1	nd	nd	nd
CBI (**12**)	3	4200[a]	1.65×10^{-4}	335
	2	236	2.94×10^{-3}	335
	1	12.3	5.47×10^{-2}	345
N-BOC-CBI (**16**)[b]	3	133	5.22×10^{-3}	256
	2	12.5	5.51×10^{-2}	256
	1	nd	nd	nd

[a] Previously estimated to be 930 h (pH 3), ref 22. [b] Data taken from ref 15. nd = not done.

solvolysis become easily measurable (Figure 3 and Table 1). For **5**, the acid-catalyzed solvolysis half-life ($t_{1/2}$) was established to be 80 h, 824 h, and ca. 30 500 h (3.3 days, 34 days, and ca. 3.5 years) at pH 1, 2, and 3, respectively. Above pH 3 including pH 7, no measurable solvolysis was observed. These observations are analogous to those made in measurements of the solvolysis rate of CBI (**12**) itself,[22] and **5** and **18** were slightly less reactive, being 4−8× more stable than **12**. Both **5** and **18**, like CBI (**12**), are much more stable than the corresponding *N*-acyl derivatives **4** and *N*-BOC-CBI (**16**). At pH 3, **5** proved to be approximately 250× more stable than **4** and **16**. This remarkable stability of **5** and **18** even at pH 1 (Table 1) is consistent with enhanced stabilization, not reactivity, derived from a fully engaged vinylogous amide.

DNA Alkylation Properties of 5. The DNA alkylation properties of both enantiomers of **5** were examined within w794 duplex DNA,[3,4] a 144 base pair segment of duplex DNA for which comparative results are available for related agents. Following treatment of ^{32}P-end-labeled duplex DNA with a range of agent concentrations (10^{-1}−10^{-4} M **5**) at 37 °C for 3, 5, and 14 days, the unbound agent was removed by ethanol precipitation of the DNA. Subsequent redissolution of the DNA, thermolysis (100 °C, 30 min) to induce strand cleavage at sites of alkylation, followed by denaturing, high-resolution polyacrylamide gel electrophoresis (PAGE), revealed no detectable sites of alkylation with either enantiomer of **5**. The comparisons with CBI-TMI and duocarmycin SA which readily alkylate DNA at 10^{-6} to 10^{-8} M (Figure 1, Supporting Information) even at 4 °C (1−6 h)[1,3,4,10,11,13,22] versus no detectable DNA alkylation by **5** even at 37 °C (10^{-1} M, 14 days) demonstrate that removal of the amide linkage and its replacement with a methylene leads to a $>10^6$-fold decrease in alkylation efficiency

Table 2. In Vitro Cytotoxic Activity

agent	IC_{50} (L1210) (nM)
natural enantiomer	
(+)-DSA (**1**)	0.01
(+)-CBI-TMI (**4**)	0.03
(+)-**5**	1400
(+)-*N*-BOC-CBI (**16**)	80
(+)-CBI (**12**)	1000
unnatural enantiomer	
(−)-DSA (**1**)	0.10
(−)-CBI-TMI (**4**)	2.0
(−)-**5**	8400
(−)-*N*-BOC-CBI (**16**)	900
(−)-CBI (**12**)	11000
(±)-*N*-methyl-CBI (**18**)	14000

for the site 5′-AATT**A** for the natural enantiomer and to a similar (>10^5) decrease in efficiency at the unnatural alkylation site (5′-TA**A**TTTT). Comparison even with the simple derivative, *N*-BOC-CBI (**16**) which alkylates DNA at $10^{-2}-10^{-3}$ M (37 °C, 1−3 days)[13b] and does so much less selectively than **4**, revealed a >10^2-fold relative decrease in alkylation efficiency with **5**.[23] Thus, removal of the linking amide and its replacement with a linking methylene renders the agents incapable of alkylating DNA in a perceptible manner.

To ensure that the loss of DNA alkylation properties was not due to a loss of noncovalent binding properties, the binding constant of **5** with poly[dA]·poly[dT] was established through competitive displacement of prebound ethidium bromide and measurement of the resulting decrease in fluorescence. This method for establishing the absolute binding constant has been described in detail for a complete series of related CC-1065 agents.[24] The decrease in fluorescence was linear throughout the titration with **5** and provided a K_b of 1.90×10^6 M^{-1}. This places **5** precisely between the related noncovalent binding agents $CDPI_2$ ($K_b = 3.2 \times 10^5$ M^{-1}) and $PDE\text{-}I_2$ ($K_b = 1.6 \times 10^7$ M^{-1}) indicating that the removal of the linking amide carbonyl with **5** did not substantially or adversely effect the noncovalent minor groove binding properties of the agent.

Cytotoxic Activity. Consistent with trends observed in the DNA alkylation studies, the cytotoxic potency of the two enantiomers of **5** was approximately 10^4-10^5 times less potent than **4** or **1**, 10−100× less potent than simple *N*-acyl derivatives including *N*-BOC-CBI (**16**) which lack the DNA binding subunit(s) but which still contain a small N^2 acyl group, and comparable in potency to CBI (**12**) itself which lacks both the N^2 acyl group and a DNA binding subunit (Table 2). This level of cytotoxic potency is comparable to that observed with analogues of CC-1065 that may bind to DNA but are incapable of DNA alkylation.[24] Consistent with prior studies, the natural enantiomer of **5** was slightly more potent than the unnatural enantiomer (6×). Thus, the removal of the linking amide and its replacement with a methylene reduced the cytotoxic potency 10^4-10^5× providing an agent that is less effective than even simple *N*-acyl derivatives and comparable to the nonpotent unsubstituted NH derivatives of the alkylation subunits.

Discussion and Conclusions. The remarkable chemical stability of **5**, its 10^4-10^5× reduction in cytotoxic potency, and its inability to alkylate DNA even under extraordinary conditions (37 °C, 2 weeks, 10^{-1} M versus 4 °C, 1−6 h, 10^{-7} M for **4**) highlight the essential role of the linking N^2 amide. Not only was **5** >10^6× less effective than **4**, its direct comparison analogue, but it was >10^2× less effective than even *N*-BOC-CBI (**16**) and *N*-Ac-CBI (**17**) and comparable in properties to CBI (**12**). These observations have clear implications on the source of catalysis for the DNA alkylation reaction. They are inconsistent with expectations of catalysis derived from C4 carbonyl protonation (acid catalysis) but are fully consistent with catalysis derived from a DNA binding-induced conformational change (χ_1 dihedral angle) that disrupts the cross-conjugated vinylogous amide stabilization activating the agents for nucleophilic attack.

Experimental Section[25]

***N*-[(5,6,7-Trimethoxyindol-2-yl)methyl]-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (5):** mp 215−217 °C dec; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.46 (br s, 1H, NH), 8.24 (dd, $J = 1.4$, 7.5 Hz, 1H, C5−H), 7.39 (dt, $J = 1.4$, 7.5 Hz, 1H, C6−H), 7.34 (dt, $J = 1.4$, 7.5 Hz, 1H, C7−H), 6.73−6.76 (m, 2H, C8−H, C4′−H), 6.32 (d, $J = 2.2$ Hz, 1H, C3′−H), 5.85 (s, 1H, C3−H), 4.46 (d, $J = 15.8$ Hz, 1H, C2′−C*H*H−NCBI), 4.42 (d, $J = 15.8$ Hz, 1H, C2′−CH*H*−NCBI), 4.02 (s, 3H, OCH_3), 3.86 (s, 6H, OCH_3), 3.67 (dd, $J = 5.4$, 10.5 Hz, 1H, C1−H*H*), 3.46 (d, $J = 10.5$ Hz, 1H, C1−H*H*), 2.72 (dt, $J = 5.0$, 5.2, 7.7 Hz, 1H, C9a−H), 1.48 (dd, $J = 4.4$, 7.7 Hz, 1H, C9−*H*H), 1.28 (t, $J = 4.4$ Hz, 1H, C9−H*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 184.0, 167.7, 149.5, 139.4, 138.7, 138.1, 133.6, 131.9, 130.9, 126.6, 126.2, 125.1, 123.4, 120.6, 103.4, 96.9, 95.2, 61.4, 61.0, 56.3, 55.0, 43.0, 34.0, 30.3, 24.1; IR (film) ν_{max} 3185, 2934, 1599, 1546, 1465, 1217 cm^{-1}; UV (pH 2 and 3 buffer) λ_{max} 345 nm (ϵ 22600), UV (CH_3OH) λ_{max} 342 nm (ϵ 21100); FABHRMS (NBA/NaI) *m/z* 439.1619 ($M^+ + Na$, $C_{25}H_{24}N_2O_4$ requires 439.1643).

(+)-(8a*R*,9b*S*)-5: tan solid, $[\alpha]^{23}_D$ +94 (*c* 0.002, CH_2Cl_2).

(−)-(8a*S*,9b*R*)-5: tan solid, $[\alpha]^{23}_D$ −90 (*c* 0.002, CH_2Cl_2).

Solvolysis Reactivity. A sample of **5** (0.05 mg) was dissolved in CH_3OH (1.5 mL) and mixed with pH 3, 2, or 1, aqueous buffer (1.5 mL). The pH 3 buffer contained 4:1:20 (v/v/v) 0.1 M citric acid, 0.2 M Na_2HPO_4, and H_2O, respectively. The pH 2 buffer contained 4:1:20 (v/v/v) 1.0 M citric acid, 0.2 M Na_2HPO_4, and H_2O, respectively. The pH 1 buffer contained 67:25:8 (v/v/v) 0.2 M HCl, 0.2 M KCl, and H_2O, respectively. The solution was sealed and kept at 25 °C protected from light. The UV spectra was measured at hourly time intervals during the first 6−12 h, every day for the next week and once a week for the next 4 months. The decrease in the long wavelength absorption at 345 nm (pH 3 and 2) or 354 nm (pH 1) was monitored. The solvolysis rate constant and the half-life were calculated from data recorded at the long wavelength from the least-squared treatment ($r = 0.99$) of the slope of the plot of time versus $\ln[(A_f - A_i)/(A_f - A)]$.

Similarly, *N*-methyl CBI (**18**, 0.05 mg) was dissolved in CH_3OH (1.5 mL) and mixed with pH 3, 2, or 1 aqueous buffer (1.5 mL). The solution was sealed and kept at 25 °C protected from light. The UV spectra was measured at regular intervals as described above. The decrease in the long wavelength absorption at 343 nm (pH 3 and 2) was monitored. The solvolysis rate constant and the half-life were determined as detailed above ($r = 0.99$).

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Supporting Information Available: Experimental details for the preparation of **5**, **7**−**11**, and **15** and for the DNA alkylation studies and a gel figure comparing the DNA alkylation of **1**, **4**, and **5** are provided (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(23) To date, efforts to detect DNA alkylation by **12** or related simple alkylation subunit NH derivatives have not revealed detectable alkylation in our[1] or related studies.[2]

(24) Boger, D. L.; Coleman, R. S.; Invergo, B. J.; Zarrinmayeh, H.; Kitos, P. A.; Thompson, S. C.; Leong, T.; McLaughlin, L. W. *Chem.-Biol. Interact.* **1990**, *73*, 29.

(25) Experimental details for the preparation of **5**, **7**−**11**, **14**, and **18** are provided in Supporting Information.