Synthesis and Evaluation of Duocarmycin and CC-1065 Analogues Containing Modifications in the Subunit Linking Amide

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The preparation and evaluation of **6** and **7**, analogues of the duocarmycins and CC-1065 in which the subunit linking amide has been replaced with an amidine and thioamide, are described. Consistent with the increased electron-withdrawing properties and conjugation of thioamides relative to amides, **7** showed increased solvolysis reactivity ($t_{1/2}$, 160 h versus 230 h) at pH 3, attributable to a diminished vinylogous amide stabilization of the reacting alkylation subunit. Amidine **6** proved to be even more unstable ($t_{1/2}$, 12 h) despite the diminished electron-withdrawing properties, but underwent preferential N^2 amidine linkage hydrolysis rather than solvolysis of the alkylation subunit, attributable to preferential N^2 vinylogous amide versus amidine conjugation. The natural isomers (+)-**6** and (+)-**7** exhibited an identical DNA alkylation selectivity as (+)-CBI-TMI and (+)-duocarmycin SA but were less efficient (10−100×). Biological studies of (+)-**6** and (+)-**7** (0.75 and 1.1 nM, respectively) indicated the analogues retained good cytotoxic activities (L1210), but were less potent than (+)-duocarmycin SA (0.01 nM, 100×) and (+)-CBI-TMI (0.02 nM, 50×). The enhanced properties of the linking amide versus amidine or thioamide established the N^2 amide as the optimal linking unit examined to date and revealed that it provides a beautiful balance between competing amide (reactivity) and vinylogous amide (stability) conjugation.

The duocarmycins and CC-1065 (**1**−**3**) are the parent members of an exceptionally potent class of antitumor antibiotics which derive their biological activity through the sequence selective alkylation of duplex DNA.[1−6] A key feature shared by **1**−**3** is the amide linking the DNA binding and alkylation subunits. The important contribution of the N^2 atom to the unusual stability of the alkylation subunit via vinylogous amide conjugation has been described, and studies have revealed an apparent appropriate balance of reactivity versus stability with the presence of the linking N^2 amide.[7−9] Replacement of the linking amide with a methylene in CBI-TMI (**4**) provided an analogue **5** possessing a fully engaged vinylogous amide which exhibited extraordinary stability ($t_{1/2}$ = 3.5 years, pH 3), a loss of virtually all biological activity, and the inability to alkylate DNA even under extreme reaction conditions (37 °C, 0.1 M, 2 weeks).[10] Alternatively, removal of the nitrogen atom provided an exceptionally reactive carbocycle alkylation subunit ($t_{1/2}$ = 4.0 h, pH 7).[8] These studies establish the N^2 site as the source of stabilization and that disruption of the vinylogous amide conjugation via a binding-induced conformational change within minor groove AT-rich sites of DNA activates the agents for nucleophilic attack (DNA alkylation catalysis).[11] To further probe the critical role of the N^2 amide, herein we report the synthesis and evaluation of the CBI-

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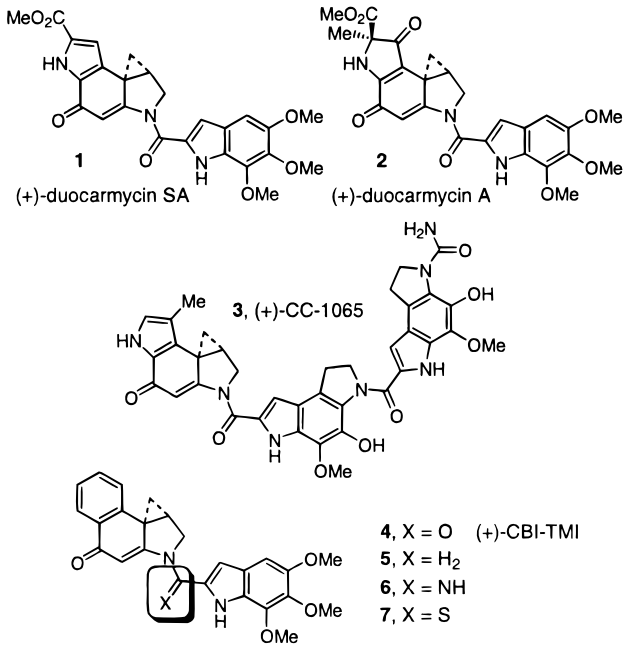

Figure 1.

TMI analogues **6** and **7** in which the linking amide has been replaced with an amidine and thioamide.

Synthesis. The initial synthesis of thioamide **7** employed the seco precursor **8** first enlisted for the preparation of **5**.[10] Thus, treatment of **8**[10] with base (DBU, 3 equiv, 25 °C, 30 min) yielded the thioamide linked analogue **7** (30%), Scheme 1. A modification of a procedure for the preparation of dithioesters from thioamides[12] was enlisted to generate amidine **6** by substituting the use of ammonia for hydrogen sulfide. Thus, activation of thioamide **8** (CH_3I, 15 equiv, CH_3CN, 25 °C, 24 h) followed by treatment with excess ammonia (THF, −78 °C) yielded the desired amidine linked precursor **9** in excellent yield (91%). Suppression of spirocyclization to **6** was achieved by slow warming of the reaction mixture over an extended period of time (20 h). Base initiated cyclization of **9** (DBU, 3 equiv, 25 °C, 30 min) provided the amidine linked analogue **6** (34%).

Alternatively, the synthesis of **9** was achieved by first coupling[13] the benzyl protected alkylation subunit precursor **11** with 5,6,7-trimethoxyindole-2-carboxylic acid yielding the benzyl protected *seco*-CBI-TMI **12** (53%). Thionation using Lawesson's reagent ($(p$MeOPhPS$_2)_2$, C_6H_6, 80 °C, 1.5 h) proceeded in improved yield (88%) versus the corresponding free phenol (57%)[10] for which the more efficient conversion is attributed to the increased solubility of **12** in benzene. Thioamide to amidine conversion using the protocol detailed above proceeded in excellent yield (95%). Final removal of the benzyl group was achieved with TFA[14] (70 °C, 80%) or upon hydrogenation (Pd−C, CH_3OH, 100%).

Solvolysis of 6 and 7. Acid-catalyzed solvolysis studies of the alkylation subunits of CC-1065 and the duocarmycins and their analogues have provided insights into their chemical and biological properties including the demonstration of the source and extent of the alkylation subunit stabilization. Replacement of the linking amide with an amidine potentially could lead to enhanced stabilization due to its decreased electron-withdrawing effect, or the increased basicity of the amidine itself may facilitate hydrolysis of the linkage site competitive with solvolysis of the alkylation subunit. Conversely, the slightly more powerful electron-withdrawing properties of thioamides versus amides ($\sigma_p = 0.00$ and 0.12, respectively, for $-NHC(X)CH_3$, X = S, O)[15] would be expected to diminish the stabilizing vinylogous amide conjugation resulting in enhanced reactivity. Analogous predictions may be reached by considering the relative extent of nitrogen conjugation in amides, amidines, and thioamides established by measuring rotational barriers about the C−N bond of *N*,*N*-dimethylacetamide derivatives ($CH_3C(=X)-N(CH_3)_2$, X = O, NH, S).[16] These studies established the thioamide as having the highest barrier of rotation followed by the amide and amidine (20.7, 18.1, and 12.8 kcal/mol, respectively), indicative of enhanced thioamide conjugation relative to amide and amidines which would compete with and diminish the stabilizing vinylogous amide conjugation.

The solvolysis of **4**, **6**, and **7** were conducted at pH 3 and monitored by UV (Table 1). Amide **4** (CBI-TMI) and thioamide **7** behaved analogous to agents previously studied in which a hypochromic shift was followed by UV. Consistent with expectations, the thioamide **7** ($t_{1/2}$, 160 h) exhibited an increased reactivity relative to the amide ($t_{1/2}$, 230 h) which proved to be the most stable agent in

Scheme 1

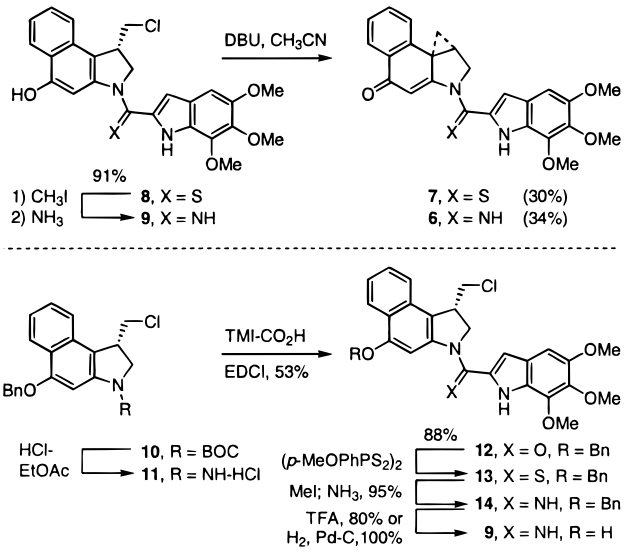

Table 1. Solvolysis Studies

agent	$t_{1/2}$ (h)[a]	k (h^{-1})	λ_{max}
CBI-TMI (**4**)	230	3.05×10^{-3}	359
6	12	5.78×10^{-2}	306[b]
7	160	4.44×10^{-3}	366

[a] Values determined at pH 3. [b] CH_3OH.

The solvolysis of **4**, **6**, and **7** were conducted at pH 3 and monitored by UV (Table 1). Amide **4** (CBI-TMI) and thioamide **7** behaved analogous to agents previously studied in which a hypochromic shift was followed by UV. Consistent with expectations, the thioamide **7** ($t_{1/2}$, 160 h) exhibited an increased reactivity relative to the amide ($t_{1/2}$, 230 h) which proved to be the most stable agent in

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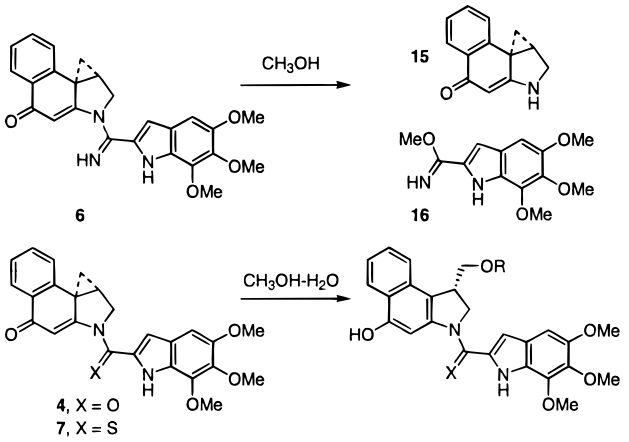
Scheme 2



the series. In contrast to simple expectations, the amidine **6** ($t_{1/2}$, 12 h) was the least stable analogue in the series. Moreover, and unlike the behavior of **4** and **7**, the initial UV traces of amidine **6** indicated a large hypochromic shift (decrease in intensity) indicative of protonation followed by the slower appearance of two peaks (λ_{max} = 312 and 335 nm) consistent with the formation of CBI. Under acidic conditions, protonation would be expected to occur at the imino nitrogen (pK_a 10.7),[17] thus activating the agent to hydrolysis rather than solvolysis of the alkylation subunit.

Preparative scale solvolysis reactions were conducted to determine and confirm the product profile. Treatment of **6** in MeOH with CF_3SO_3H (0.24 equiv, 72 h) provided CBI (**15**, 87%) as the only product (Scheme 2). Even conducting the reaction in the absence of an acid catalyst (60 h) yielded CBI (**15**, 95%) and the *O*-methylimidate **16** (71%). This latter observation suggests that the hydrolytic instability of amidine **6** may be due to more than imino nitrogen protonation under acidic conditions, but additionally derived from preferential vinylogous amide conjugation within the alkylation subunit rather than amidine conjugation itself. In contrast to **6**, both **4** and **7** underwent typical acid-catalyzed solvolysis with no evidence of hydrolysis of the linking amide or thioamide.

DNA Alkylation Properties of 6 and 7. The DNA alkylation properties of both enantiomers of **6** and **7** were examined within w794 duplex DNA, a 144 base pair segment of duplex DNA for which comparative results are available for related agents.[1,2] Following treatment of 5′-^{32}P-labeled duplex DNA with a range of agent concentrations at 25 °C for 24 h, the unbound agents were removed by EtOH precipitation of the DNA. Subsequent redissolution of the DNA in buffer, thermolysis (100 °C, 30 min) to induce strand cleavage at sites of alkylation, followed by denaturing, high-resolution polyacryamide gel electrophoresis (PAGE) next to Sanger sequencing standards revealed the DNA cleavage and alkylation sites.

A representative comparison of **6** and **7** alongside (+)-duocarmycin SA (**1**), (+)-CBI-TMI (**4**) and (−)-CBI-TMI is illustrated in the figure in Supporting Information. There are a number of important conclusions that can be made from the comparisons. First, both the thioamide and amidine linked compounds alkylated DNA in a manner analogous to CBI-TMI and retained the same DNA alkylation sequence selectivity. Only adenine-N3 alkylation was detected under the conditions of limiting agent and excess DNA. Therefore, changes in the linking amide structure had no effect on the DNA alkylation sequence selectivity of the agents.

Table 2. In Vitro Cytotoxic Activity

agent	IC_{50}, nM (L1210)
natural enantiomer	
(+)-duocarmycin SA	0.01
(+)-CBI-TMI (**4**)	0.02
(+)-**6** (amidine)	0.75 (0.75)[a]
(+)-**7** (thioamide)	1.10 (1.00)[a]
unnatural enantiomer	
(−)-duocarmycin SA	0.10
(−)-CBI-TMI (**4**)	0.90
(−)-**6** (amidine)	70 (180)[a]
(−)-**7** (thioamide)	100 (210)[a]

[a] The value in parentheses is the IC_{50} value established for the corresponding seco precursors **8** or **9**.

In previous studies of the CBI-based analogues, the natural enantiomer consistently proved to be 50−100× more potent and effective at alkylating DNA than the unnatural isomer.[18] The thioamide and amidine-linked compounds exhibited this same trend, with the unnatural isomers being 100-fold less effective at alkylating DNA than the natural enantiomers.

The only significant difference in the agents rested with the relative efficiency of DNA alkylation. Both the thioamide **7** and the amidine **6** alkylated DNA 10−100× less effectively than CBI-TMI. In part, this difference presumably reflects the relative stability of the agents and is similarly reflected in the cytotoxic activity of the agents described below. Interestingly, both the DNA alkylation efficiency and the cytotoxic potency of the amidine **6** indicates that its DNA alkylation reaction effectively competes with amidine hydrolysis under the assay conditions.

Cytotoxic Activity. The cytotoxic activities of the thioamide and amidine analogues and their seco derivatives were established and are summarized in Table 2. Previous studies have shown that the seco and ring-closed agents possess identical activities, and a similar finding was observed for the modified derivatives **6** and **7**.[1] Further consistent with previous studies, the natural enantiomers exhibited more potent cytotoxic activity (100×) than their unnatural counterparts. Consistent with their relative DNA alkylation effectiveness and relative stabilities, the modified analogues **6** and **7** were less potent than (+)-DSA (100×) and (+)-CBI-TMI (50×), establishing the amide as the optimum linkage unit.

Discussion and Conclusions. The role of the linking amide shared by **1**−**3** has been further probed with the preparation and evaluation of **6** and **7**. The amide to amidine and thioamide conversion led to an agent more susceptible to solvolysis in the case of the thioamide ($t_{1/2}$, 230 and 160 h, respectively), and one far more susceptible to hydrolysis of the linking site in the case of the amidine ($t_{1/2}$, 12 h). The former is consistent with the greater

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thioamide conjugation reducing the effective vinylogous amide stabilization of the alkylation subunit. The ease in which the latter hydrolysis of the subunit linking amidine was observed even at neutral pH indicates preferential vinylogous amide conjugation and stabilization at the expense of the stability of the linking amidine. Both observations are consistent with the predicted relative conjugation derived from estimates of barriers to rotation:[16] thioamide (20.7 kcal/mol) > amide (18.1 kcal/mol) > vinylogous amide (12.2−14.5 kcal/mol)[19] > amidine (12.8 kcal/mol). The analogues showed identical sequence selectivity patterns as (+)-duocarmycin SA and (+)-CBI-TMI, but both were less efficient (10−100×) at alkylating DNA. Consistent with these observations, the cytotoxic activity of **6** and **7** was 50−100× less potent than (+)-CBI-TMI and (+)-duocarmycin SA. The enhanced properties of the amide versus amidine or thioamide establish it as the optimal linking unit examined to date, representing a beautiful balance between reactivity and stability resulting from competing amide and vinylogous amide conjugation.

Experimental Section

***N*²-[(5,6,7-Trimethoxyindol-2-yl)iminocarbonyl]-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (6).** Pale yellow solid: mp 140−142 °C dec; ^{1}H NMR (acetone-d_6, 500 MHz) δ 10.51 (br s, 1H), 8.99 (br s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 7.51 (t, J = 7.6 Hz, 1H), 7.34 (t, J = 7.6 Hz, 1H), 7.12 (d, J = 7.6 Hz, 1H), 6.88 (s, 1H), 6.74 (d, J = 1.5 Hz, 1H), 5.37 (s, 1H), 4.43 (dd, J = 5.1, 11.2 Hz, 1H), 3.95 (d, J = 11.2 Hz, 1H), 3.93 (s, 3H), 3.84 (s, 3H), 3.80 (s, 3H), 3.04 (dt, J = 4.9, 5.1, 7.8 Hz, 1H), 1.93 (t, J = 4.9 Hz, 1H), 1.80 (dd, J = 4.9, 7.8 Hz, 1H); ^{13}C NMR (acetone-d_6, 125 MHz) δ 184.0, 163.8, 151.1, 141.7, 140.9, 140.2, 133.8, 132.4, 132.3, 132.2, 126.9, 126.8, 126.7, 124.5, 122.8, 106.7, 106.1, 98.9, 61.5, 56.6, 55.4, 34.3, 24.9, the two remaining peaks were not observed and are believed to be obscured by the solvent peak at 30 ppm; IR (film) ν_{max} 3284, 1614, 1562, 1410, 1317, 1121 cm^{-1}; UV (CH_3OH) λ_{max} 306 nm (ϵ 15700); FABHRMS (NBA/NaI) m/z 452.1574 (M^+ + Na, $C_{25}H_{23}N_3O_4$ requires 452.1586).

(+)-(8a*R*,9b*S*)-6. Pale yellow solid: $[\alpha]_D^{23}$ +30 (*c* 0.0002, CH_3OH).

(−)-(8a*S*,9b*R*)-6. Pale yellow solid: $[\alpha]_D^{23}$ −30 (*c* 0.0002, CH_3OH).

***N*²-[(5,6,7-Trimethoxyindol-2-yl)thiocarbonyl]-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (7).** Yellow semisolid: ^{1}H NMR (acetone-d_6, 500 MHz) δ 10.30 (br s, 1H), 8.04 (d, J = 7.8 Hz, 1H), 7.60 (t, J = 7.8 Hz, 1H), 7.42 (t, J = 7.8 Hz, 1H), 7.22 (d, J = 7.8 Hz, 1H), 6.99 (d, J = 2.2 Hz, 1H), 6.87 (s, 1H), 5.83 (s, 1H), 4.64 (dd, J = 4.9, 12.0 Hz, 1H), 4.33 (d, J = 12.0 Hz, 1H), 3.96 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.12 (dt, J = 4.9, 5.2, 7.8 Hz, 1H), 2.13 (t, J = 4.9 Hz, 1H), 2.00 (dd, J = 4.9, 7.8 Hz, 1H); ^{13}C NMR (acetone-d_6, 125 MHz) δ 192.2, 184.5, 161.7, 151.6, 142.2, 141.7, 140.1, 138.1, 133.4, 133.0, 128.4, 127.3, 127.0, 124.5, 123.1, 112.4, 108.1, 99.0, 61.5, 60.5, 56.5, 34.1, 32.4, 28.9, 25.9; IR (film) ν_{max} 3298, 1732, 1615, 1372, 1299, 1236, 1112, 1047 cm^{-1}; UV (pH 3 buffer) λ_{max} 366 nm (ϵ 15300), UV (CH_3OH) λ_{max} 365 nm (ϵ 14700); FABHRMS (NBA/NaI) m/z 447.1391 (M^+ + H, $C_{25}H_{22}N_2O_4S$ requires 447.1379).

(+)-(8a*R*,9b*S*)-7. Yellow semisolid: $[\alpha]_D^{23}$ +580 (*c* 0.0005, EtOAc).

(−)-(8a*S*,9b*R*)-7. Yellow semisolid: $[\alpha]_D^{23}$ −576 (*c* 0.0005, EtOAc).

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Supporting Information Available: Experimental details for the preparation of **6**, **7**, **9**, and **12**−**14**, solvolysis studies of **4**, **6**, and **7** and the characterization of **16**, experimental details for the DNA alkylation studies, a gel figure comparing the DNA alkylation of **1**, **4**, **6**, and **7**, and ^{1}H NMR spectra of **6**, **7**, **8**, **9**, **12**, **13**, **14**, and **16** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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