CC-1065 TRANSFORMATIONS

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This report defines the transformations that antitumor antibiotic CC-1065 underwent under basic and acidic conditions. The isolation, purification, characterization, and biological properties of a cyclopropapyrroloindole fragment, and an acidic fragment, PDE-I dimer, from a mild alkaline fragmentation and the phenolic product, AAP, resulting from alkylation of acetic acid by the cyclopropyl function are described.

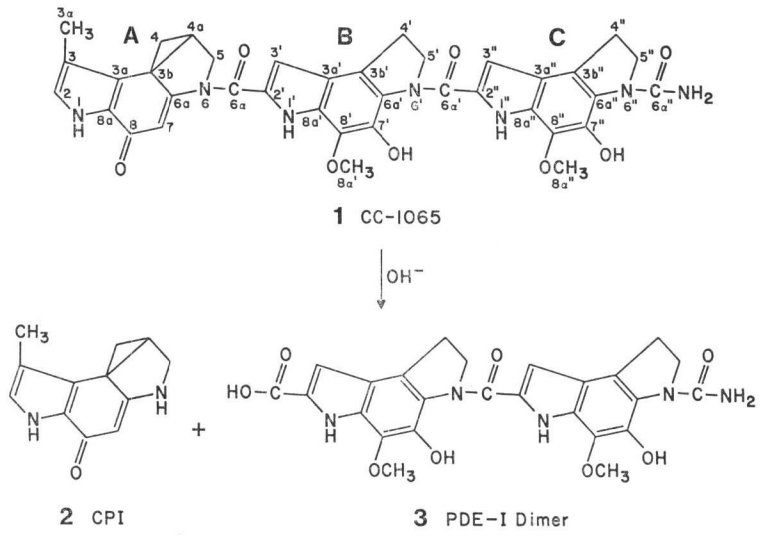
In 1980 we reported[1,2] the structure **1** for CC-1065, a highly novel type of extremely potent cytotoxic antitumor antibiotic[3] isolated from *Streptomyces zelensis*[4]. The molecular geometry and functionality of CC-1065 provided an efficient binding and subsequent alkylation of DNA[2,5~7] which resulted in a remarkable potency against a spectrum of mouse tumors[8]. Unfortunately, CC-1065 also caused a lethal delayed toxicity in non-tumored mice and rabbits dosed ip and iv with the agent[9]. This delayed toxicity prevented further development of CC-1065 itself as an antitumor agent but prompted efforts to modify the CC-1065 structure. Structural modification was complicated by the high reactivity of the cyclopropyl-conjugated dienone system. Early efforts to prepare derivatives had generated complex mixtures as the molecule had degraded under basic or acidic reaction conditions[1].

This paper defines the transformations that CC-1065 underwent under basic and acidic conditions. These transformations coupled with those described for the synthesis of the left-hand segment of CC-1065[10] may facilitate the preparation of a variety of analogs from CC-1065.

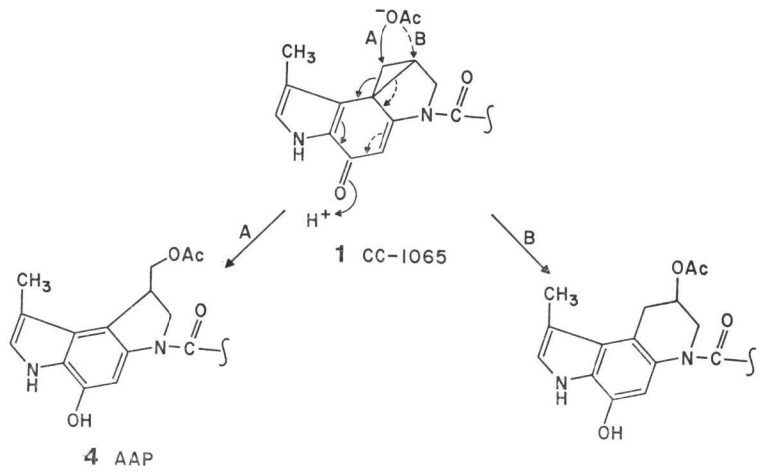
Alkaline Fragmentation

Scheme 1 depicts the alkaline fragmentation of CC-1065. The A unit of CC-1065 is attached to the BC units by an amide bond which, as part of a vinologous imide system, proved more susceptible to mild alkaline cleavage than normal amides such as that between the B and C units. Mild alkali on CC-1065 also led to the formation of dark insoluble products, complicating the isolation of the fragments which displayed similar chromatographic mobilities on reverse phase systems with neutral and basic solvents. As the acidic BC fragment **3**, called PDE-I dimer because it is composed of two PDE-I units[11], was too insoluble and polar for silica gel chromatography, we were fortunate that the cyclopropapyrroloindole (CPI) unit **2**, and PDE-I dimer were separable by virtue of their aqueous solubility in acidic and alkaline media, respectively. Essentially pure but brownish red crystalline CPI was obtained by chromatography on silica gel. Final purification was conveniently and efficiently achieved by counter current chromatography (CCC) with the Ito multilayer coil separator-extractor[12]. Racemic CPI has been synthesized by M. A. WARPEHOSKI (unpublished Upjohn data) and P. MAGNUS[13]. The highly insoluble PDE-I dimer was purified by precipitation and trituration techniques. These fragments completely lacked the biological potency of CC-1065, both *in vitro* and *in vivo*. CPI and PDE-I dimer both displayed and ID_{50} of approximately 0.2 μg/ml vs. L1210 cells in culture in sharp contrast to the ID_{50} of approximately 0.00001 μg/ml displayed by CC-1065. Both fragments also lacked activity aga-

Scheme 1. Alkaline fragmentation.



Scheme 2. Addition of acetic acid.



inst P388 leukemia in mice at the highest doses tested* indicating that these fragments retained less than 0.2% of the potency of CC-1065. This finding supports earlier speculation[14] that both the alkylating cyclopropyl portion and the BC portion are required for biological potency. This loss of biological activity is explicable in terms of their interaction with DNA. CD studies on the interaction of these fragments with CT-DNA established that PDE-I dimer, with no alkylating capability, did bind to DNA but CPI did not bind.

Addition of Acetic Acid

Scheme 2 depicts two pathways, designated A and B, for the reaction between CC-1065 and acetic acid. The only product isolated, acetic acid product or AAP, had structure **4**, the product ex-

* CPI at 10 mg/kg administered ip on days 1, 5 and 9 against 10^6 cells of ip inoculated tumor on day 0. PDE-I dimer at 100 mg/kg administered ip on day 1 against 10^6 cells of ip inoculated tumor on day 0.

Table 1. ^{13}C NMR shifts.

Tentative assignment	Multiplicity	**1** in DMF-d_7 (ppm)	**2** in DMF-d_7 (ppm)	**3** in DMSO-d_6 (ppm)	**4*** in DMF-d_7 (ppm)
3α	q	9.7	9.9		11.1
4	t	21.7	24.0		66.7
4a	d	21.8	23.2		39.8
4″	t	27.3		26.7	27.1
4′	t	28.3		27.7	28.1
3b	s	32.3	32.3		109.8
5″	t	50.2		49.5	50.0
5′	t	54.0		53.5	53.8
5	t	55.6	51.3		55.2
8α′	q	60.3		60.1	60.1
8α″	q	60.6		60.4	60.4
3′	d	106.6		106.4	104.4
3″	d	106.9		107.2	106.6
7	d	111.6	95.0		98.0
3	s	114.1	113.4		112.1
3a′	s	118.4		117.4	118.1
3a″	s	118.8		117.8	118.7
3b″	s	119.1		118.4	118.9
3b′	s	122.0		121.3	121.3
2	d	124.0	121.3		124.6
2′	s	128.0		127.4	127.9
8a	s	128.6	125.2		125.4
2″	s	128.6		127.6	128.2
6a′	s	130.0		129.0	129.8
6a″	s	130.1		129.2	130.3
8a′	s	130.6		129.6	125.4
3a	s	131.4	130.9		133.8
8a″	s	131.8		131.7	130.8
7′	s	133.4		132.5	132.8
7″	s	134.1		133.3	133.1
8′	s	139.3		138.2	138.7
8″	s	139.7		138.5	139.0
6α″	s	158.7		157.2	158.4
6a	s	161.7	172.1		137.2
6α′	s	162.0		160.8	160.0
6α	s	162.1		162.6	161.3
8	s	176.4	177.5		143.7

* Acetate Me at 20.5 ppm; acetate carbonyl at 170.9 ppm.

pected from pathway A. NMR data, ^{13}C and ^{1}H, unambiguously established that the carbon bearing OAc carried 2 H's. Purification and biological evaluation of products derived from CC-1065 was challenging. The potency of CC-1065 is so great that residual traces contaminating transformation products would mask the true activity of these products. At the same time, the properties of CC-1065 and its transformation products did not lend themselves to conventional purification schemes. The tendency of these substances to strongly bind to a variety of materials coupled with only slight solubility in any volatile solvent including water precluded conventional chromatographic and solvent partition methods. Chromatographic systems useful for CC-1065 purification[8] afforded only partial resolution of the AAP mixture. Consequently, initial samples of AAP were isolated by precipitation and trituration techniques and characterized. By TLC bioautography[8], 0.3 μg of CC-1065 afforded a clear round zone

Fig. 1. CD spectra of **1** (●, 1.85×10^{-5} M), **2** (△, 5.0×10^{-5} M) and **4** (○, 1.85×10^{-5} M) in DMF.

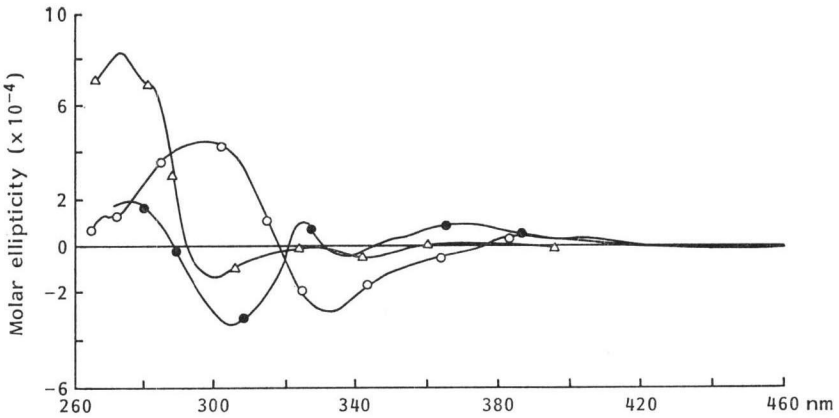
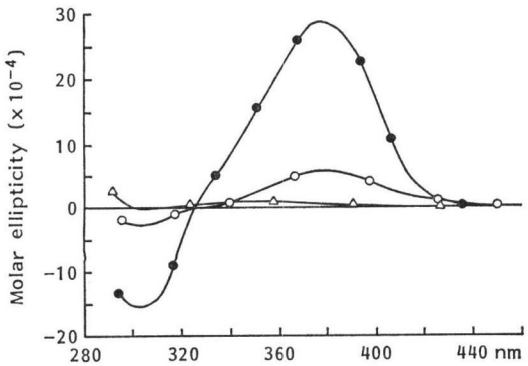


Fig. 2. CD difference spectra (mixture minus separate components) induced by CT-DNA in the electronic transitions of **1**(●), **3** (△) and **4** (○).



whereas 60 μg of AAP afforded a trace of a crescent-shaped zone at the same Rf indicating that less than 0.5% of CC-1065 was present in the AAP sample. Comparing the potency of such AAP (ID_{50} 0.002 μg/ml) with that of CC-1065 (ID_{50} 0.00001 μg/ml) in inhibiting the growth of L1210 cells in culture (3-day growth, continuous drug contact) was also consistent with 0.5% CC-1065 in the AAP sample. Against P388 leukemia in mice, AAP of this quality displayed more activity than would be expected from at inert material containing 0.5% of CC-1065. Administered ip on days 1, 5 and 9, AAP at 780 μg/kg afforded a 59% increased life span (ILS) whereas CC-1065 gave a 64% ILS at 12.5 μg/kg. It thus appears that AAP displayed modest antitumor activity. We subsequently developed chromatographic procedures applicable to purification of AAP by using reverse phase chromatography (C18) with aqueous DMF as eluant. Chromatographically purified AAP displayed an ID_{50} of 0.009 μg/ml vs. L1210 cells in culture indicating that no more than 0.1% CC-1065 was present. This sample, administered ip as a single dose of 6.3 mg/kg on day 1 afforded a 68% ILS against P388 leukemia in mice whereas a single dose of 200 μg/kg of CC-1065 afforded an ILS of 95%.

Characterization

NMR spectroscopy had contributed vital information to the structure determination of CC-1065 and provided a convenient method for assigning structures to transformation products. Tentative assignments for all the carbons, numbered as shown for CC-1065 in Scheme 1, of CC-1065, CPI, PDE-I dimer and AAP are listed in Table 1. The structures assigned to CPI, PDE-I dimer and AAP were supported by FAB mass spectrometry. The absorption patterns of CC-1065, AAP, CPI and PDE-I dimer are similar in that all compounds in DMF show a broad band near 350 nm as the main spectral

feature above 270 nm. However, the structures can be distinguished on the bases of the position and intensity (molar absorptivity) of this band as follows: CC-1065 (367 nm, 47,350); AAP (359 nm, 49,600); PDE-I dimer (353 nm, 32,600) and CPI (344 nm, 14,800). Based on these data and ignoring wavelength shifts, a simple intensity relationship exists; each tricyclic ring structure contributes about 15,000 to the molar absorptivity in this region of the spectrum. Such a simple relationship does not exist in the case of the CD data. Fig. 1 shows that the spectra differ considerably from structure to structure (PDE-I dimer is optically inactive). The perturbational or conformational effects responsible for these spectral differences are not known at this time.

All of the compounds except CPI interact with CT-DNA and show induced CD spectra as a result (Fig. 2). The wavelength of the induced CD maxima and the molar ellipticity at that wavelength are as follows: CC-1065 (378 nm, 286,000); AAP (378 nm, 56,000) and PDE-I dimer (348 nm, 10,000). The magnitude of the CD spectra indicate that the asymmetry induced in AAP and PDE-I dimer upon binding to CT-DNA is less than that acquired by CC-1065. We suspect that this is because AAP and PDE-I dimer do not bind covalently to CT-DNA as does CC-1065[2,5,6,15] (T. A. SCAHILL and D. H. SWENSON, unpublished Upjohn data) and hence are not held as rigidly to the helix as is CC-1065. Evidently, the B and C rings of CC-1065 are necessary binding groups since CPI which has the potential to covalently bind to CT-DNA does not (according to the zero induced CD curve found for this compound).

Experimental

As a precaution, experimental procedures were carried out in subdued light under an atmosphere of nitrogen. Column chromatography was carried out on silica gel (EM Reagents, Kieselgel 60, 230 ~400 mesh) or C18 (Waters, Prep C18, 55~105 μm). Fractions were monitored by a TLC densitometric assay described[8] for CC-1065. TLC was carried out on silica gel plates (Whatman LK6DF) or C18 reversal phase plates (Whatman LKC18D). ^{13}C NMR spectra were recorded on a Varian XL200 spectrometer and chemical shifts reported (Table 1) in ppm relative to internal TMS. CD spectra were recorded on a Jasco 500 C spectrometer in a 1.0-cm cell. CT-DNA induced CD difference spectra were determined in a 5.0-cm cell on 0.37×10^{-5} M solutions of **1**, **3** and **4** in 0.01 M phosphate buffer (pH 7.2) after 24 hours of incubation with 11.1×10^{-5} M CT-DNA.

Alkaline Fragmentation: CPI and PDE-I Dimer (2 and 3)

CC-1065 (1.00 g, 1.42 mmol) was stirred in 440 ml of 0.2 N NaOH with initial cooling. After 3 hours at room temperature the dark solution was chilled and brought to pH 2 with conc HCl. After brief standing in the ice bath (30 minutes) the mixture was filtered through a Celite pad on a fine fritted glass funnel and the pad was washed with H_2O. The isolation of **2** from the filtrate is described below as is the isolation of **3** from $KHCO_3$ extracts of the pad.

CPI (2)

The filtrate was adjusted to pH 5 with NH_4OH and lyophilized. The residue was extracted with several portions of MeOH totalling 150 ml. The methanolic extract was treated with one ml of NH_4OH, evaporated onto 7 g of silica gel and chromatographed on a column of silica gel (100 g slurried in $CHCl_3$ - MeOH - NH_4OH, 95: 5: 1). The column was developed with this solvent system while collecting 44 ml fractions. Evaporation of fractions 9~15 left 106 mg (0.53 mmol, 37% yield) of **2** as a brownish-red crystalline residue, essentially homogeneous by TLC. Silica gel TLC Rf 0.22 (methylethylketone - acetone - H_2O, 80: 17: 3); Rf 0.45 ($CHCl_3$ - MeOH - NH_4OH, 90: 10: 0.5). Trituration of the residue with acetone afforded 100 mg of crystalline but still pigmented **2**. CCC was utilized to separate **2** from the small amount of pigment present. Initially, small samples of **2** were partitioned between the phases

of several solvent systems and the **2** concentration in the upper and lower phases was estimated by TLC densitometry. The system $CHCl_3$ - cyclohexane - MeOH - H_2O, 5: 2: 10: 5, evenly partitioned the **2** between the upper and lower phases and was used for CCC in the Ito multilayer coil separator-extractor (P.C., Inc.). The column (2.6 mm i.d.) had an approximate capacity of 330 ml. The organic (lower) phase was used as mobile phase at a flow rate of 2 ml/minute while the coil was rotated at 800 rpm; approximately 87% of the stationary phase was retained. Collected fractions (20 ml) of the mobile phase were monitored by TLC densitometry. After approximately 40 ml of eluate, the next 40 ml displayed purplish-red color. However, the initial 280 ml of eluate displayed negligible 360 nm absorption and held only 4 mg of residue. The next 120 ml of eluate contained essentially colorless, homogeneous **2**. Evaporation and acetone trituration of the residue afforded 97 mg of CPI as a cream colored solid. UV λ_{max}^{MeOH} nm (ε) 232 (11,500), 278 (24,400), 359.5 (14,450). CD in MeOH: nm (molar ellipticity) 360 (+6,500), 305 (+5,000), 290 (−20,500), 270 (+68,500), 230 (−19,000). FAB-MS: *m/z* 201 (M+H).

PDE-I Dimer (**3**)

The brownish-black loaded Celite pad was extracted* with aqueous $KHCO_3$ (1.00 g in 75 ml) and washed with H_2O. The filtrate was acidified to pH 2 with 1 N HCl and chilled for several days. The still somewhat gelatinous precipitate was collected on a fine fritted glass funnel, washed with H_2O and dried affording 0.326 g of crude **3**. Acetone trituration of the residue left after reduced pressure evaporation of DMF extracts (125 ml) of the crude acid afforded 0.313 g (0.60 mmol, 42%) of **3**. This sample was then extracted with 50 ml of 1 N NH_4OH and the clarified extract was evaporated to dryness under reduced pressure. Two 10 ml portions of AcOH were added and evaporated to dryness and the residue was then triturated with 95% EtOH affording 0.272 g of **3** after washing with additional 95% EtOH. The 0.313 g and 0.272 g samples were equivalent by TLC densitometric assay scanned with 340 nm UV light. C18 TLC Rf 0.42 (MeOH - 0.5 N brine - conc NH_4OH, 60: 40: 1). UV λ_{max}^{DMF} nm (ε) 296 (22,300), 353 (32,600). CD in DMF: zero rotation between 460 and 270 nm. FAB-MS: *m/z* 522 (M+H).

AAP (**4**)

A solution of 110 mg (0.156 mmol) of CC-1065 in 3.9 ml of DMF was cooled in an ice bath and diluted with 78 ml of AcOH. The brown solution was stored at room temperature for 28 hours, poured into 1.6 liters of cold H_2O, and stored in a refrigerator for 2 days. Suspended solids were then collected on a small bed of Celite in a suction funnel and washed with H_2O. The damp cake was dried under vacuum and extracted with 620 ml of acetone. A second smaller extract contained negligible 360 nm absorption. The acetone extract was evaporated to dryness and the resulting residue was dissolved in 3 ml of DMF, chilled, diluted with 1.5 ml of H_2O and chromatographed on a column of C18. The column was poured from 20 g C18 slurried in MeOH and washed with MeOH, aqueous MeOH, H_2O and finally DMF - H_2O, 2: 1. The column was eluted with DMF - H_2O, 2: 1 collecting fractions which were monitored by TLC densitometry scanned with 360 nm UV light. C18 TLC Rf 0.33 (DMF - H_2O, 2: 1). CC-1065 displayed Rf 0.46 on this system. **4** appeared in the eluate after an initial 132 ml had been collected. Although reasonably homogeneous **4** was present in the next 40 ml of eluate, center cut fractions (26 ml), which contained 88% of the **4**, crystallized on standing in the refrigerator. After dilution with an equal volume of H_2O and further chilling, the solid **4** in these fractions was collected, washed with H_2O, and dried affording 37 mg (0.048 mmol, 31%). UV λ_{max}^{DMF} nm (ε) 302 (sh 30,650), 359 (49,600). CD in DMF: nm (molar ellipticity) 370 (+9,000), 325 (+9,400), 305 (−33,000). FAB-MS: *m/z* 763 (M) and 764 (M+H).

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* Further extraction of the spent pad with DMF yielded a brownish-black extract leaving 0.68 g of black residue on evaporation. Analysis of this residue by TLC densitometry on several silica gel and C18 chromatographic systems indicated the absence of CC-1065, CPI, PDE-I dimer, or other 360 nm absorbing substances. The nature of this by-product has not been established.

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