N-(2-Hydroxyethyl)doxorubicin from Hydrolysis of 3′-Deamino-3′-(3-cyano-4-morpholinyl)doxorubicin

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The susceptibility of 3′-deamino-3′-(3-cyano-4-morpholinyl)doxorubicin to hydrolysis at pH 7, 4, and 2 has been compared with that of the typically stable morpholine analogue. At pH 7, 74% of the cyanomorpholine was unchanged after 24 h at room temperature, but at pH 2 only 10% remained. Products identified were aglycon (8%) and N-(2-hydroxyethyl)doxorubicin (7%). Most of the losses were to unidentified polar products not eluted from HPLC. Authentic hydroxyethyl was synthesized from doxorubicin by reductive alkylation with glycolaldehyde. Antitumor potency was comparable to that of doxorubicin rather than that of cyanomorpholine.

The intense antitumor potency of the cyanomorpholino derivative **3**[1] of doxorubicin (**1**) and the unprecedented activity[2-6] against doxorubicin-resistant tumors suggest an altered mechanism of action relative to **1**. One possibility is that the α-cyano amine in analogue **3** is a biochemically reactive functional group, adding further to the multiple mechanisms that may be employed by **1**. There are hypothetical precedents for α-cyano amine as an alkylating function for DNA in the literature on the saframycins and naphthyridinomycins.[7-9] Hence, it is of interest to explore the chemical reactivity of **3**. We now describe the partial loss of **3** under hydrolytic conditions as preliminary evidence of reactivity.

At pH 7 in 50% aqueous solution, the stability of **3** was reasonably good. After 22 h at room temperature, 74% of the initial **3** remained, judging from reverse-phase HPLC analysis with measurement of the absorbance at 254 nm. The results are shown in Table I. Only small amounts of new products were observed (<5% of the initial absorbance). Most of the loss (17% of the initial absorbance) appeared to occur on the HPLC column, presumably by nonelution of unidentifed products.[10] At pH 2, on the other hand, breakdown of **3** was almost complete. Only 10% remained after 22 h. Both diastereoisomers of **3** were degraded, the initially predominant one at a slightly faster rate (Table I, footnote c). The two observed products were doxorubicinone (8%) and a typically basic anthracycline (7%) subsequently identified as N-(2-hydroxyethyl)doxorubicin (**2**). Most of the drug (67% of the initial absorbance) was lost on the HPLC column. Again, we assume these losses are due to nonelution of unidentified products. This seemed likely because the losses increased with the increased breakdown at pH 2. Results for the intermediate case at pH 4 are also given in Table I.

Table I also shows a comparison with the morpholino derivative **4** lacking the α-CN. At any pH (7, 4, or 2), breakdown of **4** was essentially negligible. In this case, the evident overall losses in chromophore may as well be due to experimental error[11] as to nonelution of unidentified products from HPLC. The results with **4** clearly show that the hydrolytic breakdown of **3** must be associated with the α-cyano amine.

The two observed products required explanation. Normally, basic anthracyclines do not undergo glycoside cleavage to doxorubicinone at pH 2 at room temperature. The more facile acid cleavage of nonbasic anthracyclines—as with **3**—has been reported, however.[12,13]

Table I. Drug Stability after 22 h[a]

drug	pH	drug recovery, %	hydrolysis products, %	overall absorbance loss,[b] %
MRA	7	90	<1	4
	4	88	<1	12
	2	96	<1	4

drug	pH	drug recovery, %	hydrolysis products, %		overall absorbance loss,[b] %
			2	aglycon	
MRA-CN[c]	7	74[c]	0.8	0.3	17
	4	54[c]	8	0.1	37
	2	10[c,d]	7	8	67

[a] Drug stock solutions were prepared (0.6 mg/mL) in CH_3CN (or CH_3OH) and were diluted 1:1 with phosphate buffer (pH 7), citrate buffer (pH 4), and 0.1 N HCl–KCl (pH 2). The buffered solutions were stored at 23 °C for 22 h, and 0.010-mL aliquots were analyzed by reverse-phase HPLC in 0.1 M NaH_2PO_4–CH_3CN (75:25). Percentages were calculated as the amount of compound detected at time/total detected at start. [b] Losses were presumably due to nonelution of unknowns from HPLC. [c] Diastereoisomers **a**/**b** were 55/42% at start and, after 22 h were 41/33% (pH 7), 29/25% (pH 4), and 4/6% (pH 2). [d] From the extensive decay at pH 2 it was evident that the rate was not linear with time; at 4.5 h, **a** had decreased from 55 to 28% and **b** from 42 to 26%.

No doubt the basic compounds are protected toward acid attack by the positive charge on the protonated amine—as

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(10) Incomplete HPLC elution of **3** was not an explanation for the overall losses in chromophore, because the detection of **3**, like other neutral anthracyclines, was shown to be linear with the amount injected and was readily calibrated.
(11) Experimental error in the measurements for **4** (estd ±3%) was greater than for **3**. Calibration of **4** was more difficult because, like other basic anthracyclines, detectability increased with the amount injected.

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Table II. Biological Screening Data

compd	antitumor efficacy at opt dose vs. leukemia P388 in mice[a] day 1 % T/C (mg/kg)[f]	day 1 % T/C (mg/kg)[g]	inhibn of colony formation in HeLa cells[b] ID_{50},[g] ng/mL	inhibn of synth in leukemia L1210 cells[c] ED_{50},[h] μM DNA	RNA	ΔT_m[d] of isolated helical DNA in soln,[h] °C	log P[e] (octanol–phosphate buffer at pH 7.4)[h]
1	182 (7.5)	273 (10)	19	1.6[i]	0.58[i]	12.8[i]	0.07[i]
3	262 (0.012)[i,j]	233 (0.025)[k]		0.0030[i]	0.000 53[i]	8.7[i]	1.98[i]
2	157 (7.5)	205 (6.6)	31	5.2	0.81	10.7	0.19
5				0.65	0.12		

[a] Mice injected ip with 10^6 P388 cells on day 0 and then treated ip. T/C = average survival time of treated mice/control mice = antitumor efficacy and must be ≥120% for active result. [b] ID_{50} = drug concentration for 50% inhibition of colony growth after 24-h exposure to exponentially growing cells, followed by plating (50 cells/mL) in fresh growth medium without drug, incubation at 37 °C in a humidified atmosphere of 95:5 air/CO_2 for 7–10 days, and scoring. [c] ED_{50} = drug concentration for 50% inhibition of the incorporation of [^{3}H]thymidine in the DNA or [^{3}H]uridine in the RNA of actively growing L1210 cells in culture, after 3 h of exposure. Drugs were initially dissolved in Me_2SO, and the solution was diluted to a final concentration of 1% Me_2SO. [d] ΔT_m = T_m of DNA–drug complex − T_m of DNA (calf thymus): concentration of drug, 5.2×10^{-6} M; concentration of DNA (*P*), 5.2×10^{-5} M in 0.01 M phosphate buffer (pH 7) containing 10^{-5} EDTA and 5% Me_2SO. [e] Log *P* = log of ratio of concentration in organic phase/partitioned concentration in H_2O phase, measured from UV absorbances. [f] Results from Adria Laboratories. [g] Results from Farmitalia Carlo Erba. [h] Results from SRI. [i] Data from ref 1. [j] Compared with 262%, T/C for 1 = 252%; ref 1. [k] Toxic deaths, 10/30 after 3 months; T/C = 168% at 0.0125 mg/kg.

with **4** at pH 2. The *N*-(2-hydroxyethyl) analogue **2** was identified by mass spectral analysis and then by synthesis of an authentic sample. The synthesis was carried out by reductive alkylation of **1** with glycolaldehyde and $NaBH_3CN$, followed by chromatographic purification. Among the several byproducts, oxazolidino structure **5** was of interest as another example of aldehyde condensation with NH and 4′-OH in these reactions. As a degradation product of **3**, structure **2** from fragmentation of the cyanomorpholine was difficult to rationalize.[16] Conceivably, protonation of the morpholino ring O at pH 2 could be followed by loss of the proton on the α carbon with elimination of the β alkoxyl to form **6**. Further protonation at the enamine terminal carbon would be favored by conversion to the iminium species **7**, which is readily hydrolyzed. This process would be distinct from the simpler ejection of CN that was proposed[7–9] for the saframycins and naphthyridinomycins. Such mechanisms may of course operate simultaneously. They are of interest because they may be related to the biological action of **3**. Further studies will be required to fully account for the hydrolytic losses of **3**.

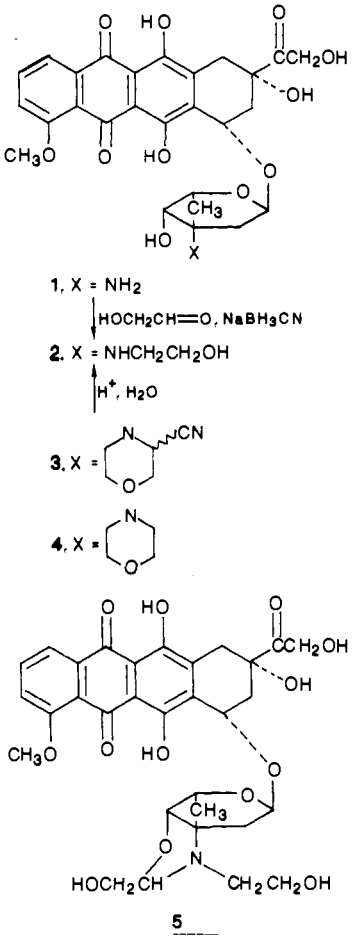
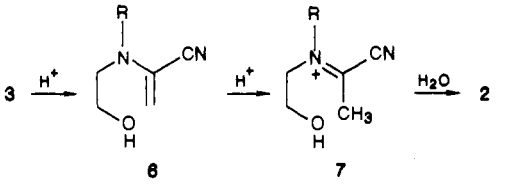


Table III. Growth Inhibition in Sensitive and Resistant Cells[a]

compd	IC_{50}, μM P388/S[b]	P388/ADR[c]	resistance index[d]
1	0.068	8.2	121
3	0.000 16	0.000 30	1.9
2	0.23	3.9	17

[a] After 48-h exposure to drug; procedure as in ref 4; preliminary presentation of data in ref 6. [b] The cells routinely used in screening. [c] A P388 subline resistant to 1, as in ref 4. [d] Resistance index = ratio of IC_{50} values for P388/ADR ÷ P388/S.



Biological Results

It was clear that hydrolysis of **3** to **2** was not an activation process. Screening data are presented in Table II. In two independent tests against leukemia P388 in the mouse, **2** was poorer than **1** in efficacy (% T/C), comparable to **1** in potency, and hence 300–600 times less potent than cyanomorpholine **3**. Consistent with this, **2** was significantly less active than **1** in HeLa cells as an inhibitor of colony formation and in L1210 cells as an inhibitor of DNA/RNA synthesis. Relative to **3**, the loss of potency in cell culture was nearly 2000 times. Like few *N*-alkyl analogues, **2** approached **1** in polarity, as measured by the octanol–buffer partition coefficient (log *P*). It bound effectively to DNA, as measured by elevation of the T_m. Synthetic byproduct **5** was of interest because it was more potent than **1** in L1210 cells and 10 times more potent than **2**.

(12) Complete glycosyl cleavage was encountered in attempts at 14-bromination of *N*-(trifluoroacetyl)daunorubicin, presumably due to the HBr released; T.H.S., unpublished result.
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Activity against the sensitive and resistant sublines of leukemia P388 is compared in Table III. The sensitive line showed the 3-fold decreased potency of **2** relative to **1**, but the resistant line showed that **2** was twice as potent as **1**. The significant comparison is of the resistance index, showing that **2** was intermediate between **1** and the non-cross-resistant cyanomorpholine **3**. It is not clear why the usual doxorubicin cross resistance should be even partly deleted with an *N*-alkyl derivative such as **2**. The resistance index (17) of **2** was the same as that (14) recently reported[6,14] for *N*-(cyanomethyl)doxorubicin, the simplest of the α-cyano amines. It was clear, however, that the capacity of **3** to overcome cross resistance was significantly diminished in going from **3** to **2**, just as was antitumor activity in other screens.

Relationships between chemical properties (structure, reactivity) and biological properties (antitumor efficacy, potency, cross resistance) are of continuing interest in this series.

Experimental Section

Chemical and biological methods were the same as previously described in this series.[1,4,14,15] Reverse-phase HPLC analyses were done on a Spectra-Physics SP-8100LC using a Waters Z-Module Radial Pak Nova C-18 5-μm column, with a flow rate of 2 mL/min and monitoring at 254 nm. DCI-MS and NDCI-MS were determined on a Ribermag R10-10C GC-MS with NH_3 as the reagent gas; in the assignments, the term sugar refers to that portion of the parent structure, without the glycosyl O.

***N*-(2-Hydroxyethyl)doxorubicin Hydrochloride (2·HCl).** A stirred solution of 0.580 g (1.00 mmol) of **1**·HCl and 0.120 g (2.00 mmol) of glycoaldehyde (crystalline dimer, Aldrich) in 30 mL of CH_3CN–H_2O (2:1) was treated with a solution of 0.042 g (0.67 mmol) of $NaBH_3CN$ in 1.5 mL of CH_3CN–H_2O (2:1). The mixture was stirred at room temperature in the dark for 1 h and poured into 50 mL of 0.1 N acetic acid. The solution was washed with $CHCl_3$, basified with solid $NaHCO_3$, and extracted repeatedly with 25-mL portions of $CHCl_3$–CH_3OH (4:1) and then $CHCl_3$. The combined extracts were dried and evaporated to 0.400 g of a solid residue containing the following: 30–40% of **2** free base, R_f = 0.15 by TLC in $CHCl_3$–CH_3OH–H_2O (40:10:1); along with 30–40% of the 13-dihydro derivative of **2** (identified by MS), R_f = 0.04; 10% of **1**, R_f = 0.10; and 10–15% of unidentified byproducts R_f = 0.45, 0.55, and 0.65. The solid was redissolved in 2 mL of CH_2Cl_2–CH_3OH (6:1) and added to a column (1.5 × 27 cm) of silica gel in CH_2Cl_2–CH_3OH (98:2). The column was eluted with CH_2Cl_2–CH_3OH in 100-mL lots (98:2, 96:4, 94:6, 92:8 (200 mL), 90:10, 88:12, 86:14, 84:16) and finally 1000 mL (80:20). Eluate fraction A (840 mL) afforded 78 mg consisting of lipophilic byproducts, fraction B (60 mL) 18 mg from which 12 mg of **2** was obtained by preparative TLC, and fraction C (240 mL) 75 mg of **2**. A suspension of the combined 87 mg in 10 mL of H_2O was stirred and acidified to pH 4.5 with 1.22 mL of 0.1 N HCl added dropwise. The resultant solution was lyophilized, and the residual hydrochloride was dissolved in 2 mL of CH_3OH and precipitated with 15 mL of ether to yield 0.084 g (13%). Purity was 89% by reverse-phase HPLC in 0.1 M NaH_2PO_4–CH_3CN (75:25), containing 1% of the 13-dihydro derivative and 6% of the *N*,*N*-bis(2-hydroxyethyl) analogue (identified by MS of a purified fraction). Presence of 2% of **1** was analyzed by TLC. For **2**, UV–vis (MeOH) λ_{max} 234 nm ($\epsilon \times 10^{-3}$ 37.2), 252 (25.7), 289 (8.66), 478 (12.0), 530 (6.74); NDCI-MS, *m*/*z* 587 (M); DCI-MS, *m*/*z* 192 (sugar + H_2O), 174 (sugar). Anal. ($C_{29}H_{33}NO_{12}$·HCl·1.5 H_2O) C, H, N.

Preparative TLC ($CHCl_3$–CH_3OH, 6:1) of fraction A above afforded an 8-mg fraction that was identified as ***N*-(2-hydroxyethyl)-*N*,4′-*O*-(2-hydroxyethylidene)doxorubicin (5)** by NDCI-MS, *m*/*z* 629 (M); DCI-MS, *m*/*z* 234 (sugar + H_2O), 216 (sugar). Other fractions were mixtures of **5** with the 13-dihydro derivative.

Hydrolysis of 3. A solution of 5.0 mg of **3** in 3.5 mL of CH_3OH was diluted with 3.5 mL of 0.1 M H_3PO_4 and stored at 23 °C in the dark. After 3, 5, and 24 h, aliquots were analyzed by reverse-phase HPLC in pH 4 0.05 M citrate buffer–CH_3OH (35:65). Disappearance of the well-resolved diastereoisomers of **3** (55% **a**, 42% **b**) was accompanied by the appearance of two new products, with an overall decrease in total absorbance. The solution after 24 h was diluted with 10 mL of H_2O and extracted with 10 mL of $CHCl_3$–CH_3OH (95:5). The dried extract was evaporated to yield 0.4 mg containing 77% of doxorubicinone along with unreacted **3** diastereoisomers (8% **a**, 13% **b**), identified by HPLC. The aqueous portion was neutralized with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$–CH_3OH (95:5; 2 × 8 mL). These extracts were dried and evaporated to yield 2.4 mg containing 10–20% of **2** (identified by MS and by TLC and HPLC comparison with synthetic **2**) and 9% of doxorubicinone.

Essentially identical results were observed in CH_3OH solution diluted with 0.1 M citric acid (pH 2). In undiluted CH_3OH solution, **2** underwent no change after 24 h. After 6 weeks in CH_3OH at −10 °C, only minor contaminants were formed (3%, 2%, 1%, unidentified).

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(16) Note added in proof: The complete acid decomposition of cyanomorpholine in contrast to cyanopiperidine was recently noted. Barton, D. H. R.; Billion, A.; Boivin, J. *Tetrahedron Lett.* **1985**, *26*, 1229.

Book Reviews

Synthesis and Applications of Isotopically Labeled Compounds 1985. Second International Symposium, Kansas City, Missouri, 3–6 September 1985. Edited by R. R. Muccino. Elsevier, New York. 1986. xxxiv + 557 pp. 17 × 24.5 cm. ISBN 0-444-42612-4.

This volume relates the work presented at the Second International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds held at Kansas City, MO, during Sept 3–6, 1985. The conference gathered 337 participants from 19 countries to review current and future directions on isotope research via 178 oral and poster presentations.

The text is arranged in essentially the chronological order of the conference proceedings. Whereas the first Kansas City Symposium in 1982 stressed the complimentary nature of radioactive and stable isotopes, the emphasis of this most recent meeting was on the newer applications of labeled compounds. Therefore, this book reviews such topics as the preparation and use of labeled peptides, radioligands in receptor studies, applications of ^3H and ^{15}N NMR, and clinical applications of NMR imaging. Aside from the purely technical papers, the volume also contains the historically valuable reflections of Professor Melvin Calvin during his award address. As a pioneer in the synthesis and applications of isotopically labeled compounds, Professor Calvin's after-dinner discourse was a memorable highlight of the 4-day meeting. An author and subject index and a useful list of conference attendees' names and addresses are also included in the book.