JOURNAL OF
MEDICINAL
CHEMISTRY

Volume 32, Number 6 June 1989

Communications to the Editor

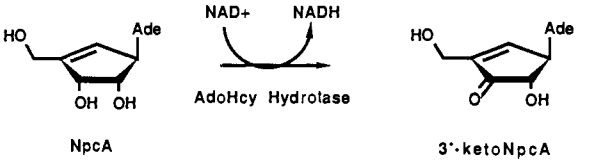
Oxidation of Neplanocin A to the Corresponding 3′-Keto Derivative by *S*-Adenosylhomocysteine Hydrolase

Sir:

In recent years, *S*-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) has emerged as a target for the design of antiviral agents.[1,2] De Clercq and Cools[3] have established a close correlation between the antiviral potency of a variety of adenosine analogues, including neplanocin A (NpcA, Scheme I), and their inhibitory effects on AdoHcy hydrolase. Inhibition of AdoHcy hydrolase results in increased levels of intracellular AdoHcy, a product inhibitor of *S*-adenosylmethionine (AdoMet) dependent methyltransferases.[4] We have previously proposed that the antiviral activity of AdoHcy hydrolase inhibitors results from the inhibitory effects of elevated cellular AdoHcy levels on methyltransferases necessary for viral mRNA maturation.[2]

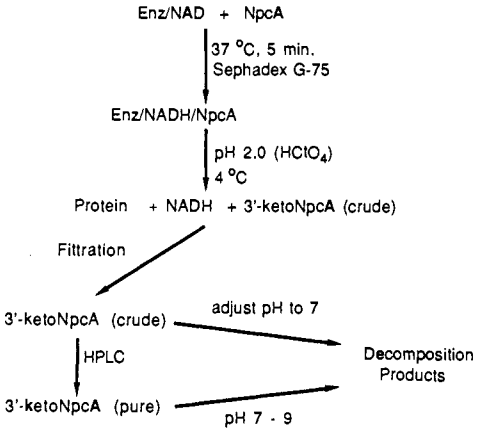
Our research group has shown that NpcA is a potent inhibitor of AdoHcy hydrolase isolated from beef liver[4] and *Alcaligenes faecalis*.[5] The two mechanisms proposed for NpcA-induced inactivation of AdoHcy hydrolase both involve reduction of enzyme-bound NAD^+ to NADH as the initial step.[6,7] Since this step is analogous to the first step in the normal catalytic mechanism described by Palmer and Abeles,[8] it has been reasonably assumed that reduction

Scheme I



NpcA 3′-ketoNpcA

Scheme II. Methodology for the Isolation and Purification of 3′-KetoNpcA



of NAD^+ to NADH is accompanied by oxidation of the NpcA to the corresponding 3′-ketocyclopentenyl derivative (3′-ketoNpcA, Scheme I). However, questions still remain concerning the fate of 3′-ketoNpcA. Does this intermediate depurinate in the active site, resulting in the formation of an electrophile that is subsequently attacked by water, homocysteine, or an enzyme nucleophile? If 3′-ketoNpcA is stable, does it remain tightly bound to the enzyme; or does it dissociate, leaving behind the inactive NADH form of the enzyme?

In this communication, we describe the isolation and characterization of 3′-ketoNpcA formed by AdoHcy hydrolase catalyzed oxidation of NpcA. The fact that 3′-ketoNpcA is stable enough to be isolated from AdoHcy hydrolase suggests that inactivation of the enzyme does not result from depurination of 3′-ketoNpcA followed by covalent modification at the active site as proposed by Wolfson et al.[7] We also report that 3′-ketoNpcA is a weak, competitive inhibitor of the NAD^+ form of bovine liver

(1) De Clercq, E. *Biochem. Pharmacol.* **1987**, *36*, 2567.
(2) Keller, B. T.; Borchardt, R. T. *Antiviral Drug Development*: a Multidisciplinary Approach; De Clercq, E., Walker, R. T., Eds.; Plenum Press: New York, 1988; pp 123–138.
(3) De Clercq, E.; Cools, M. *Biochem. Biophys. Res. Commun.* **1985**, *129*, 306.
(4) Borchardt, R. T.; Keller, B. T.; Patel-Thombre, U. *J. Biol. Chem.* **1984**, *259*, 4353.
(5) Matuzewska, B.; Borchardt, R. T. *Arch. Biochem. Biophys.* **1987**, *256*, 50.
(6) Matuzewska, B.; Borchardt, R. T. *J. Biol. Chem.* **1987**, *262*, 265.
(7) Wolfson, G.; Chisholm, J.; Tashjian, A. H., Jr.; Fish, S.; Abeles, R. H. *J. Biol. Chem.* **1986**, *261*, 4492.
(8) Palmer, J. L.; Abeles, R. H. *J. Biol. Chem.* **1979**, *254*, 1217.

0022-2623/89/1832-1415$01.50/0

AdoHcy hydrolase and that, upon formation, this oxidation product remains tightly bound to the NADH form of this enzyme. Inactivation results from the reduction of NAD^+ to NADH (which we refer to as a cofactor depletion mechanism) and the high affinity of 3′-ketoNpcA for the NADH form of the enzyme.

The 3′-ketoNpcA was prepared by oxidation of NpcA with AdoHcy hydrolase (Scheme II).[6,9] Freshly prepared bovine liver AdoHcy hydrolase[10] was incubated for 5 min at 37 °C with a slight excess of [8-^{3}H]NpcA.[11] The excess [8-^{3}H]NpcA was removed by size-exclusion chromatography on Sephadex G-75. Attempts to denature the enzyme/3′-ketoNpcA complex at neutral pH with sodium dodecyl sulfate (0.02–0.8%) resulted in release of only 20–30% of the tritiated material; this method was not pursued further. The enzyme/3′-ketoNpcA complex was denatured and the 3′-ketoNpcA released from the protein by treatment with 0.01 N $HClO_4$.[12] The denatured protein was removed by filtration; analysis of the acidic filtrate by HPLC revealed the presence of a new peak (elution time 11.6 min) which was not present when either NpcA (elution time 12.2 min) or AdoHcy hydrolase alone was acidified. Scintillation counting confirmed that this new peak, which accounted for 80–90% of the tritiated material, was enzymatically transformed NpcA, presumably the 3′-keto derivative. The remainder of the radiolabeled material was adenine (14.8 min) and an unidentified material eluting at 18 min. If the acidic filtrate was made neutral or alkaline, the presumed 3′-ketoNpcA decomposed to adenine and/or an unidentified degradation product in variable proportions. The amount of adenine liberated appears to depend on the buffer used to purify the enzyme/3′-ketoNpcA complex by gel filtration (Tris buffer causing more depurination than phosphate buffer). Homogeneous 3′-ketoNpcA (Figure 1A) was obtained by preparative HPLC and stored as an acidic buffered solution (pH 2.3) at 4 °C.

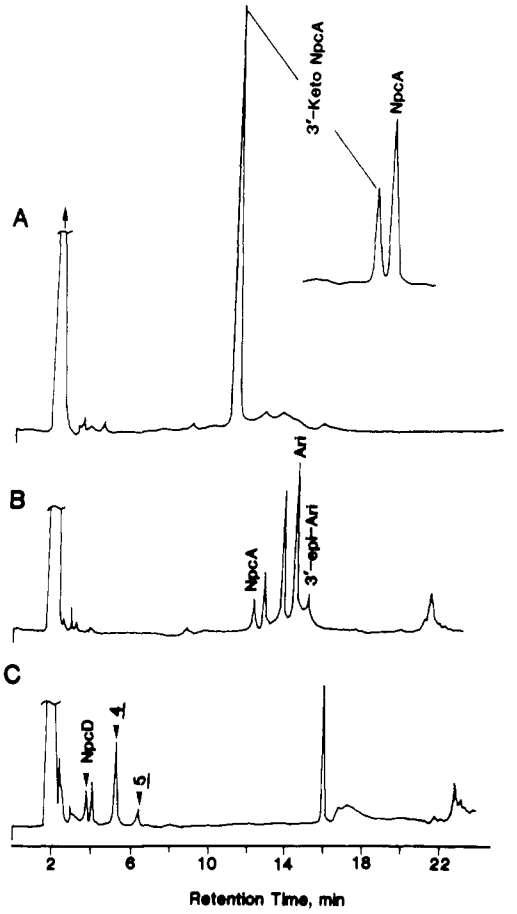


Figure 1. HPLC chromatograms of 3′-ketoNpcA and its reduction/deamination products. High-pressure liquid chromatography (HPLC) was performed on a Shimadzu SLC-6A chromatograph using a C-18 reversed-phase column (15 × 0.46 cm, ODS Hypersil) and gradient elution using the following system: 3% solvent A for 2 min and then 3%–20% solvent A for 23 min. Solvent A was acetonitrile and solvent B was 50 mM sodium phosphate and 10 mM sodium heptanesulfonate (HSA) adjusted to pH 3.20 with H_3PO_4. Absorbance was monitored at 259 nm. A: purified 3′-ketoNpcA. The inset shows coinjection with NpcA. B: products from reduction of purified 3′-ketoNpcA with $NaBH_3CN$. The peaks labeled NpcA, Ari, and 3′-epi-Ari were identified by coinjection with authentic samples. C: products from deamination of reduced 3′-keto-NpcA with adenosine deaminase. The peaks labeled NpcD, 4, and 5 were identified by coinjection with samples prepared by deamination of the reference materials.

Reaction of [8-^{3}H]NpcA with AdoHcy hydrolase does not allow for preparation of large quantities of [8-^{3}H]-3′-ketoNpcA. Attempts were made to scale up the oxidation of NpcA under potentially catalytic conditions with AdoHcy hydrolase (e.g. by adding exogenous NAD^+ to reactivate the enzyme) or with commercially-available alcohol dehydrogenases; however, these protocols were uniformly unsuccessful (data not shown). Since [8-^{3}H]-3′-ketoNpcA was not available in sufficient quantities to characterize its structure by conventional spectroscopic methods (e.g. NMR, mass spectroscopy), we resorted to chemical methods for characterization. The product of oxidation was shown to be the 3′-ketonucleoside by reduction with $NaBH_3CN$ yielding known compounds. The identities of these reduction products were further confirmed by deamination with adenosine deaminase.

Reduction with $NaBH_3CN$ is not selective for 1,2- vs 1,4-hydride addition[13] nor, in this case, is it stereospecific.

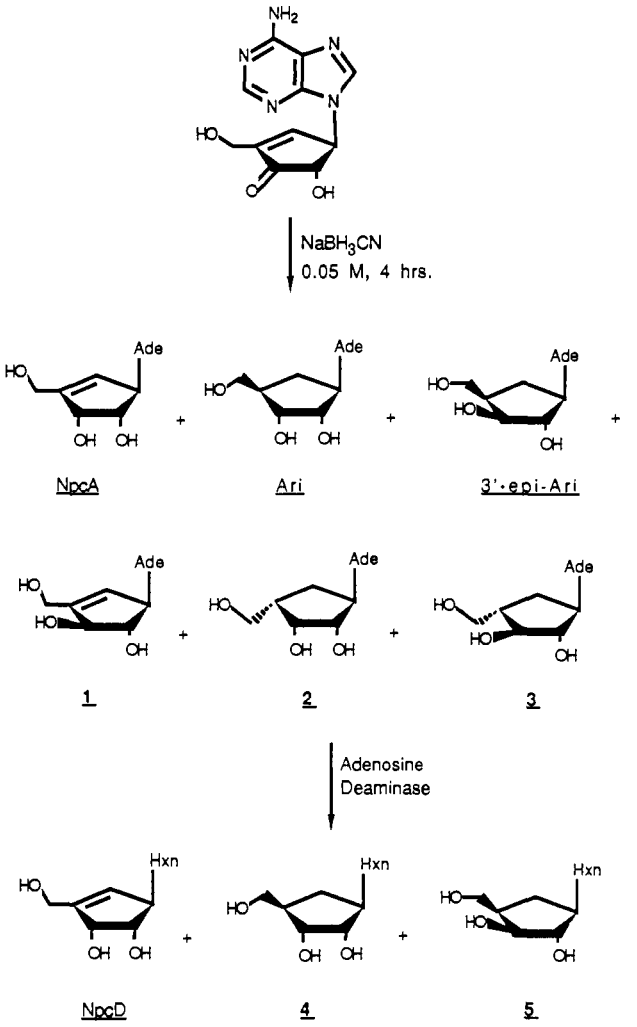
(9) To 2–3 mg of the purified bovine liver AdoHcy hydrolase[10] in 2–4 mL of 10 mM Tris buffer (pH 6.8) was added a slight molar excess of [^{3}H]NpcA. After a 5-min incubation at 37 °C, residual inhibitor was removed on a Sephadex G-75 column (15 × 1.3 cm) eluting with either 10 mM $NaHPO_4$ or Tris buffer (both pH 7.4). Absorbance was monitored at 214 nm. The peak corresponding to the AdoHcy hydrolase/3′-ketoNpcA complex was concentrated (Amicon PM30 membrane filter) and denatured as described in ref 12.

(10) Narayanan, S. R.; Borchardt, R. T. *Biochem. Biophys. Acta* 1988, *965*, 22.

(11) Neplanocin A (10 mg, 38 μmol) was placed in a screw-cap culture tube. Frozen [^{3}H]H_2O (0.2 mL, 1 Ci, ICN Radiochemicals, Inc., Irvine, CA) was then transferred in under vacuum. The culture tube was capped, placed in a steel pipe, and heated to 100 °C for 6 h in a sand bath. The solution was then cooled and lyophilized to remove [^{3}H]H_2O. Unlabeled H_2O (3 × 0.5 mL and 1 × 1.0 mL) was added and lyophilized. The activities of the last two H_2O samples were similar and minimal. The [8-^{3}H]NpcA (specific activity 35 dpm/pmol) showed a single peak by HPLC that coeluted with NpcA.

(12) The AdoHcy hydrolase/3′-ketoNpcA complex purified by Sephadex G-75 chromatography was acidified with perchloric acid to pH 2.3. The precipitated protein was removed with a protein concentrator (Amicon PM30 or YMT membrane) at 4 °C. The filtrate, containing the 3′-ketoNpcA, was applied to a C-18 reversed-phase HPLC column and eluted with the gradient system described in the legend of Figure 1. In order to decrease the salt content of the purified 3′-ketoNpcA, the collected fractions were concentrated by rotary evaporator and then recycled through the C-18 column using the same gradient but with solvent B diluted 8-fold with distilled water. Acetonitrile was removed by concentrating the eluate with a rotary evaporator, and then the purified 3′-ketoNpcA was stored at 4 °C at pH 2.3 in NaH_2PO_4/HSA solution (ca. a 1:5 dilution of solvent B). Concentration to dryness in a lyophilizer or rotary evaporator resulted in decomposition of the 3′-ketoNpcA.

Scheme III. Reduction/Deamination Products of 3′-KetoNpcA[a]



[a] Ade = 9-adenyl, Hxn = 9-hypoxanthyl. Only those deaminated products which were identified are depicted.

Therefore, reduction of 3′-ketoNpcA is expected to yield a mixture of six possible products (Scheme III): NpcA, Ari, 3′-epi-Ari, 3′-epi-NpcA (**1**) and two other diastereomers of aristeromycin (**2** and **3**). $NaBH_3CN$ (Aldrich Chemical Co., Milwaukee, WI) reduction of the tritiated product isolated from AdoHcy hydrolase catalyzed oxidation of NpcA resulted in the formation of a complex mixture from which five peaks could be resolved by HPLC (Figure 1B). Collection and scintillation counting demonstrated that the tritium content (data not shown) of fractions of the HPLC eluate corresponded to the chromatogram obtained by UV monitoring of the eluate. NpcA is unchanged after treatment with $NaBH_3CN$. Of the five separable products, three were identified as NpcA, Ari, and 3′-epi-Ari by coelution with authentic samples. Formation of Ari and 3′-epi-Ari particularly demonstrates the presence of a 3′-carbonyl in the product isolated from the reaction of AdoHcy hydrolase and NpcA.

As definitive identification of 3′-ketoNpcA depended on chemical characterization of its reduction products, we chose to confirm their identities by a further transformation. The mixture of products obtained by $NaBH_3CN$ reduction of the presumed 3′-ketoNpcA was neutralized and allowed to react with adenosine deaminase (Type III; Sigma Chemical Co., St Louis, MO; 4 IU, 2 h, 22 °C). This resulted in deamination of four of the reduction products (Figure 1C). Three of these products were identified (scheme III) as deaminated NpcA (NpcD), deaminated Ari (**4**), and deaminated 3′-epi-Ari (**5**) by coinjection with samples prepared by deamination of the authentic compounds. Again, scintillation counting confirmed that each of the deamination products arose from the [8-^{3}H]NpcA used to inactivate AdoHcy hydrolase. This two-stage characterization of the product of AdoHcy hydrolase catalyzed oxidation of NpcA provides strong evidence supporting the formation of 3′-ketoNpcA as the oxidation product.

Since depurination of 3′-ketoNpcA generated by AdoHcy hydrolase catalyzed oxidation of NpcA has been suggested to be a critical step in the irreversible inactivation of the enzyme,[7] we decided to investigate the chemical stability of the purified 3′-ketoNpcA. As noted above, adenine was produced in variable amounts (5–60%) when crude solutions of 3′-ketoNpcA were neutralized. Another unidentified degradation product formed in inverse proportion to adenine. The decomposition of the purified 3′-ketoNpcA in phosphate buffer was monitored by HPLC. The amount of 3′-ketoNpcA remaining was determined by collecting the fraction eluting from 11–13 min and determining the tritium content (the small amount of 3′-ketoNpcA available from the AdoHcy hydrolase catalyzed oxidation of NpcA made it impractical to use peak area measurements). The 3′-ketoNpcA decayed with apparent first-order kinetics at rates of 0.004 day^{-1} (pH 2.3, 4 °C), 0.09 day^{-1} (pH 7.0, 22 °C), and 0.5 day^{-1} (pH 9.0, 22 °C). The major decomposition product had an elution time of 17 min; only traces of adenine (<10%, elution time 14.8 min) were formed in these experiments. As indicated above, treatment of the crude 3′-ketoNpcA with Tris buffer solution during isolation leads to more significant decomposition to adenine. Collectively, these data show that 3′-ketoNpcA is surprisingly stable in acidic aqueous solution, and that the depurination which was proposed by Abeles and coworkers[7] to be an integral part of k_{cat} inactivation of AdoHcy hydrolase is probably an artifact of the workup procedure, which involved neutralization of the crude reaction mixture.

In addition to determining the physicochemical properties of 3′-ketoNpcA, we were also interested in determining its effects on the catalytic activity of AdoHcy hydrolase. Since 3′-ketoNpcA could only be isolated from the inactivated AdoHcy hydrolase by $HClO_4$ denaturation, it appears that the 3′-ketonucleoside binds tightly to the NADH form of the enzyme. However, 3′-ketoNpcA was observed to be a weak, reversible inhibitor of the NAD^+ form of AdoHcy hydrolase (K_i = 0.8 μM) as measured in the hydrolytic direction with [^{3}H]AdoHcy.[14] In an independent study, we have shown that 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine,[15] a NpcA analogue synthesized in this laboratory,[16] is oxidized to the corresponding 3′-ketonucleoside by AdoHcy hydrolase or manganese dioxide.[17,18] Like 3′-ketoNpcA, this 3′-ketonucleoside is reasonably stable in acidic solution but decomposes quickly in neutral or alkaline media. This 3′-

(13) Hutchins, R. O.; Kandasamy, D. *J. Org. Chem.* **1975**, *40*, 2530.

(14) Richards, H. H.; Chiang, P. K.; Cantoni, G. L. *J. Biol. Chem.* **1978**, *253*, 4476.

(15) Narayanan, S. R.; Keller, B. T.; Borcherding, D. R.; Scholtz, S. A.; Borchardt, R. T. *J. Med. Chem.* **1988**, *31*, 500.

(16) Borcherding, D. R.; Scholtz, S. A.; Borchardt, R. T. *J. Org. Chem.* **1987**, *52*, 5457.

(17) Paisley, S. D.; Hasobe, M.; Borchardt, R. T. *Nucleotides Nucleosides* In press.

(18) Paisley, S. D.; Wolfe, M. S.; Borchardt, R. T. *FASEB J.* **1988**, *2*, A1119 (Abstr 4783).

ketonucleoside is also a weak, competitive inhibitor of AdoHcy hydrolase and does not produce time-dependent inactivation of the enzyme.

The data presented above, as well as the observation in our laboratory that AdoHcy hydrolase inactivated by NpcA or the synthetic analogue of NpcA can be reactivated by incubation with NAD^+,[6,15] strongly suggest that these inhibitors inactivate the enzyme by a cofactor depletion mechanism, which simply involves conversion of the enzyme from the NAD^+ form (catalytically active) to the NADH form (catalytically inactive), and tight-binding of the 3′-ketoNpcA to this NADH form. These data do not support the k_{cat} mechanism proposed by Wolfson et al.[7] for the NpcA-induced inactivation of AdoHcy hydrolase.

Other AdoHcy hydrolase inactivators may act through similar mechanisms to that of NpcA. For example, 4′,5′-unsaturated 5-fluoroadenosine inactivators convert enzyme-bound NAD^+ to NADH.[19] The subsequent loss of fluoride anion in this case may be due to Michael addition of water, forming a product which has a high affinity for the NADH form of AdoHcy hydrolase. Further studies on the mechanism of AdoHcy hydrolase inactivators should afford the opportunity to rationally design potent and selective inhibitors of this enzyme.

Acknowledgment. This research was supported by grants from the National Institutes of Health (Grant No. GM-29332) and Glaxo, Inc. NpcA was a gift from Dr. Junichi Murase (Toyo Jozo Co., Tagata-gun, Shizuoka, Japan), aristeromycin (Ari) was a gift from Dr. John Montgomery (Southern Research Institute, Birmingham, AL), and 1α-adenin-9-yl-2α,3β-dihydroxy-4α-(hydroxymethyl)cyclopentane (3′-epi-Ari) was a gift from Dr. Robert Vince (University of Minnesota, Minneapolis, MN). We thank Dr. Robert P. Hanzlik (University of Kansas) for assisting in the synthesis of ^{3}H-NpcA and Dr. Mathias P. Mertes (University of Kansas) for helpful discussions, specifically for suggesting the term "cofactor depletion mechanism".

(19) McCarthy, J. R.; Jarvi, E. T.; Matthews, D. P.; Edwards, M. L.; Prakash, N. J.; Bowlin, T. L.; Mehdi, S.; Sunkara, P. S.; Bey, P. *J. Am. Chem. Soc.* **1989**, *111*, 1127.

Steven D. Paisley, Michael S. Wolfe
Ronald T. Borchardt*
Departments of Pharmaceutical Chemistry and Medicinal Chemistry
The University of Kansas
Lawrence, Kansas 66045
Received February 1, 1989

E-Ring Desoxy Analogues of Etoposide

Sir:

Etoposide (1, VP-16, Vepesid), a clinically efficacious antineoplastic drug used for the treatment of testicular cancer and one of the most active single agents against small cell lung cancer, is the least toxic of all chemotherapeutic agents.[1] As a semisynthetic derivative of the naturally occurring lignan podophyllotoxin,[2] a potent inhibitor of microtubule assembly,[3] VP-16 apparently exerts its cytotoxic effect by DNA strand scission, retaining little of the antimitotic potency of its aglycon. In 1983, independent studies by Ross[4] and Long[5] demonstrated that DNA breakage is associated with etoposide-induced DNA/protein cross-links and correlates with both the cytotoxicity and the ability of several analogues to inhibit the catenation activity of eukaryotic topoisomerase II (topo II). During this same period, the investigations of Sinha[6] pointed to the relevance of the etoposide *o*-quinone **2** in the mechanism of action, since VP-16 gives rise to a stable phenoxy radical following in vitro peroxidative activation, and because the intermediate formed during microsomal activation irreversibly binds to both nucleic acids and proteins. Sinha's later bioactivation studies[7] have confirmed this *o*-quinone and identified the E-ring phenoxy radical by ESR spectroscopy. Liu and co-workers[8] have found that inhibition of topo II by VP-16 blocks DNA religation by stabilizing the initially formed cleavable complex, thereby leading to DNA scission. Early structure–activity relationship (SAR) data revealed that a free 4′-hydroxyl group in 1 was essential for DNA breakage[9] and antitumor activity,[10] whereas 4′-methoxy derivatives were mostly inactive. A free 4′-phenol may confer both hydrogen-bonding capability for interaction with topo II and/or DNA and also would greatly enhance the biooxidation process. Therefore, in order to probe the significance of the *o*-quinone and topo II based mechanisms, we now report the first synthesis and biological evaluation of etoposide desoxy E-ring analogues **6**, **7**, and **9**.[11]

The synthesis of the title compounds was achieved by using both total and semisynthetic techniques. Oxidation of etoposide (1) to *o*-quinone **2** using the method of Nemec,[12] followed by condensation with methoxylamine hydrochloride in pyridine, regioselectively delivers monoquinone oxime **3**. Hydrogenolysis of **3** gives aminophenol **4** (70% overall), which yields diazo phenoxide **5** (79%) after treatment with $NaNO_2$ in HOAc/THF. Reduction of **5** ($NaBH_4$/MeOH, 41%) provides 3′-desmethoxyetoposide (**6**, 3′-DesMeOVP). Efficient preparation of 4′-deshydroxyetoposide (**7**, 4′-DesOHVP) proceeded via

(1) Issell, B. F.; Muggia, F. M.; Carter, S. K. *Etoposide (VP-16) Current Status and New Developments*; Academic Press: New York, 1984.
(2) Keller-Juslen, C.; Kuhn, M.; von Wartburg, A. *J. Med. Chem.* **1971**, *14*, 936.
(3) Cassady, J. M.; Douros, J. D. *Anticancer Agents Based on Natural Product Models*; Academic Press: New York, 1980; Chapter 9 by Jardine, I.
(4) Wozniak, A. J.; Ross, W. E. *Cancer Res.* **1983**, *43*, 120.
(5) (a) Long, B. H.; Minocha, A. *Proc. Am. Assoc. Cancer Res.* **1983**, *24*, 321. (b) Minocha, A.; Long, B. H. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 165. (c) Long, B. H.; Brattain, M. G., see Chapter 5 in ref 1.
(6) (a) Sinha, B. K.; Trush, M. A. *Biochem. Pharmacol.* **1983**, *32*, 3495. (b) Sinha, B. K.; Myers, C. E. *Ibid.* **1984**, *33*, 3725. (c) Sinha, B. K.; Trush, M. A.; Kalyanaraman, B. *Ibid.* **1985**, *34*, 2036. (d) Haim, N.; Nemec, J.; Roman, J.; Sinha, B. K. *Ibid.* **1987**, *36*, 527.
(7) (a) Haim, N.; Roman, J.; Nemec, J.; Sinha, B. K. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 215. (b) Haim, N.; Nemec, J.; Roman, J.; Sinha, B. K. *Cancer Res.* **1987**, *47*, 5835. (c) Katki, A. G.; Kalyanaraman, B.; Sinha, B. K. *Chem.-Biol. Interact.* **1987**, *62*, 237.
(8) (a) Chen, G. L.; Yang, L.; Rowe, T. C.; Halligan, B. D.; Tewey, K. M.; Liu, L. F. *J. Biol. Chem.* **1984**, *259*, 13560. (b) Ross, W.; Rowe, T.; Glisson, B.; Yalowich, J.; Liu, L. F. *Cancer Res.* **1984**, *44*, 5857.
(9) Loike, J. D.; Horwitz, S. B. *Biochemistry* **1976**, *15*, 5443.
(10) (a) Long, B. H.; Musial, S. T.; Brattain, M. G. *Biochemistry* **1984**, *23*, 1183. (b) Long, B. H. *NCI Monogr.* **1987**, *4*, 123. (c) Long, B. H.; Stringfellow, D. A. *Adv. Enzyme Regul.* **1988**, *27*, 223.
(11) All new compounds in this communication gave satisfactory analytical and spectroscopic data in full accord with the assigned structures. More complete synthetic details will be described shortly.
(12) Nemec, J. U.S. Patent 4 609 644, Sept. 2, 1986.

0022-2623/89/1832-1418$01.50/0