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MECHANISM OF ACTION OF THE NITROSOUREAS—V

FORMATION OF O^6-(2-FLUOROETHYL) GUANINE AND ITS PROBABLE ROLE IN THE CROSSLINKING OF DEOXYRIBONUCLEIC ACID

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Abstract—DNA which has been exposed to 2-haloethylnitrosoureas has been shown to contain the chemical crosslink 1-(N^3-deoxycytidyl), 2-(N^1-deoxyguanosinyl)-ethane [W. P. Tong, M. C. Kirk and D. B. Ludlum, *Cancer Res.* **43**, 3102 (1982)]. We have hypothesized that this structure is formed by an initial attack of a 2-haloethyl group on the 6 position of guanine followed by an intramolecular rearrangement and secondary crosslinking reaction with cytosine. We have now shown that DNA which had been reacted with *N*-(2-fluoroethyl)-*N'*-cyclohexyl-*N*-nitrosourea contained O^6-(2-fluoroethyl)guanine and have thus demonstrated that the first step in the proposed mechanism occurs. Furthermore, O^6-(2-fluoroethyl)guanosine hydrolyzed to N^1-(2-hydroxyethyl)guanosine, showing that the necessary intramolar rearrangement occurs. These two observations greatly strengthen the proposed route to DNA crosslinking by the 2-haloethylnitrosoureas.

The 2-haloethylnitrosoureas have proven to be useful antitumor agents for the treatment of lymphomas, brain tumors, and certain other malignancies. Although their mechanism of cytotoxicity is not fully established, it is known that they modify DNA extensively and cause interstrand crosslinking which is probably cytotoxic [1–3].

Recently, we reported the discovery of a crosslinked dinucleoside in DNA exposed to *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU)§. This dinucleoside has the structure of 1-(N^3-deoxycytidyl),2-(N^1-deoxyguanosinyl)-ethane (dCyd-CH_2CH_2dGuo) and is probably responsible for at least some of the interstrand crosslinking which has been observed [4].

We have postulated that this crosslink is formed through an initial attack of a haloethyl group on the 6 position of guanine. The O^6-(2-haloethyl)guanine thus formed could undergo rearrangement and react with its base-pairing partner, cytosine, in the opposite DNA strand [4]. This would account for the important observation of Erickson *et al.* [5] that MER^+ cells, i.e. cells which can remove methyl groups from the 6 position of guanine, are resistant to the 2-haloethylnitrosoureas. Presumably, such cells would remove haloethyl groups from the 6 position of guanine before they could rearrange and form crosslinks.

Although the route, which we have proposed, to the crosslinked structure, dCydCH_2CH_2dGuo, seems plausible, direct evidence for the formation and rearrangement of O^6-(2-haloethyl)guanine has not been available. In this paper, we show that O^6-(2-fluoroethyl)guanine is a product of the reaction between DNA and the cytotoxic nitrosourea, *N*-(2-fluoroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (FCNU).

To examine the properties of O^6-(2-haloethyl)-guanine nucleosides, we have synthesized and characterized O^6-(2-fluoroethyl)guanosine. This compound hydrolyzed to N^1-(2-hydroxyethyl)guanosine, probably through the cyclic intermediate, N^1,O^6-ethanoguanosine. These data greatly strengthen the hypothesis that DNA interstrand crosslinking by the nitrosoureas occurs through an initial attack of the haloethyl group on the O^6 position of guanine followed by rearrangement and subsequent crosslinking.

MATERIALS AND METHODS

Materials. FCNU was obtained from Mr. Leonard Kedda, Drug Research and Development, National Cancer Institute, Division of Cancer Treatment. Fluoroethanol and guanosine were obtained from the Aldrich Chemical Co. (Milwaukee, WI), a 50% dispersion of sodium hydride in oil came from the Alfa Division of the Ventron Corp. (Danvers, MA), and calf thymus DNA was obtained from the Worthington Biochemical Corp. (Freehold, NJ).

Synthesis of O^6*-(2-fluoroethyl)guanosine.* This synthesis follows the procedure we used previously to synthesize O^6-substituted guanosines [6] and involves the displacement of Cl by the FCH_2CH_2O group from sodium fluoroethoxide.

2-Fluoroethanol (2 ml) and sodium hydride (80 mg) were added to 50 mg of 6-chloro-2-aminopurine-9-riboside in a 10-ml round-bottomed

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§ Abbreviations: BCNU, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; dCydCH_2CH_2dGuo, 1-(N^3-deoxycytidyl),2-(N^1-deoxyguanosinyl)-ethane; FCNU, *N*-(2-fluoroethyl)-*N*-cyclohexyl-*N*-nitrosourea; and FAB, fast ion bombardment.

flask, the solution was stirred at room temperature for 5 min and then at 55° for 50 min. Unreacted 2-fluoroethanol was removed under vacuum, and the residue was dissolved in 5 ml of water. This solution was adjusted to neutral pH with dilute HCl, filtered, and separated on a G-10 column (2 × 100 cm) which was eluted with water at a flow rate of 0.98 ml/min; 10-min fractions were collected. Fractions 77–94 contained 830 A_{280} units of the expected product, O^6-(2-fluoroethyl)guanosine; fractions 31–37 contained 39 A_{254} units of a material shown to be N^1-(2-hydroxyethyl)guanosine, as described in Results.

Rearrangement of O⁶-(2-fluoroethyl)guanosine under basic conditions. Fifty A_{280} units of O^6-(2-fluoroethyl)guanosine were dissolved in 25 mM ammonium hydroxide and heated to 100° for 30 min. This solution was cooled and separated on a G-10 column (1.5 × 42 cm) eluted with water at a flow rate of 1 ml/min; again, 10-min fractions were collected. Essentially all of the O^6-(2-fluoroethyl)guanosine was converted to N^1-(2-hydroxyethyl)guanosine, which appeared in fractions 7–10. A trace of starting material appeared in fractions 19–21.

Depurination and rearrangement of O⁶-(2-fluoroethyl)guanosine under acidic conditions. Three hundred A_{254} units of O^6-(2-fluoroethyl)guanosine were dissolved in 2 ml of 0.1 N HCl and incubated overnight at 37°. The solution was then separated on a small G-10 column (1.5 × 42 cm) as described above. Fractions 40–45 contained 75 A_{280} units of the expected O^6-(2-fluoroethyl)guanine, while fractions 8–10 contained 30 A_{254} units of N^1-(2-hydroxyethyl)guanosine. Fraction 6 contained 15 A_{292} units of a compound tentatively identified as O^6,N^1-ethanoguanosine. In addition, there were several smaller unidentified peaks, and the remainder of the material was recovered as unchanged O^6-(2-fluoroethyl)-guanosine.

Reaction of FCNU with DNA. One milligram of FCNU dissolved in 20 μl of 95% ethanol was added to 1 ml of calf thymus DNA solution, 8 mg/ml, in 25 mM sodium cacodylate buffer, pH 7.0. A control solution of DNA was treated with ethanol alone.

These solutions were incubated for 6 hr at 37°; then 50 μl of 6 M NaCl was added and the DNA was precipitated with 2 vol. of 95% ethanol. After the DNA was washed extensively with ethanol, it was redissolved in water and reprecipitated in the same way. Traces of ethanol were removed *in vacuo*, and DNA was redissolved in 1.75 ml of water. Then, 0.2 ml of 1 N HCl was added, and the solution was incubated for 18 hr at 37° to liberate modified purine bases.

The HCl was partially neutralized with 1 N NH_4OH and adjusted so that the pH of the solution remained at approximately 1. A sample of an O^6-methylguanine solution (50 μl, approximately 0.06 A_{283} units) was added as an internal standard. the mixture was then passed through a small DEAE-Sephadex column containing about 0.7 ml of gel to remove oligonucleotides. As indicated by the O^6-methylguanine marker, purine-containing solutions go through this column unchanged so that chromatographic analysis can be performed directly on the filtrate.

High pressure liquid chromatography. High pressure liquid chromatography (HPLC) was performed on a modular apparatus consisting of a Milton–Roy 5000 psi minipump, a Rheodyne model 7020 injector, a Perkin–Elmer LC-55B UV detector, and a Laboratory Data Control Fluoromonitor III interfaced with a Sigma 10 Data System. Analyses were performed on a Spherisorb ODS 5 μm (4.6 × 250 mm) column eluted as described in the footnote to Table 1. The Spherisorb column was protected with a guard column packed with CO : Pell ODS resin from Whatman. All separations were performed at room temperature.

Spectrometry. Ultraviolet spectra were obtained on all derivatives on a Beckman model 35 spectrophotometer in 0.1 N HCl, in 0.1 M sodium cacodylate buffer, pH 7.0, and in 0.1 M NaOH.

Mass spectroscopy was performed, on a Varian MAT 311A instrument, by Mr. Marion Kirk of the Southern Research Institute, Birmingham, AL.

RESULTS

O^6-(2-Haloethyl)guanine nucleosides have apparently not been observed previously. We chose to synthesize O^6-(2-fluoroethyl)guanosine since it is probable that the fluoroethyl derivatives are more stable than the corresponding chlorethyl compounds and that the ribonucleoside is less subject to depurination than the deoxyribonucleoside.

As described in Materials and Methods, fluoroethanol reacted smoothly with 6-chloro-2-aminopurine-9-riboside in the presence of sodium hydride to yield the expected O^6-(2-fluoroethyl)guanosine. This reaction mixture was separated on the G-10 column, and fractions which contained a material with the characteristic spectrum of an O^6-substituted guanosine were pooled and concentrated by lyophilization. HPLC analysis under the conditions shown in Table 1 yielded a single symmetrical peak.

Table 1. High pressure liquid chromatography of nucleoside derivatives*

Compound	Retention time (min) System A	System B
N^1-(2-Hydroxyethyl)guanine	5.4	
N^1-(2-Hydroxyethyl)guanosine	5.7	
O^6-(2-Hydroxyethyl)guanine	8.6	23.0
O^6-(2-Hydroxyethyl)guanosine	10.6	
O^6-(2-Fluoroethyl)guanine	16.0	29.2
O^6-(2-Fluoroethyl)guanosine	19.2	
Guanine		14.0
Adenine		20.0
N^7-(2-Hydroxyethyl)guanine		14.9
N^7-(2-Fluoroethyl)guanine		18.0
O^6-Methylguanine		27.0

* Spherisorb ODS 5 μm (4.6 × 250 mm) column eluted at a flow rate of 1 ml/min with: System A, 8% acetonitrile in 50 mM KH_2PO_4, pH 4.5, or System B, gradient of 5 to 25% acetonitrile in 5 mM sodium heptane sulfonate and 2.5 mM sodium chloride, pH 3.5, run over a 40-min period.

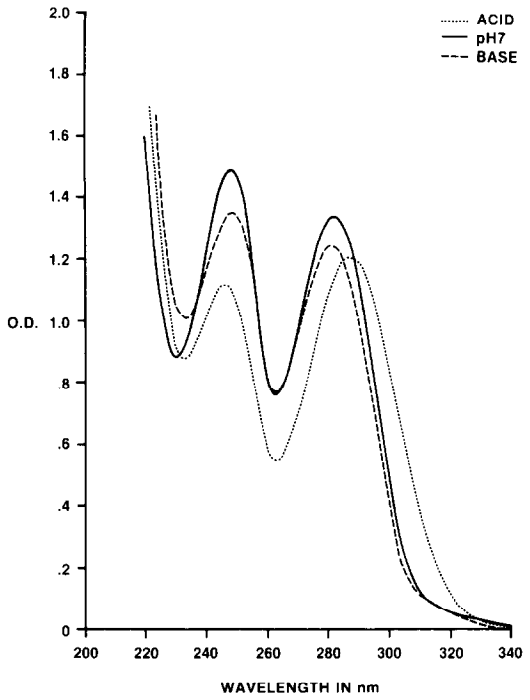

Fig. 1. Ultraviolet spectra of O^6-(2-fluoroethyl)guanosine.

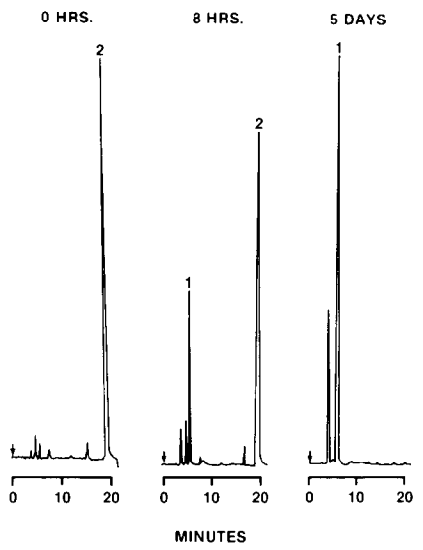

Fig. 3. Rearrangement and hydrolysis of O^6-(2-fluoroethyl)guanosine at 37°. O^6-(2-Fluoroethyl)guanosine was incubated in 0.1 M phosphate buffer, pH 7.4, and, at the indicated times, 100-μl aliquots were analyzed by HPLC as described in Materials and Methods. After 5 days, most of the O^6-(2-fluoroethyl)guanosine (peak 2) had been converted to N^1-(2-hydroxyethyl)guanosine (peak 1).

That this purified material had the expected structure, O^6-(2-fluoroethyl)guanosine, was verified by ultraviolet and mass spectrometry. Ultraviolet spectra in acid, base, and neutral solutions are shown in Fig. 1; these are typical of O^6-substituted guanosines.

The nature of this substituent was verified by electron impact mass spectrometry which revealed M^+ 329 and M − ribose 197, corresponding to the proposed structure. Together with its manner of synthesis (displacement of Cl by the FCH_2CH_2O group from

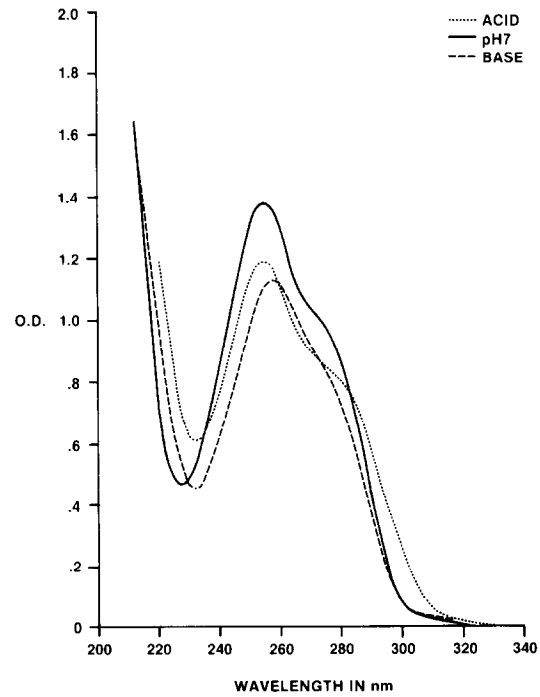

Fig. 2. Ultraviolet spectra of N^1-(2-hydroxyethyl)-guanosine.

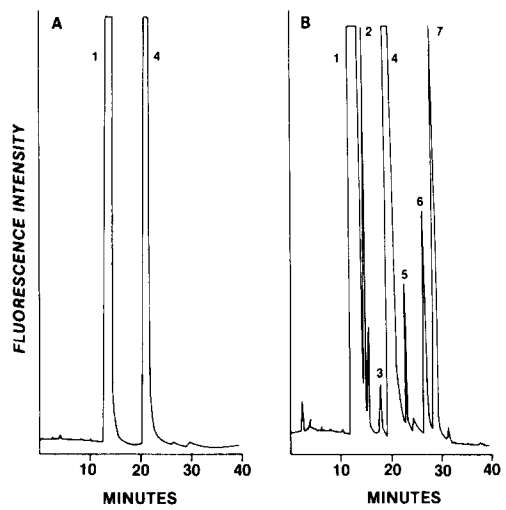

Fig. 4. HPLC separation of purines released from control DNA (left) and DNA which had been reacted with FCNU (right). Derivatives were separated on a Spherisorb column as described in Materials and Methods. The new derivative, O^6-(2-fluoroethyl)guanine, is represented by peak 7. Other peaks: peak 1, guanine; peak 2, N^7-(2-hydroxyethyl)guanine; peak 3, N^7-(2-fluoroethyl)guanine; peak 4, adenine; peak 5, O^6-(2-hydroxyethyl)guanine; and peak 6, O^6-methylguanine (marker).

sodium fluoroethoxide), these data establish this derivative as O^6-(2-fluoroethyl)guanosine.

Under basic conditions, this compound rearranged quickly at 100° to a new derivative which could be separated from the parent compound on a G-10 column. Essentially all of the product appeared in a new peak which was pure by the HPLC criteria of Table 1 and which had the ultraviolet spectrum shown in Fig. 2. This spectrum is typical of a 1-substituted guanosine; that the substituent was a 2-hydroxyethyl group was established by mass spectrometry. Measurements with positive FAB revealed (M + 1) 328 and with negative FAB, (M − 1) 326. Quite evidently, hydrolysis under basic conditions proceeded through the rearrangement reaction described below.

This rearrangement also occurred during depurination under acidic conditions. When the parent compound was heated in 0.1 N HCl at 37°, and the products were separated on a G-10 column, approximately one-fourth of the parent compound was recovered as the expected O^6-(2-fluoroethyl)-guanine. An additional 10% was converted to N^1-(2-hydroxyethyl)guanosine, and an additional small amount was isolated as an unstable derivative which might have been O^6,N^1-ethanoguanosine since it decomposed to N'-(2-hydroxyethyl)guanosine.

Again, these derivatives were purified on a G-10 column and shown to consist of single symmetrical peaks on HPLC. The structure of the base, O^6-(2-fluoroethyl)guanine, was again confirmed by ultraviolet and mass spectrometry. The latter measurement, performed with an electron impact technique, revealed M^+ 197, (M − HF) 177, and (M − CH_2F) 164.

The rate of rearrangement and hydrolysis of O^6-(2-fluoroethyl)guanosine to N^1-(2-hydroxyethyl)-guanine was then followed at pH 7.4 and 37°. As shown in Fig. 3, HPLC analysis revealed a slow conversion of the original O^6-(2-fluoroethyl)-guanosine peak to N^1-(2-hydroxyethyl)guanosine; the half-life for the reaction was approximately 18.5 hr.

With the HPLC marker, O^6-(2-fluoroethyl)-guanine, in hand, it was a relatively simple matter to show that FCNU produced this modification in DNA. DNA at a concentration of 8 mg/ml was reacted with FCNU at a final concentration of 1 mg/ml for 6 hr at 37°. The DNA was then precipitated and washed free of unreacted nitrosourea as described in Materials and Methods; its content of modified purines was measured after depurination as described above.

As shown in Fig. 4, control DNA in the left-hand panel, which had not been exposed to FCNU, contained only the normal purines, guanine and adenine. As shown in the right-hand panel of that figure, however, DNA which had been exposed to FCNU contained the new derivative, O^6-(2-fluoroethyl)guanine, as well as other derivatives identified earlier: N^1-(2-hydroxyethyl)guanine [7], N^1-(2-fluoroethyl)guanine [8], and O^6-(2-hydroxyethyl)guanine [6]. Presumably, the FCNU-treated DNA may also have contained some of the cross-linked material, dCydCH_2CH_2dGuo, but this derivative would not have been released by the depurination reaction.

DISCUSSION

It now seems probable that the nitrosoureas produce some, if not all, of their cytotoxicity by cross-linking DNA. Although difficult to prove with certainty, an intuitive feeling that the crosslink would be difficult to repair, as well as evidence that resistant cells contain fewer crosslinks than sensitive cells [9], have contributed to this conclusion.

Data showing that the nitrosoureas transfer haloethyl groups to DNA have suggested a mechanistic route to a cross-linking reaction—that haloethyl groups could react a second time with the opposite strand of DNA to tie the two strands together [10]. However, the chemical nature of the crosslink has proved elusive.

Recently, we obtained evidence for the existence of a crosslinked dinucleoside, 1-(N^3-deoxycytidyl),2-(N^1-deoxyguanosinyl)-ethane, in DNA which had been reacted with BCNU [4]. Favorable steric considerations make this a likely candidate for an interstrand crosslink.

We suggested that this crosslink may form by an initial attack of the haloethyl group on the O^6 position of guanine followed by a rearrangement reaction and subsequent crosslinking with cytosine in the opposite DNA strand.

To establish this mechanism, it was necessary to demonstrate that O^6-haloethylguanines were actually formed by the attack of a haloethyl nitrosourea on DNA. Suspecting that O^6-(2-chloroethyl)guanine might be too reactive to isolate conveniently, we chose to work with a 2-fluoroethylnitrosourea, FCNU, which would generate O^6-(2-fluoroethyl)guanine (previous studies have shown that fluoroethyl nucleosides are more stable than chloroethyl nucleosides [8]). The data presented here show conclusively that O^6-(2-fluoroethyl)-guanine was indeed formed when this nitrosourea interacted with DNA.

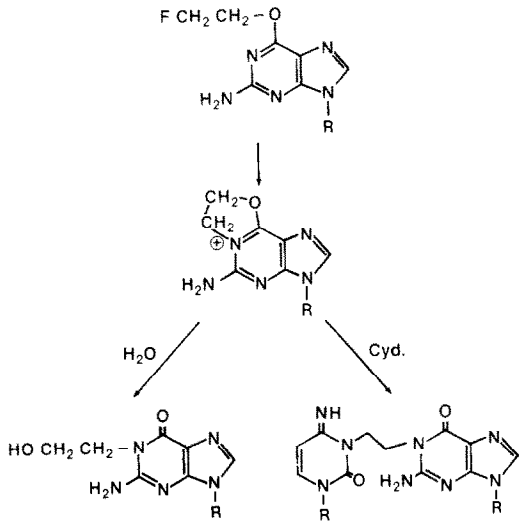

Fig. 5. Scheme for the secondary reactions of O^6-(2-fluoroethyl)guanine nucleosides. Cyclization to the intermediate, N^1,O^6-ethanoguanine nucleoside, can be followed by hydrolysis or other nucleophilic attack.

Thus, the first step in our suggested route to the crosslinked nucleoside, dCydCH_2CH_2dGuo, has been shown to occur.

In preparing the necessary HPLC marker, O^6-(2-fluoroethyl)guanine, we also obtained the data above which show that O^6-(2-fluoroethyl)guanosine rearranged and hydrolyzed to N^1-(2-hydroxyethyl)guanosine. An apparently similar rearrangement was observed by Montgomery and coworkers [11] when the treatment of O^6-(2-hydroxyethyloxy)purine with thionyl chloride in chloroform gave N^1-(2-chloroethyl)hypoxanthine.

The half-life for this rearrangement reaction was of the right order of magnitude to explain the time course of DNA crosslinking observed by Kohn [12] and by Lown *et al.* [3]. These workers have shown that DNA, incubated with nitrosoureas and then washed free of unreacted compound, continues to undergo crosslinking reactions for a matter of hours.

The key steps in the hydrolysis and crosslinking reactions are shown in Fig. 5. O^6-(2-Fluoroethyl)guanine is formed by an initial attack of a fluoroethyl carbonium ion or similar reactive species on the O^6 position of guanine. This compound then probably cyclizes to the reactive N^1,O^6-ethanoguanine nucleoside which can undergo either hydrolysis or crosslinking reactions. In DNA, crosslinking would probably be greatly favored over hydrolysis by steric considerations.

We are seeking further evidence to relate this particular crosslinking reaction to cytotoxicity, but the data presented here strongly support our proposed mechanism for the formation of the dCydCH_2CH_2dGuo crosslink.

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REFERENCES

1. D. B. Ludlum and W. P. Tong, in *Nitrosoureas in Cancer Treatment* (Eds. B. Serrou, P. S. Schein and J.-L. Imbach), p. 21. Elsevier/North-Holland, Amsterdam (1981).
2. K. W. Kohn, L. C. Erickson and G. Laurent, in *Nitrosoureas in Cancer Treatment* (Eds. B. Serrou, P. S. Schein and J.-L. Imbach), p. 33. Elsevier/North-Holland, Amsterdam (1981).
3. J. W. Lown, L. W. McLaughlin and Y-M. Chang, *Bioorg. Chem.* **7**, 97 (1978).
4. W. P. Tong, M. C. Kirk and D. B. Ludlum, *Cancer Res.* **42**, 3102 (1982).
5. L. C. Erickson, G. Laurent, N. A. Sharkey and K. W. Kohn, *Nature, Lond.* **288**, 727 (1980).
6. W. P. Tong and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **100**, 351 (1981).
7. C. T. Gombar, W. P. Tong and D. B. Ludlum, *Biochem. Pharmac.* **29**, 2639 (1980).
8. D. B. Ludlum and W. P. Tong, *Biochem. Pharmac.* **27**, 2391 (1978).
9. C. B. Thomas, R. Osieka and K. W. Kohn, *Cancer Res.* **38**, 2448 (1978).
10. B. S. Kramer, C. C. Fenselau and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **56**, 783 (1974).
11. J. R. Piper, A. G. Laseter, T. P. Johnston and J. A. Montgomery, *J. med. Chem.* **23**, 1136 (1980).
12. K. W. Kohn, *Cancer Res.* **37**, 1450 (1977).