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ANTIREVERSE TRANSCRIPTASE ACTIVITY OF GLIOTOXIN ANALOGS

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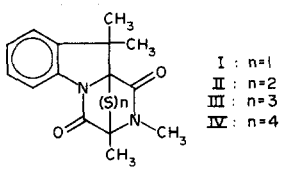
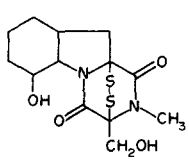
Abstract—Gliotoxin analogs, containing 1, 2, 3 or 4 sulfur atoms in their epithiodioxopiperazine moiety, were found to inhibit the RNA-directed DNA polymerase (reverse transcriptase) activity associated with RNA tumor viruses [murine (Moloney) leukemia virus] and to suppress the transformation of normal mouse (MO) cells by murine (Moloney) sarcoma virus. The antireverse transcriptase activity of the gliotoxin analogs depended on the number of sulfur atoms in the epithiodioxopiperazine ring in as far as the tetra- and trisulfides proved more inhibitory than the disulfide which, in turn, proved more inhibitory than the monosulfide. Inhibition of the reverse transcriptase reaction by the gliotoxin analogs could not be attributed to chelation of Mn^{2+} or Zn^{2+}, the necessary cofactors, since the gliotoxin analogs retained their inhibitory effects at supra-optimal Mn^{2+} and Zn^{2+} concentrations. However, the ability of the gliotoxin analogs to inhibit the reverse transcriptase reaction was abolished in the presence of a large excess of reducing agents such as dithiothreitol. It would appear, therefore, that the antireverse transcriptase activity of the gliotoxin analogs directly depends on an intact di-, tri- or tetra-sulfide bridge in the epithiodioxopiperazine ring.

Current evidence suggests that the reverse transcriptase (RNA-directed DNA polymerase) associated with RNA tumor viruses is required to initiate, but not to maintain, the transformation of the cell by the RNA tumor virus. Inhibitors of reverse transcriptase may prevent this malignant transformation and, therefore, could be useful in the prophylaxis of the onset or relapse of neoplastic diseases [1].

According to their mechanism of action, reverse transcriptase inhibitors could be divided into agents which bind directly to the enzyme (e.g. rifamycins and streptovaricins), substrate analogs (e.g. ara-CTP: 1-β-D-arabinofuranosylcytosine 5'-triphosphate), template-primer analogs (e.g. oligothymidylate derivatives, 2'-modified polyribonucleotides), agents which bind to the template (e.g. daunomycin, distamycins, ethidium bromide, tilorone and fluoranthene derivatives) and agents which sequester essential cofactors such as Zn^{2+} (e.g. *ortho*-phenanthroline) [1].

Herein we report on the antireverse transcriptase effects of several newly synthesized epipolythiodioxopiperazines (I, II, III, IV). These compounds are analogs of gliotoxin (V), a fungal product that is known to inhibit the multiplication of poliovirus and some other viruses [2–5]. The antiviral activity of gliotoxin and related products (e.g. aranotin, chetomin) appears to reside in the epithiodioxopiperazine moiety (VI) of the molecule [5–9]. Only the native oxidized form (VI) is active; the reduced form (VII) is not [7].





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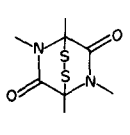

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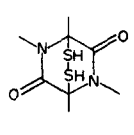


VII

Gliotoxin (V) has been found to inhibit the endogenous RNA-directed DNA polymerase activity of Rous sarcoma virus by 75 per cent at a concentration of 50 μg/ml, while DNA-directed DNA polymerase activity of the same virus was not inhibited (S. Mizutani and H. M. Temin, personal communication, 1975). Neither did gliotoxin analog II inhibit the DNA-directed RNA polymerase activity of *E. coli* [10].

MATERIALS AND METHODS

The synthesis of compounds I, II, III and IV has been described recently [10, 11]. Stock solutions of the compounds were prepared at 10 mg/ml in Me_2SO (dimethylsulfoxide). The methodology for measuring the DNA-polymerase activity of murine (Moloney) leukemia virus has been described previously [12], as has been the technique for monitoring the transformation of MO-cells by murine (Moloney) sarcoma virus [13]. In the DNA-polymerase assays, murine (Moloney) leukemia virus served as source of both the enzyme and its template.

A full description of the reagents and viruses is provided, see [13]. Two batches of ([^{3}H]methyl)dTTP were employed (specific radioactivity: 30 Ci/m-moles and 50 Ci/m-moles, respectively). Both samples were obtained from the Radiochemical Centre, Amersham, U.K., through IRE (Institut des Radio-Eléments, Fleurus, Belgium). The first batch was used in the

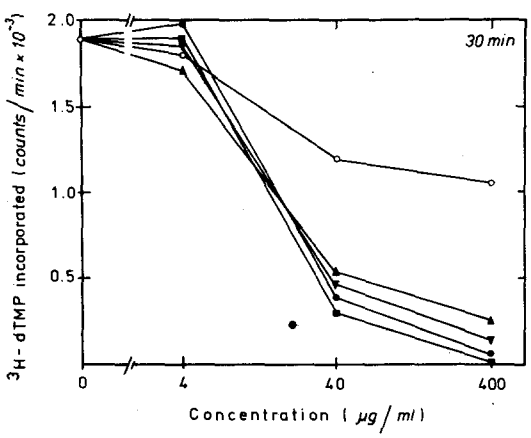
initial experiments (reports in Fig. 1), whereas the second batch was used in later experiments (shown in Figs 2–4).

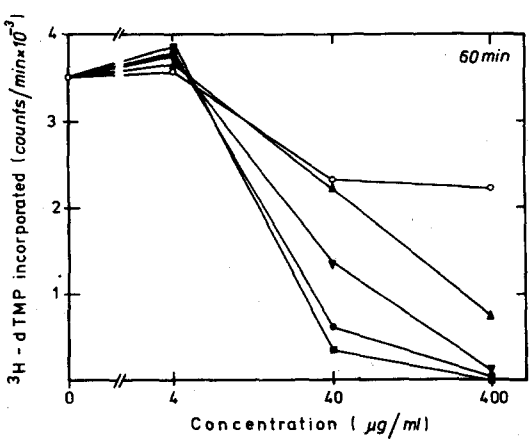
Murine (Moloney) leukemia virus was supplied by Electro-Nucleonics Laboratories (Bethesda, MA, U.S.A.). Two different virus stocks were employed. The first virus stock, which was used in our initial experiments (Fig. 1), originated from JLSV5 cells (mouse BALB/c bone marrow cells infected with Moloney murine leukemia virus; catalog no. 1021, lot no. 541–39–5A). The second virus stock, which was used in further experiments (Figs 2–4), originated from NIH-3T3 cells (NIH Swiss mouse embryo cells infected with Moloney murine leukemia virus; catalog no. 1024, lot no. 719–24–9).

The differences in origin of the murine leukemia virus stocks (as well as the differences in specific radioactivity of the ([^{3}H]methyl)dTTP batches) employed in the reverse transcriptase assays may explain why the extent of DNA synthesis (amount of [^{3}H]dTMP incorporated) was considerably lower in the initial experiments (Fig. 1) than in the later experiments (Figs 2–4).

RESULTS

As shown in Fig. 1, all four gliotoxin analogs tested inhibited the DNA polymerase activity of murine (Moloney) leukemia virus. The extent of inhibition





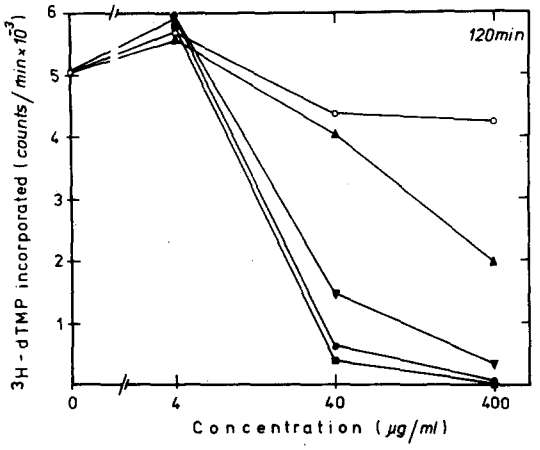


Fig. 1. Effect of compound I (▲), compound II (▼), compound III (●), compound IV (■) on DNA polymerase activity associated with murine (Moloney) leukemia virus. The standard assay mixture (250 μl) contained 40 mM Tris–HCl (pH 7.8), 50 mM NaCl, 4 mM $MnCl_2$, 1.6 mM dithiothreitol, 0.0125% (v/v) Triton X-100, 0.64 mM each of dATP, dCTP and dGTP, 0.035 mM [^{3}H]methyl)dTTP, 20 μl of virus stock suspension and varying concentrations of the gliotoxin analogs as indicated in the abscissa. Since stock solutions of the gliotoxins had been prepared in Me_2SO, the effect of Me_2SO on DNA polymerase activity was also determined (○). Final concentrations of Me_2SO in the assay mixtures were 0.2, 2 and 4%. The corresponding gliotoxin concentrations were 4, 40 and 400 μg/ml, respectively. The assay mixtures were incubated at 37°. Fifty μl aliquots were withdrawn at 30 min (first panel), 60 min (second panel) and 120 min (third panel), and tested for acid-precipitable radioactivity as described before [12]. Note that no carbopol [12] was added to the assay mixtures.

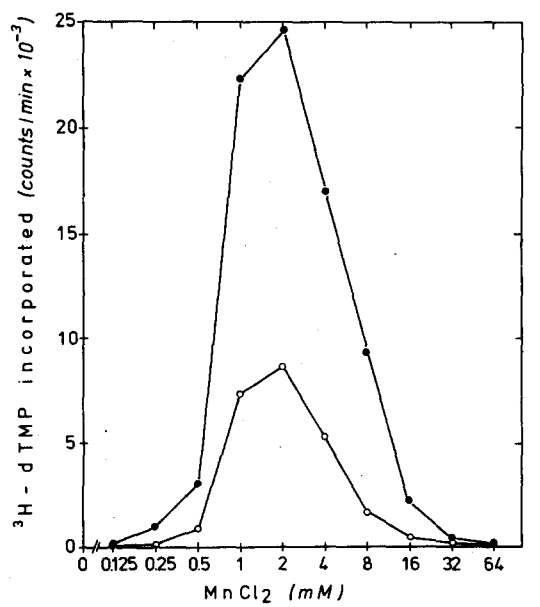


Fig. 2. Influence of various $MnCl_2$ concentrations on DNA synthesis in the presence (○) and absence (●) of compound IV (40 μg/ml). Except for $MnCl_2$, the assay mixtures were composed as described in the legend to Fig. 1. Final concentration of DMSO was 2% (v/v). The reaction mixtures were incubated at 37° for 60 min, at which time 100 μl aliquots were withdrawn and assayed for acid-precipitable radioactivity.

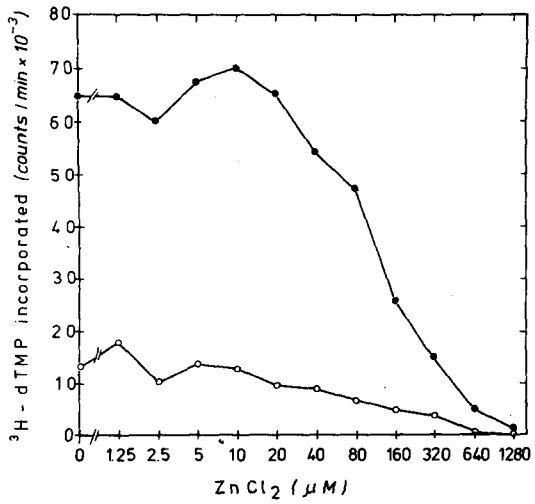


Fig. 3. Influence of various $ZnCl_2$ concentrations on DNA synthesis in the presence (○) and absence (●) of compound IV (40 μg/ml). Except for $ZnCl_2$, which was added at the concentrations indicated in the abscissa, and for dithiothreitol, which was omitted, the assay mixtures were composed as described in the legend to Fig. 1. Final concentration of DMSO was 2% (v/v). The reaction mixtures were incubated at 37° for 60 min, at which time 100 μl aliquots were withdrawn and assayed for acid-precipitable radioactivity.

depended on the number of sulfur atoms in the epithiodioxopiperazine ring. The tetra- and trisulfides proved more effective than the disulfide which, in turn, proved more effective than the monosulfide. The differences in antireverse transcriptase activity among the gliotoxin analogs could most clearly be observed at a concentration of 40 μg/ml. At 4 μg/ml, none of the compounds exerted an inhibitory effect. At 400 μg/ml, the di-, tri- and tetrasulfides nearly annihilated the DNA polymerase activity, whereas the monosulfide partially reduced the polymerase activity.

To determine whether the gliotoxin analogs, by virtue of their epithiodioxopiperazine ring, might inhibit the reverse transcriptase reaction by sequestering essential divalent cations, we examined the antireverse transcriptase activity of gliotoxin analog IV in the presence of various concentrations of Mn^{2+} and Zn^{2+}. Mn^{2+} was tested since it is the divalent cation present in the standard reaction mixture. Zn^{2+} was chosen because there is strong evidence that the RNA-directed DNA polymerase, like other DNA polymerases (e.g. *E. coli* DNA polymerase I [14]) are zinc metalloenzymes (15–19). Agents which are known to chelate zinc (e.g. *ortho*-phenanthroline) inhibit the reverse transcriptase, while nonchelating isomers (e.g. *meta*-phenanthroline) do not [17, 18]. Figure 2 shows that the Mn^{2+} concentration required for maximal DNA synthesis was not altered upon addition of gliotoxin analog IV and that gliotoxin IV inhibited DNA synthesis equally well at all Mn^{2+} concentrations tested. These findings suggest that gliotoxin IV does not act by binding Mn^{2+}. Similarly, inhibition of DNA synthesis by gliotoxin analog IV could not be overcome by adding Zn^{2+} to the reaction mixture, even if Zn^{2+} was added at concentrations which themselves inhibited the polymerase reaction (Fig. 3). Thus, gliotoxin IV apparently does not act by chelating Zn^{2+}.

Other divalent cations which were examined for a possible antagonistic or synergistic effect with gliotoxin compound IV included Cu^{2+}, Ni^{2+} and Pb^{2+}. Cu^{2+} has previously been reported to potentiate the antireverse transcriptase activity of thiosemicarbazones [20]. In accord with previous observations [20], addition of $PbCl_2$, $NiSO_4$ and especially $CuSO_4$ led to a significant inhibition of the reverse transcriptase activity. The gliotoxin analog IV retained its inhibitory effect on the DNA polymerase reaction in the presence of increasing Pb^{2+}, Ni^{2+} and Cu^{2+} concentrations; its activity was neither reduced nor potentiated by the simultaneous presence of Pb^{2+}, Ni^{2+} or Cu^{2+} (data not shown).

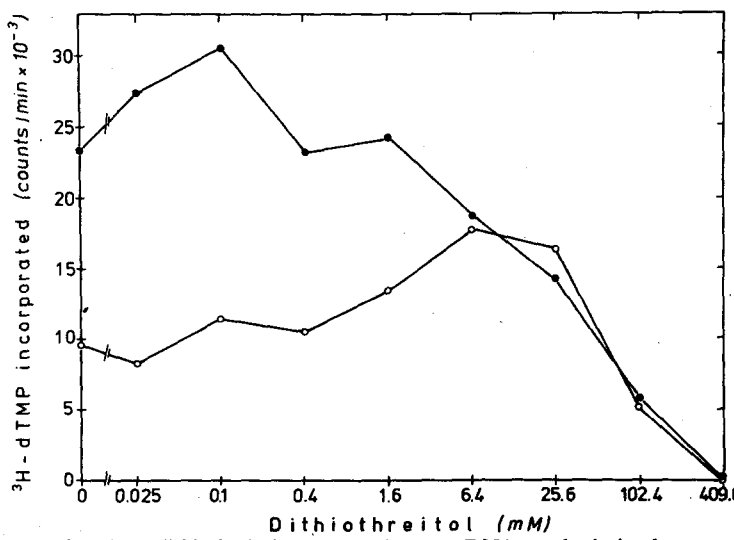


Fig. 4. Influence of various dithiothreitol concentrations on DNA synthesis in the presence (○) and absence (●) of compound IV (40 μg/ml). Except for dithiothreitol, the assay mixtures were composed as described in the legend to Fig. 1. Final concentration of DMSO was 2% (v/v). The reaction mixtures were incubated at 37° for 60 min, at which time 100 μl aliquots were withdrawn and assayed for acid-precipitable radioactivity.

Table 1. Effect of compounds I, II, III and IV on the development of murine (Moloney) sarcoma virus-induced foci in MO-cells

Compound	Number of foci Concentration of compound (μg/ml) 0	0.1	1	10	100
I	—	—	73	52	N
II	—	54	23	N	TOX
III	—	39	6	TOX	TOX
IV	—	41	11	TOX	TOX
	Concentration of Me_2SO (% v/v) 0.001	0.01	0.1		1
Me_2SO	—	NT	70	62 (TOX)	TOX
Control	71	—	—	—	—

Data represent average values for four observations. Mouse MO-cells were seeded in microtiter plates at 40,000 cells per well. After two-and-a-half to 3 hr, when the cells were firmly attached to the plastic, medium was removed and the cells were inoculated with murine (Moloney) sarcoma virus. After 1 hr adsorption at 37°, the cells were washed (three times) and exposed to varying concentrations of the compounds (in Eagle's minimal essential medium supplemented with 10% fetal bovine serum). After 24 hr incubation at 37°, the cells were washed, trypsinized and seeded on semi-confluent MO-cell monolayers (in 60 mm petri dishes). Foci of transformed cells were enumerated microscopically 7 days later. To evaluate the cytotoxic effects of the compounds, MO-cells were examined microscopically after they had been exposed for 24 hr with either of the compounds tested. Me_2SO was assayed for toxicity and inhibition of focus formation at the concentrations present in the gliotoxin samples (0.001, 0.01, 0.1 and 1% at gliotoxin concentrations of 0.1, 1, 10 and 100 μg/ml, respectively). TOX: microscopic alteration of cell morphology; N: normal cell morphology.

Since dithiothreitol is generally included in our reverse transcriptase assay mixture to optimize reaction velocity and facilitate virus disruption, one might argue that the target of inhibition by the gliotoxin analogs is the enzyme assay instead of the enzyme itself, for it has been shown that compound II is capable of oxidizing dithiothreitol by virtue of its epithiodioxopiperazine moiety [21]. That substances which catalyze an oxidation of dithiothreitol may indeed cause an inhibition of the RNA-directed DNA polymerase reaction has recently been demonstrated for 6-(*p*-hydroxyphenylazo)-uracil (HPUra) [22]. Inhibition of the DNA polymerase reaction by the gliotoxin analogs could not be attributed to an inactivation of dithiothreitol, since gliotoxin compound IV remained equally effective in inhibiting DNA synthesis when assayed in the complete absence of dithiothreitol as in the presence of dithiothreitol concentrations up to 1.6 mM (Fig. 4). However, if the concentration of dithiothreitol was increased to 6.4 or 25.6 mM, compound IV was not further capable of inhibiting the reverse transcriptase reaction (Fig. 4). These results clearly suggest that only the oxidized form of gliotoxin IV and probably all other gliotoxin analogs is active in inhibiting reverse transcriptase activity.

Support that the inhibition observed with the gliotoxin analogs in our *in vitro* reverse transcriptase assay truly reflected an inhibition of the enzyme *per se* stemmed from *in vivo* observations. As shown in Table 1, compounds II, III and IV significantly reduced the ability of murine (Moloney) sarcoma virus to induce foci in MO-cells. Although the gliotoxin analogs proved toxic for the cells at concentration ≥ 10 μg/ml, inhibition of malignant transformation was achieved at non-toxic concentrations (0.1–1 μg/ml). The order of *in vivo* activity of the gliotoxin compounds closely paralleled their relative *in vitro* activity, thus, in order of decreasing activity: tetrasulfide $\simeq$ trisulfide > disulfide > monosulfide.

DISCUSSION

The gliotoxin analogs I, II, III and IV can be considered as effective inhibitors of the *in vitro* reverse transcriptase activity of RNA tumor viruses. *In vivo*, they inhibit the transforming ability of RNA tumor viruses. Our studies reveal a remarkable structure-function relationship in the antireverse transcriptase activity of the gliotoxin analogs. The tetra- and trisulfides appeared more active than the disulfide which, in turn, proved more effective than the monosulfide.

The antiviral activity of gliotoxin (V) was first mentioned in 1964 by Rightsel *et al.* [2]. The compound was found to inhibit the cytopathic effect of poliovirus in cell culture at concentrations as low as ≤ 0.014 μg/ml. However, gliotoxin failed to protect mice against an intracerebral poliovirus challenge, when administered orally at the maximum tolerated dose (10 mg/kg/day) [2]. In later studies, the inhibitory effect of gliotoxin on poliovirus replication was attributed to a specific inhibition of viral RNA synthesis: at concentrations which did not affect cellular RNA synthesis, gliotoxin effectively and irreversibly suppressed viral RNA synthesis [4]. More recently, gliotoxin, acetylaranotin and their derivatives were found to inhibit the RNA polymerase of Coxsackie virus at concentrations which were several orders of magnitude lower than those required to inhibit the RNA polymerase of the host cell [8].

Trown and Bilello [7] postulated that gliotoxin and other epidithiopiperazinediones would inhibit the viral RNA-directed RNA polymerase via formation of a mixed disulfide with an essential sulfhydryl group of the enzyme. A similar mechanism may be invoked to explain the inhibitory effects of compounds I, II, III and IV on the RNA-directed DNA polymerase activity of RNA tumor viruses, the more that II has been shown to react with thiols [21]. The critical dependence of the antireverse transcriptase activity on the number of sulfur atoms in the epithiodioxopiperazine ring (Fig. 1) as well as the loss of inhibitory activity upon reduction of the (tetra) sulfide bridge by dithiothreitol (Fig. 4) would seem to favor the hypothesis that the epithiodioxopiperazine moiety of the gliotoxin analogs directly interacts with the RNA-directed DNA polymerase.

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REFERENCES

1. R. G. Smith and R. C. Gallo, *Life Sci.* **15**, 1711 (1974).
2. W. A. Rightsel, H. G. Schneider, B. J. Sloan, P. R. Graf, F. A. Miller, Q. R. Bartz, J. Ehrlich and G. J. Dixon, *Nature, Lond.* **204**, 1333 (1964).
3. N. M. Larin, M. P. Copping, R. H. Herbst-Laier, B. Roberts and R. B. M. Wenham, *Chemotherapia* **10**, 12 (1965).
4. P. A. Miller, K. P. Milstrey and P. W. Trown, *Science, N.Y.* **159**, 431 (1968).
5. J. K. McDougall, *Archs Ges. Virusforsch.* **27**, 255 (1969).
6. P. W. Trown, *Biochem. biophys. Res. Commun.* **33**, 402 (1968).
7. P. W. Trown and J. A. Bilello, *Antimicrob. Ag. Chemother.* **2**, 261 (1972).
8. K. C. Murdock, *J. med. Chem.* **17**, 827 (1974).
9. H. C. J. Ottenheijm, J. A. M. Hulshof and R. J. F. Nivard, *J. Org. Chem.* **40**, 2147 (1975).
10. H. C. J. Ottenheijm, J. D. M. Herscheid, G. P. C. Kerkhoff and T. F. Spande, *J. Org. Chem.* **41**, 3433 (1976).
11. H. C. J. Ottenheijm, G. P. C. Kerkhoff, J. W. H. A. Bijen and T. F. Spande, *Chem. Commun.* 768 (1975).
12. E. De Clercq and P. J. Claes, *Biochim. biophys. Acta* **331**, 328 (1973).
13. E. De Clercq, A. Billiau, J. Hobbs, P. F. Torrence and B. Witkop, *Proc. natn. Acad. Sci. U.S.A.* **72**, 284 (1975).
14. C. F. Springgate, A. S. Mildvan, R. Abramson, J. L. Engle and L. A. Loeb, *J. biol. Chem.* **248**, 5987 (1973).
15. D. S. Auld, H. Kawaguchi, D. M. Livingston and B. J. Vallee, *Proc. natn. Acad. Sci. U.S.A.* **71**, 2091 (1974).
16. B. J. Poiesz, G. Seal, and L. A. Loeb, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4892 (1974).
17. B. J. Poiesz, N. Battula and L. A. Loeb, *Biochem. biophys. Res. Commun.* **56**, 959 (1974).
18. D. S. Auld, H. Kawaguchi, D. M. Livingston and B. L. Vallee, *Biochem. biophys. Res. Commun.* **57**, 967 (1974).
19. D. S. Auld, H. Kawaguchi, D. M. Livingston and B. L. Vallee, *Biochem. biophys. Res. Commun.* **62**, 296 (1975).
20. W. Levinson, A. Faras, B. Woodson J. Jackson and J. M. Bishop, *Proc. natn. Acad. Sci. U.S.A.* **70**, 164 (1973).
21. H. C. J. Ottenheijm, J. D. M. Herscheid and R. J. F. Nivard, *J. Org. Chem.* **42**, 925 (1977).
22. M. Sclair, D. M. Byrd and W. A. Carter, *Res. Commun. Chem. Pathol. Pharmac.* **10**, 525 (1975).