EVALUATION OF THE ELECTROPHILICITY OF DNA-BINDING PYRROLO[2,1-c][1,4]BENZODIAZEPINES BY HPLC

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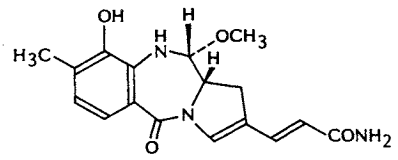
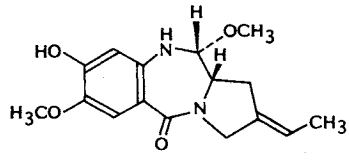
An HPLC assay is described that can be used to study the covalent bonding interaction of carbinolamine-containing pyrrolo[2,1-c][1,4]benzodiazepines with the model nucleophile thiophenol, in order to evaluate electrophilicity at the C-11-position. Preliminary experiments with anthramycin, tomaymycin and neothramycin show that their reaction with thiophenol follows second-order kinetics, but the ranking order of reactivity (neothramycin > tomaymycin > anthramycin), does not correlate with either *in vitro* cytotoxicity or *in vivo* antitumour activity. This suggests that other factors such as non-covalent DNA-interaction or drug transport play a more crucial role in biological activity than simple alkylating ability. This assay should, however, prove a useful tool in the study of structure-activity relationships for this series of compounds and provide "C-11-electrophilicity" parameters for use in Hansch analysis and related studies.

The carbinolamine-containing pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) are a family of potent DNA-binding antitumour antibiotics, best known members of which include anthramycin (**1**), tomaymycin (**2**) and the neothramycins A and B (**3**, shown as C-3-butyl ethers) (Fig. 1). These drugs form a covalent adduct (**4**) in the minor groove of DNA *via* alkylation of the exocyclic amino group of guanine[1]. Numerous studies including physical experiments, UV, fluorescence spectroscopy, NMR and, more recently, molecular modelling have established that covalent bond formation occurs at the C-11-position[2]. Furthermore, these molecules are sequence-selective, covering a region of three base pairs with a preference for purine-guanine-purine sequences[3]. The complete mechanism of covalent bond formation has not been elucidated but reaction is thought to proceed *via* either an imine species or by direct SN2 displacement of the carbinolamine hydroxyl or its methyl ether equivalent (Fig. 2)[4,5]. Recent studies in this laboratory have attempted to separate the covalent and noncovalent components of DNA interaction[6~8], with a view to using this information in the design of PBD analogues with enhanced sequence-selectivity for possible use as antitumour agents, probes of DNA structure and potential gene regulators[9]. The study reported here involves the *in vitro* reaction of PBDs with a model nucleophile, in an attempt to mimic interaction with the N-2 of guanine in the minor groove of DNA. Reaction of some PBDs with nucleophiles has been previously documented[4,10], although kinetic studies have not been previously reported. HPLC was chosen as a rapid and covenient method of monitoring the rate and extent of reaction, and ^{1}H NMR has been used to identify reaction products. Several model nucleophiles were initially investigated but only thiophenol was found to be suitable, using DMSO as a reaction solvent. The assay may be used to evaluate

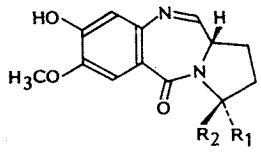
Fig. 1. Pyrrolo[2,1-c][1,4]benzodiazepines and the PBD-guanine adduct.



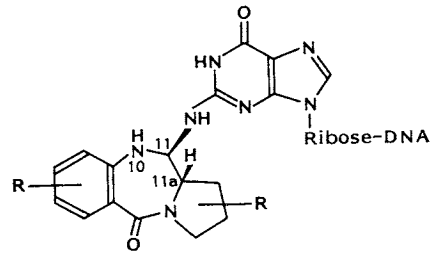
Anthramycin methyl ether (**1**)



Tomaymycin methyl ether (**2**)

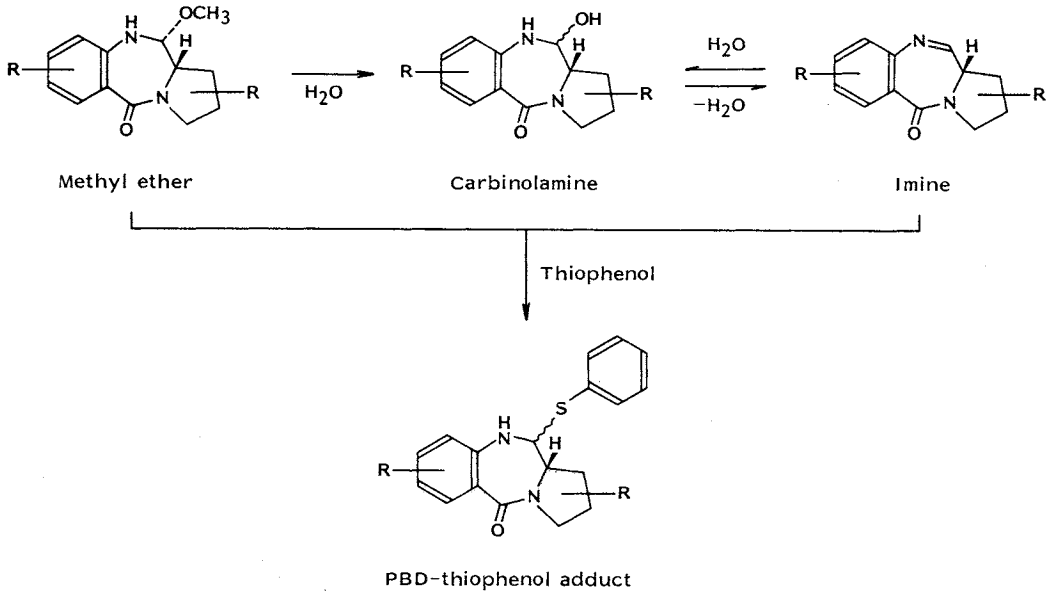


Neothramycin A butyl ether (**3**)
$R_1 = OCH_2CH_2CH_2CH_3$ $R_2 = H$
Neothramycin B butyl ether (**3**)
$R_1 = H$ $R_2 = OCH_2CH_2CH_2CH_3$



Pyrrolo[2,1-c][1,4]benzodiazepine-DNA adduct (**4**)

Fig. 2. Possible reactive species leading to the thiophenol adduct.



Methyl ether Carbinolamine Imine

Thiophenol

PBD-thiophenol adduct

and compare the C-11-electrophilicity (*e.g.* alkylating ability) of PBD analogues. An important feature is the use of a 3-cm cartridge column that allows up to three measurements per hour to be made during kinetic studies.

Materials and Methods

Apparatus

HPLC was performed with an LKB Bromma 2150 HPLC pump, a Rheodyne 7010 injector with 10 μl

loop, a Perkin Elmer "3×3" (3 cm length, 3 μm) C_{18} cartridge column, a Perkin Elmer LC90 BIO spectrophotometric UV detector and a Spectra-Physics SP4100 computing integrator.

Reagents and Materials

Anthramycin and tomaymycin methyl ethers, and neothramycins A and B (3-*O*-butyl ethers) were gifts from Hoffmann-La Roche Corporation (New Jersey, U.S.A.), Fujisawa Corporation (Osaka, Japan) and the Institute of Microbial Chemistry (Tokyo, Japan), respectively. Anthramycin and tomaymycin methyl ethers were crystalline solids shown to possess the C-11 (*R*) configuration by ^{1}H NMR. In contrast, neothramycin was shown to exist in the N-10–C-11 imine form by ^{1}H NMR. Thiophenol (analytical grade), and HPLC-grade acetonitrile, methanol and DMSO were purchased from the Aldrich Chemical Company UK. Water was purified by deionization and distillation. The HPLC mobile phase consisted of acetonitrile - water (15 : 85), de-aerated and filtered through a Nylon-66 membrane (Rainin).

HPLC Conditions

A flow rate of 1.5 ml/minute of mobile phase was used with the Perkin Elmer "3×3" cartridge column. This provided Rt's of anthramycin = 1.3 minutes; tomaymycin = 1.0 minute; neothramycin = 1.0 minute; thiophenol = 15 minutes. Anthramycin and tomaymycin were monitored at 333 nm and neothramycin at 318 nm.

Calibration

Calibration graphs (not shown) for anthramycin and tomaymycin were prepared by plotting peak areas against concentration for a number of samples in the range 0.02 to 1.2 mg/ml over which response was linear. For anthramycin: 0.7~0.18 mg/ml, CV = 6.1%; 0.12~0.02 mg/ml, CV = 28%; average CV = 21%. For tomaymycin: 1.15~0.02 mg/ml, CV = 17%.

Assay Procedure

The PBD (1.0~3.0 mg) was accurately weighed into a 10-ml glass vial followed by the addition of DMSO (2.0 ml). The vial was capped, and the PBD dissolved by immersion in an ultrasound bath (Dawe Sonicleaner) for 2 minutes. The vial was then allowed to equilibrate at 37.0°C for 5 minutes. A 50 μl sample (100 μl Hamilton syringe) was used to flush the 10 μl HPLC injection loop. A further 30 μl was then injected and the loop contents loaded onto the column. After the DMSO and PBD peaks had eluted, the integrator parameters were adjusted to record only the PBD peak area. Five or more 30 μl samples were then analyzed in succession to check reproducibility (±5%) and to determine the peak area correponding to the initial concentration of PBD. During this process of establishing baseline data, 500 μl of sample was transferred to a 1-ml Eppendorf tube which was capped. Ten ml of a solution of thiophenol of the required concentration in DMSO was freshly prepared (P1000 Gilson Pipettman, 10 ml volumetric flask). To start a kinetic run, 10 μl of this dilution was immediately pipetted (P20 Gilson Pipettman) into the 500 μl of PBD sample. The Eppendorf tube was sealed and agitated on the Rotamixer for 30 seconds. Immediately after mixing, a 30-μl sample was taken and analyzed. After the PBD and thiophenol peaks had eluted and integration had been performed, further reaction samples were chromatographed at appropriate time intervals until reaction was complete.

Results and Discussion

In the development of this assay, initial studies utilised aqueous reaction media in an attempt to represent a biological environment. However, at least two of the PBDs used in the study, anthramycin and tomaymycin, were originally in the N-10–C-11 methyl ether forms and liable to hydrolyse to carbinolamine species. Although hydrolysis appeared to be slow in H_2O - CH_3OH or H_2O - DMSO systems, addition of a trace of acid caused rapid conversion to the carbinolamine. Since the reason for developing the assay was to compare the C-11-electrophilicity of PBD analogues rather than to mimic a biological system, and also since the possibility of multiple species at the start of the reaction was unacceptable,

anhydrous DMSO was eventually chosen as the reaction solvent. This had the advantage of allowing direct comparison between the results of HPLC assays and parallel NMR investigations in DMSO-d_6. The NMR data provided confirmation that the PBDs were in one form (*e.g.* methyl ether or imine) at the beginning of the experiment.

Of several model nucleophiles tested, thiophenol was found to be the most useful, as NMR studies in DMSO-d_6 (data not shown) demonstrated that both anthramycin and tomaymycin react to form C-11-thiophenol adducts in high yield and at a rate suitable for monitoring by HPLC. No other reaction or degradation products were observed within the time scale of the experiment (24 hours). An HPLC trace for a typical kinetic experiment is illustrated in Fig. 3 which shows measurements for anthramycin before and after the addition of 4 mol equivalents of thiophenol. The shoulder of each anthramycin peak is probably due to the presence of small amounts of the C-11 (*S*) methyl ether or the equivalent C-11 (*R*) or (S) carbinolamines, even though the same sample was shown to be only one species (C-11 (*R*) methyl ether) by ^{1}H NMR. This could be due to C-11-epimerisation through interaction with the column stationary phase, or hydrolysis by the mobile phase. However, as contact time with the mobile phase is brief with a "3 × 3" cartridge column (1.0 ~ 1.5 minutes), the precision of the assay is not compromised. At least three injections were made prior to the addition of thiophenol in order to establish a baseline for integration. The peak shapes are sufficiently consistent to give reproducible integration data ($< \pm 5\%$) for consecutive injections. A calculated 4 mol equivalent of thiophenol were then added to the PBD in a small volume of DMSO. Fig. 3 shows the resulting reduction in concentration of anthramycin over approximately 6 hours. The next sample could be injected as soon as the thiophenol peak had completely eluted (20 minutes). The individual experiments involving excess thiophenol showed pseudo-first-order kinetics (Fig. 4(A)) but, by comparing experiments, the rate of reaction was found to increase proportionally with the concentration of thiophenol (Fig. 4(B)) over the range 0.02 ~ 1.5 mg/ml, indicating that the reaction was second-order overall.

Fig. 3. HPLC trace of reaction of 4 mol equiv thiophenol with anthramycin methyl ether in DMSO.

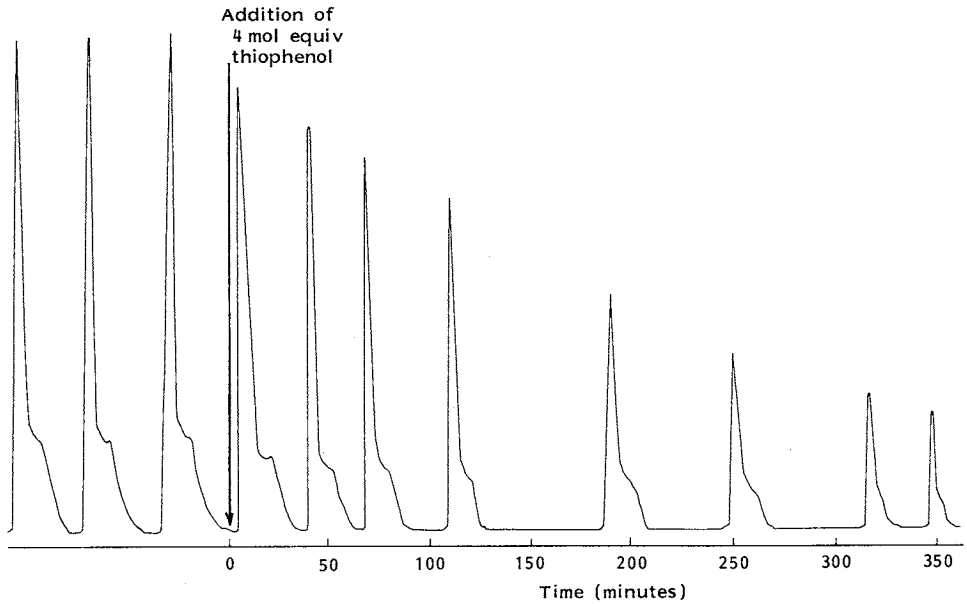


Fig. 4. The reaction of anthramycin methyl ether (AME) with thiophenol.

(A) Reaction of anthramycin methyl ether with 8, 16 and 32 mol equiv thiophenol. □ 8 mol equiv thiophenol, △ 16 mol equiv thiophenol, ○ 32 mol equiv thiophenol.

(B) Relationship between the rate of reaction of anthramycin methyl ether and thiophenol concentration.

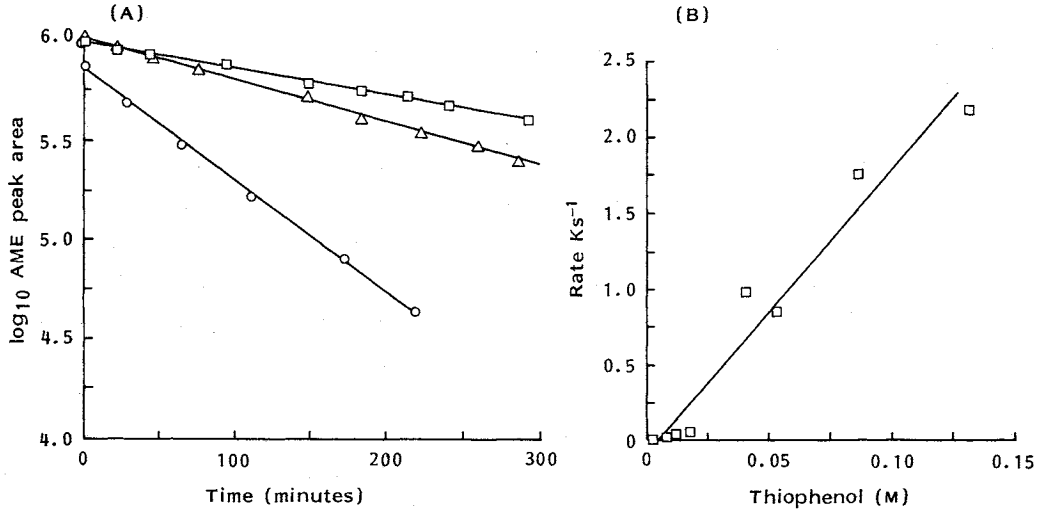


Fig. 5. Reaction of tomaymycin methyl ether (TME) with 4 and 8 mol equiv thiophenol.

△ 4 mol equiv thiophenol, □ 8 mol equiv thiophenol.

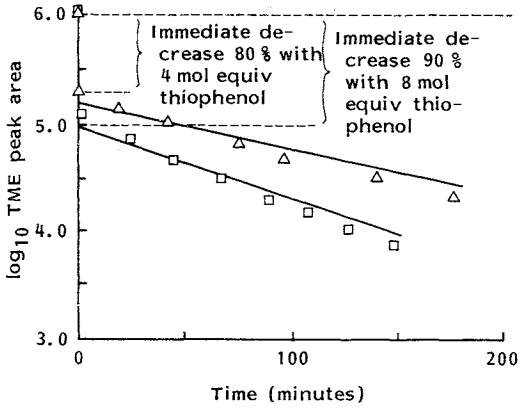
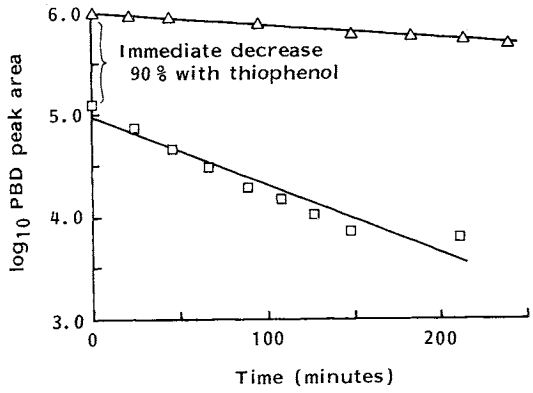


Fig. 6. Comparison of the rate of reaction of anthramycin methyl ether and tomaymycin methyl ether with 8 mol equiv thiophenol.

△ Anthramycin, □ tomaymycin.



Tomaymycin was evaluated in a similar manner but could be monitored over a short time period only, due to the speed of reaction. The rates of reaction with two different concentrations of thiophenol are shown in Fig. 5. The reaction shows an initial instantaneous reduction in concentration, followed by an exponential reduction at a faster rate than seen for anthramycin. It was found that both the initial reduction in concentration and the slope of the linear segment of the plot increase in direct proportion to thiophenol concentration. The initial reduction phase was also observed with anthramycin, but to a lesser extent and only at higher thiophenol concentrations (Fig. 4(A); 32 mol equiv). Similar HPLC studies on neothramycin showed that it was too reactive for kinetic data to be obtained. Parallel ^{1}H NMR studies in DMSO-d_6 - D_2O

showed the presence of the N-10–C-11 imine form of tomaymycin due to the elimination of methanol but this species was not observed for anthramycin.

Fig. 6 shows a direct comparison between the rates of reaction of anthramycin and tomaymycin with 8 mol equivalents of thiophenol at 37°C. The relative rates of reaction for anthramycin and tomaymycin (5.4×10^{-5}/s and 3.5×10^{-4}/s) appear to reflect their *in vitro* cytotoxicity (IC_{50} for 50% inhibition of proliferation of B16 melanoma cells on continuous exposure = 0.4 and 0.02 μM, respectively[3]. Interestingly, neothramycin is much less cytotoxic *in vitro* (IC_{50} 11.0 μM) but reaction with thiophenol is too fast to be measured in this assay. The rank order of biological potency in a number of *in vivo* screens appears to be tomaymycin > anthramycin > neothramycin[2]. The results of this investigation suggest that the PBDs are not acting as simple alkylating agents and that other factors such as non-covalent interaction with DNA and/or drug transport play a crucial role in their biological activity.

Conclusion

An HPLC assay has been developed that allows comparison of the C-11-electrophilicity of PBD analogues towards thiophenol. From the results reported here for anthramycin, tomaymycin and neothramycin, it is clear that neither *in vitro* cytotoxicity or *in vivo* biological potency correlate with C-11-electrophilicity alone, confirming that other factors such as non-covalent interactions and drug transport are essential for DNA-adduct formation and subsequent biological activity. As there is current interest in the development of compounds of this group as antitumour agents, sequence-selective DNA probes and possible gene regulators, this assay should prove useful in providing information on C-11-electrophilicity for a wide range of PBD analogues. This may lead to a more complete view of structure-activity relationships in this series. For example, it is anticipated that "C-11-electrophilicity" constants will be useful as parameters for Hansch-type analyses.

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