A quantitative assay to measure the relative DNA-binding affinity of pyrrolo[2,1-c][1,4]benzodiazepine (PBD) anti-tumour antibiotics based on the inhibition of restriction endonuclease *BamHI*

Madhu S.Puvvada, John A.Hartley[1], Terence C.Jenkins[2] and David E.Thurston*
School of Pharmacy and Biomedical Sciences, University of Portsmouth, Park Building, King Henry Ist Street, Portsmouth PO1 2DZ, [1]University College London Medical School, 91 Riding House Street, London W1P 8BT and [2]CRC Biomolecular Structure Unit, The Institute of Cancer Research, Sutton, Surrey SM2 5NG, UK

ABSTRACT

An assay has been developed (restriction endonuclease digestion assay – RED_{100}) based on inhibition of the restriction endonuclease *BamHI* that is capable of quantitative evaluation of the relative DNA-binding affinity of pyrrolo[2,1-c][1,4]benzodiazepine (PBD) antitumour antibiotics. This method provides comparable results to those obtained from thermal denaturation and ethidium bromide displacement assays but is much more sensitive, discriminating between molecules of similar structure such as DC-81, *iso*-DC-81 and neothramycin. The results reveal a trend between relative DNA-binding affinity and *in vitro* cytotoxicity for the PBDs in two tumour cell lines studied.

INTRODUCTION

The pyrrolo[2,1-c][1,4]benzodiazepines (PBDs, Figure 1) are a family of sequence-selective DNA-binding antitumour antibiotics that bind exclusively to the exocyclic N2 of guanine in the minor groove of DNA (Figure 2) (1−2). These molecules possess an (S)-configuration at the chiral C11a-position which provides them with a right-handed twist when viewed from the A-ring towards the C-ring. This feature provides the appropriate three-dimensional shape for isohelicity with the minor groove of B-form DNA, leading to a snug fit at the binding site. Well known members of the PBD family include anthramycin, tomaymycin, DC-81, neothramycin and sibiromycin. Footprinting-type studies have shown that the adducts span 3 base-pairs with a rank order of preference for 5′-Pu-G-Pu > 5′-Pu-G-Py or 5′-Py-G-Pu > 5′-Py-G-Py sequences (2−4). Recent high-field NMR and molecular modelling studies have provided detailed information about the precise three-dimensional structure of some PBD-adducts, including orientation of the molecule in the groove and

stereochemistry at the C11-position (2). So far, there is little understanding of the relationship between DNA-binding affinity, sequence-selectivity, and either *in vitro* cytotoxicity or *in vivo* antitumour activity. This is due, in part, to the lack of a sensitive and biologically relevant assay to compare the binding affinities of known PBDs. Although thermal denaturation (5) and ethidium bromide displacement (6) assays (ΔT_m and EB_{60}, respectively) have been useful in providing data, they are not sensitive enough to discriminate between weakly binding compounds of similar structure within this family.

The principle of inhibiting a restriction endonuclease by DNA-interacting ligands as a means to evaluate relative DNA-binding affinity has been investigated by a number of groups; for example cis-platin (7) and peptide nucleic acid chimeras (8) have been studied. Inhibition of restriction endonucleases by a PBD was originally established by Sumner and Bennet (9), who demonstrated significant inhibition with anthramycin. However, the relationship between the concentration of anthramycin and the degree of inhibition was not investigated, and it was not clear from the study whether the effect was unique to anthramycin or was a general effect with other members of the PBD family. In this study, several members of the PBD family of various structure have been investigated for their ability to inhibit endonuclease cleavage activity, and a concentration dependence has been established for the inhibition.

MATERIALS AND METHODS

Anthramycin and tomaymycin methyl ethers, neothramycins A and B (3-*O*-butyl ethers) and sibiromycin were gifts from Hoffmann-La Roche Corporation (New Jersey, USA), Fujisawa Corporation (Ibaraki, Japan), the Institute of Microbial Chemistry (Tokyo, Japan), and Kyowa Hakko Kogyo Co. Ltd (Tokyo, Japan), respectively. DC-81 and *iso*-DC-81 were synthesised in this laboratory (10,11).
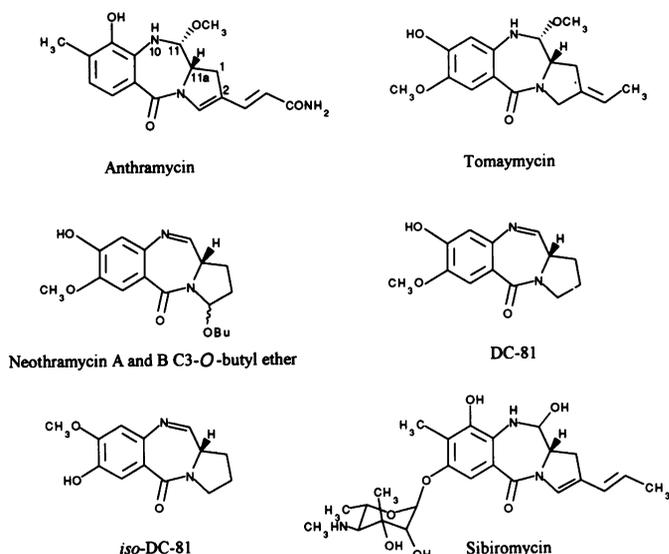

Figure 1. Structures of the pyrrolo[2,1-c][1,4]benzodiazepine (PBD) antitumour antibiotics evaluated using the RED₁₀₀ assay.
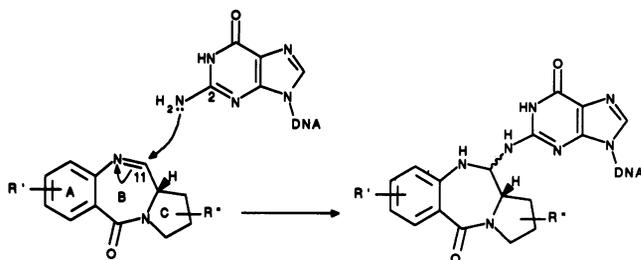

Figure 2. Mechanism of action of the PBDs: Formation of an aminal bond between the C11-position of the PBD and N2 of guanine.

Stock solutions of the PBD (1mM) were prepared by dissolving each compound in the minimum amount of methanol (Aldrich, HPLC grade) before making up to volume with sterile water. All solutions were stored at 4°C. Methanol concentrations greater than 8%^{v}/v were found to inhibit restriction endonuclease activity; therefore, this concentration of co-solvent was not exceeded in final stock solutions.

pBR322 plasmid transformed into *E.coli* was supplied by Dr K.Firman (Biophysics Department, University of Portsmouth, UK). Cells bearing the plasmid were grown, amplified with ampicillin (20μg/ml), the DNA isolated by alkaline lysis, and then purified by treatment with RNase A, phenol extraction, polyethyleneglycol (PEG) 8000 precipitation and ethanol precipitation (12). pBR322 DNA samples were stored at −20°C in Tris−HCl buffer (10mM Tris−HCl, pH7.8) containing 1mM EDTA.

Restriction endonuclease *BamH*I supplied with One-Phor-All™ (×10) restriction buffer (OPA⁺ — 100mM Tris−HCl pH 7.5, 1mM EDTA, 100mM magnesium acetate, 500mM potassium acetate) was purchased from Pharmacia. All other reagents (molecular biology grade) were purchased from Sigma.
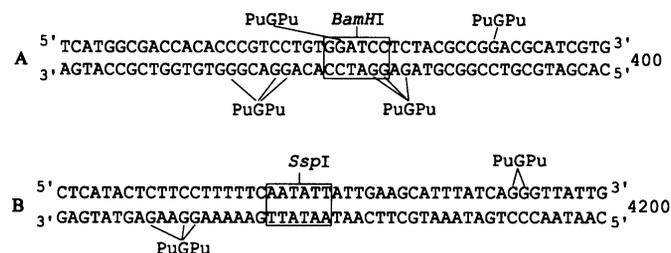

Figure 3. DNAstar sequence analysis of pBR322 (A: 350−400 bp, B: 4150−4200 bp) for *BamH*I (5'-GGATCC) and *Ssp*I (5'-AATATT) restriction sites, and PBD binding sites.

RED₁₀₀—restriction endonuclease digestion assay

PBD−DNA complexes were prepared by incubating plasmid DNA (0.5μg) with a range of concentrations of the PBD in OPA⁺ (×2) restriction buffer (pH 7.5) and bovine serum albumin (BSA) (0.1mg/ml) in a reaction volume of 49μl for 1h at 37°C. An excess of *BamH*I (20 units in 1μl) was added and the restriction digestion carried out by incubation for a further 1h at 37°C in a final volume of 50μl. *Ssp*I at concentrations equivalent to *BamH*I was also used as a control in some cases. Each digestion was stopped by incubation for 20 min at 65°C followed by 10 min at 4°C. 15μl (150ng of plasmid DNA) from each digestion was added to 10μl of loading buffer (0.25% bromophenol blue, 40% sucrose) and loaded onto a 1.0% horizontal agarose gel which was run in Tris-acetate EDTA buffer (TAE—40mM Tris base, pH 8.0, 18mM glacial acetic acid, 1mM EDTA) at 100V for 2h. The gels were stained with ethidium bromide (2μg/ml) for 30 min and then destained for 10 min in TAE buffer. The DNA bands were visualised by ultraviolet illumination and photographed (ƒ5.6/60s) using Polaroid 665 (iso 80/20°C) film. Negatives were washed with distilled water and allowed to dry prior to densitometric analysis. Each lane of a gel was scanned with a Gelscan XL densitometer and the ratios between open-circular, linear and supercoiled DNA forms were determined in order to obtain the percentage of linear DNA present. The amount of linear DNA was thus considered to be inversely related to the DNA-binding affinity of the PBD.

Thermal denaturation studies

The PBD compounds were studied in DNA thermal denaturation experiments using calf thymus (CT) DNA (type-I, highly polymerized). The CT-DNA had $A_{260}/A_{280} = 1.9$, and was satisfactorily free from protein; a molar extinction value at 260nm of $\epsilon = 6600$ M⁻¹cm⁻¹ was used. Aqueous solutions of DNA were prepared in Millipore-purified water buffered at pH 7.00±0.01 using 10mM sodium phosphate and 1mM EDTA; no added salt or support electrolyte was used. Working solutions containing 100μM of DNA alone and in the presence of μM of a PBD compound were monitored at 260nm using a Varian-Cary 219 spectrophotometer fitted with a Neslab ETP-3/RTE4 circulating water heating/cooling accessory. All PBD-DNA solutions were incubated at 37°C for 18h prior to examination. Heating was applied at 1°C min⁻¹ from 40°C until thermal denaturation of the DNA was complete, as judged from the increase in absorption. The optical absorbance versus temperature curves were sampled, normalised and analysed as previously described (5). Thermal denaturation temperatures (T_m) were

Table 1.

Compound	RED$_{100}$[a] (μM)	ΔT_m[b] (°C)	EB$_{60}$[c] (mM)	IC$_{50}$(μM)[d]	
				L1210	PC6
sibiromycin	1.9	16.3 ± 0.1	0.01	0.0029	0.000017
anthramycin	13.3	13.0 ± 0.1	0.02	0.022	0.0028
tomaymycin	182.1	2.6 ± 0.1	1.00	0.0037	0.0018
DC-81	719.6	0.7 ± 0.1	>1.00	0.38	0.33
neothramycin	1208.2	0.8 ± 0.1	>1.00	2.7	1.3
iso-DC-81	1526.1	0.6 ± 0.2	nd	2.7	2.7

[a]Concentration of PBD required to inhibit *Bam*HI cleavage by 100%. [b]Thermal denaturation studies with calf thymus DNA (ΔT_m for a 5:1 molar ratio of DNAp:PBD following incubation at 37°C for 18h). [c]Concentration of PBD required to decrease fluorescence due to ethidium bromide DNA-binding to 60% of the PBD-free control (nd = not determined). [d]Dose of PBD required to inhibit cell growth by 50% compared with PBD-free controls. Compounds were dissolved in DMSO and then diluted to provide a final concentration of 0.05 v/v% DMSO. Incubation times (37°) were: L1210, 3 days; PC6, 4 days.

determined at a relative absorbance value of 0.50 and are reported as the mean ± s.e.m. of at least four determinations. The change in T_m (ΔT_m) following interaction of CT-DNA with an added PBD was evaluated from:

$$\Delta T_m = T_{m(\text{DNA}-\text{PBD})} - T_{m(\text{DNA})}$$

and is reported (Table 1) for a fixed [DNAp]:[PBD] molar ratio of 5:1. This value was selected following trials to establish the saturation concentration of each compound, and from experience with other agents which bind via the minor groove of duplex DNA (5). The ratio selected did not lead to saturation of DNA binding.

RESULTS

The principle of the RED$_{100}$ assay is based upon the ability of a PBD to inhibit the cleavage activity of restriction endonuclease *Bam*HI. Figure 3A shows a partial sequence (350−400 bp) of pBR322 with the preferred PBD (5'-Pu-G-Pu), *Bam*HI (5'-GG-ATCC) and *Ssp*I (5'-AATATT) binding sites located by the DNAstar programme (13). The *Bam*HI cleavage sequence overlaps with several favoured PBD binding sites suggesting that ligand-binding has the potential to inhibit *Bam*HI cleavage activity. The initial objective of this study was to examine a range of [PBD]:[DNA] molar ratios for the ability to inhibit DNA linearisation by *Bam*HI. However, equivalent experiments were carried out with *Ssp*I, which cleaves at an AT-rich site that does not overlap with favourable PBD binding sites (Figure 3B). These experiments acted as a control to confirm that the measured DNA-binding affinity was associated with binding of PBDs at the restriction endonuclease cleavage site.

Figure 4 shows a typical RED$_{100}$ assay gel for anthramycin including two controls: open-circular and supercoiled DNA in lane 1, and fully linearised DNA in lane 2. As the concentration of PBD is increased (lanes 3−10), the total percentage of linear DNA produced decreases in proportion to PBD concentration. Laser densitometry was used to quantitate the amount of linear DNA formed as a percentage of the three forms of DNA. Extrapolation of a plot of percentage linear DNA formed versus PBD concentration (Figure 5) allowed the concentration required to completely inhibit enzyme-induced DNA cleavage (RED$_{100}$) to be found. One advantage of this quantitation procedure is that the amount of open-circular form of the DNA at the start of the assay is not relevant. A second advantage is that using
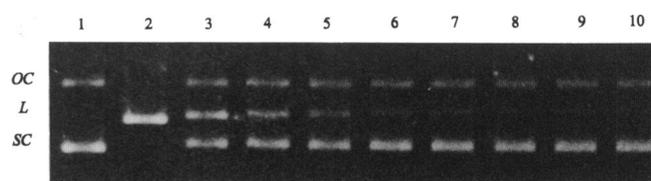

Figure 4. 1% agarose electrophoresis gel showing inhibition of *Bam*HI cleavage of pBR322 by anthramycin. Anthramycin−DNA complexes were prepared by incubating 0.5μg pBR322 with varying concentrations of anthramycin in a 49μl reaction volume containing 0.1mg/ml BSA and OPA$^+$ buffer (×2) for 1h at 37°C. Excess *Bam*HI (20 units in 1μl) was then added to give a final volume of 50μl and restriction digestion carried out by incubation for 1h at 37°C. Digestion was stopped by heating for 20 min at 65°C followed by cooling for 10 min at 4°C. DNA (150ng) from each digest was loaded onto a 1% agarose gel using bromophenol blue loading buffer and electrophoresed for 2h at 100V in TAE buffer. Lane 1: Control pBR322; lane 2: complete digest of pBR322 by *Bam*HI; lanes 3−10: anthramycin-pBR322 complexes digested by *Bam*HI with anthramycin concentrations of 1.8, 3.7, 5.5, 7.3, 9.2, 11.0, 12.8 and 14.6 μM, respectively. OC = open-circular, L = linear, and SC = supercoiled pBR322.
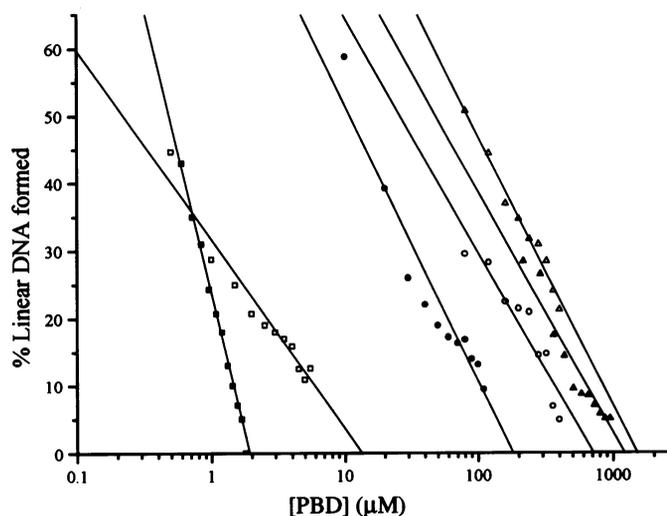

Figure 5. Graph representing the effect of PBD concentration on the inhibition of cleavage of pBR322 by *Bam*HI: sibiromycin (■), anthramycin (□), tomaymycin (●), DC-81 (○), neothramycin (▲), and *iso*-DC-81 (△). The lines represent linear regression fits to the data; slope values (and regression coefficients) are −84.2 (0.99), −28.2 (0.97), −40.9 (0.97), −34.6 (0.94), −35.9 (0.96) and −39.6 (0.99), respectively. In all cases, PBD concentrations of < 0.01μM gave no inhibition of *Bam*HI cleavage.

extrapolated values for 100% inhibition avoids any problems associated with crossed linear regression plots. The significance of the slope value for each PBD is not fully understood, although it is suggested that differences may reflect other competitive binding modes.

Inhibition experiments for the PBDs (Figure 5) show a clear rank order of inhibition of cleavage: sibiromycin > anthramycin > tomaymycin > DC-81 > neothramycin > iso-DC-81 in terms of RED_{100} value. This rank order of inhibition is identical to that observed for DNA—PBD binding as measured by ΔT_m and EB_{60} studies for sibiromycin, anthramycin and tomaymycin (Table 1). Interestingly, neither of the latter spectrophotometric methods is able to discriminate between DC-81, iso-DC-81 and neothramycin, presumably due to the similarity in the ability of these molecules to stabilize the double-stranded helix, a process that depends mainly on the strength of hydrogen bonding and van der Waals interactions between the PBD and DNA bases in the minor groove. However, DC-81 was shown to have an approximately 1.5-fold greater RED_{100} value compared to neothramycin, and an approximately two-fold difference between DC-81 and iso-DC-81 was observed (Table 1). Furthermore, the PBDs failed to inhibit SspI cleavage at concentrations equivalent to their RED_{100} concentrations for experiments with BamHI. The data in Table 1 indicate that there is a broad correlation between the RED_{100} and in vitro cytotoxicity (IC_{50}) values determined in L1210 and PC6 cell lines, with sibiromycin and iso-DC-81 being the most and least cytotoxic compounds, respectively. This trend is reflected in both the ΔT_m and EB_{60} data, although it is clear that the RED_{100} assay provides superior resolution of the DNA-modifying abilities of compounds of different structures.

DISCUSSION

The results (Table 1) show that this assay is capable of determining the rank order of DNA-binding affinity for a series of PBD molecules. Although the measurement step relates to inhibition of enzyme cleavage, the correlation with ΔT_m values, which are associated with stabilization of the DNA helix, suggest that RED_{100} values are directly related to DNA-binding affinity. There is an identical rank order of experimental RED_{100}, ΔT_m, and EB_{60} values for sibiromycin, anthramycin and tomaymycin, with sibiromycin being the most potent. Furthermore, the relative differences between tomaymycin and the two most potent compounds are reflected in all three determined parameters. For the weaker binding compounds, DC-81, neothramycin and iso-DC-81, the ΔT_m and EB_{60} values are unable to distinguish between their relative binding affinities. However, a rank order is obtained from the relative RED_{100} values, suggesting that this assay method provides greater sensitivity to differences in DNA binding. Interestingly, the rank order of PBDs based on their RED_{100} values reflects the trend of IC_{50} values across two cell lines (L1210 and PC6), except that the rank order of anthramycin and tomaymycin is reversed in each line.

The DNA-binding data provided by this assay highlight a number of features of interest concerning structure—activity relationships (SARs) for the PBDs. First, the least cytotoxic molecules, DC-81, neothramycin and iso-DC-81, with the lowest relative DNA-binding affinities, all possess imine functionalities at the N10-C11 position. Furthermore, these three compounds have saturated and relatively unsubstituted C-rings. Interestingly,

it has been previously shown that neothramycin is more electrophilic at C11 than compounds such as anthramycin or tomaymycin that contain carbinolamine methyl ether functions at N10-C11 (14).

The three most potent compounds have in common an sp^2-hybridized C2 carbon (Figure 1), although the unsaturation is endocyclic in anthramycin and sibiromycin, but exocyclic in tomaymycin. Based on the RED_{100}, ΔT_m and EB_{60} data for anthramycin and sibiromycin, endocyclic unsaturation appears to be significantly more favourable for DNA binding. This may relate to the more planar profile of molecules with C2-C3 unsaturation, allowing them to conform more easily to the shape of the minor groove (2,15). Finally, sibiromycin is the only PBD examined to possess a sugar moiety, and is the most potent of the group of compounds tested, suggesting that secondary functionalisation also plays a role in determining relative DNA-binding affinity.

The experiment with SspI was included to help develop an understanding of the mechanism of the RED_{100} assay at the molecular level. At present there is little information available regarding the mechanism of action of BamHI, although some work has been carried out on other restriction endonucleases such as EcoRV (16,17). There are two possible mechanisms to explain the inhibition of BamHI by the PBDs: (i) direct steric inhibition of enzyme recognition and/or binding at the enzyme recognition sequence due to PBD binding, and (ii) indirect interference with enzyme-mediated processes due to PBD-induced stiffening of the DNA helix (or other conformational changes) upon binding. If the latter process is operative, then binding of a PBD ligand at a location adjacent to the enzyme recognition site might be expected to inhibit DNA cleavage to some extent. The fact that SspI is not significantly inhibited by PBDs at similar concentrations to BamHI in the RED_{100} assay lends support to the first mechanism, involving direct, competitive inhibition.

In summary, the RED_{100} assay is a sensitive and apparently biologically relevant method to determine the relative DNA-binding affinities of PBDs. This assay is of potential value in future SAR studies of both naturally-occurring and synthetic compounds in this family, and should be helpful in studies of the mechanism of action of DNA-modifying PBDs at the molecular level.

ACKNOWLEDGEMENTS

REFERENCES

1. Remers, W.A. (1988) In: *The Chemistry of Antitumor Antibiotics*, Vol. 2, John Wiley and Sons, New York, pp. 28—92.
2. Thurston, D.E. (1993) In: Neidle, S. & Waring, M.J. (Eds.): *Molecular Aspects of Anticancer Drug—DNA Interactions*, Vol. 1, Macmillan Press Ltd, U.K., pp. 54—88.
3. Hurley, L.H., Reck, T., Thurston, D.E., Langley, D.R., Holden, K.G., Hertzberg, R.P., Hoover, J.R.E., Gallagher, G. Jr., Faucette, L.F., Mong, S-M., and Johnson, R.K. (1988) *Chem. Res. Toxicol.*, 1, 258—268.

4. Hertzberg, R.P., Hecht, S.M., Reynolds, V.L., Molineux, I.J., and Hurley, L.H. (1986) *Biochem.*, **25**, 1249–1258.
5. Jones, G.B., Davey, C.L., Jenkins, T.C., Kamal, A., Kneale, G.G., Neidle, S., Webster, G.D., and Thurston, D.E. (1990) *Anti-Cancer Drug Des.*, **5**, 249–264.
6. Morgan, A.R., Lee, J.S., Pulleyblank, D.F., Murray, N.L. and Evans, D.M. (1979) *Nucl. Acids Res.*, **7**, 547–569.
7. Balcarova, Z., Mrazek, J., Kleinwachter, V. and Brabec, V. (1992) *Gen. Physiol. Biophys.*, **11**, 579–588.
8. Nielson, P.E., Egholm, M., Berg, R.H. and Buchardt, O. (1993) *Nucl. Acids Res.*, **21**, 197–200.
9. Sumner, W. and Bennett, G.N. (1981) *Nucl. Acids. Res.*, **9**, 2105–2119.
10. Bose, D.S., Jones, G.B. and Thurston D.E. (1992) *Tetrahedron*, **48**, 751–758.
11. Bose, D.S., and Thurston, D.E. (1993) *Tetrahedron Letters*, **34**, 1377–1378.
12. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989) In: *Molecular Cloning*: A Laboratory Manual, Second Ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.
13. DNAstar Inc., 1228 South Park Street, Madison, WI 53715.
14. Morris, S.J., Thurston, D.E., and Nevell, T.G. (1990) *J. Antibiotics*, **43**, 1286–1292.
15. Thompson, A.S. (1992) PhD Thesis, University of Portsmouth.
16. Halford, S.E., Taylor, J.D., Badcoe, I.G., and Clarke, A.R. (1991) *Biochem.*, **30**, 8743–8753.
17. Halford, S.E., Vermote, C.L.M. (1992) *Biochem.*, **31**, 6082–6089.