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3-Nitropyrrole and 5-nitroindole as universal bases in primers for DNA sequencing and PCR

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ABSTRACT

3-Nitropyrrole and 5-nitroindole have been assessed as universal bases in primers for dideoxy DNA sequencing and in the polymerase chain reaction (PCR). In contrast to a previous report, we have found that the introduction of more than one 3-nitropyrrole residue at dispersed positions into primers significantly reduced their efficiency in PCR and sequencing reactions. Primers containing 5-nitroindole at multiple dispersed positions were similarly affected; for both bases only a small number of substitutions were tolerated. In PCR experiments neither base, when incorporated into primers in codon third positions, was as effective as hypoxanthine, which was incorporated in six codon third positions in a 20mer oligomer. However, primers containing up to four consecutive 5-nitroindole substitutions performed well in both PCR and sequencing reactions. Consecutive 3-nitropyrrole substitutions were tolerated, but less well in comparable reactions.

INTRODUCTION

A universal base, which could substitute for any of the four natural bases, would be of great utility for manipulating DNA *in vitro*. In previous work thermal stability studies have been made of oligomers containing a variety of potentially suitable analogues (1–3). However, apart from hypoxanthine (4), only 3-nitropyrrole, **1**, has been investigated in respect of enzymatic reactions with primers containing this base (5,6). We compared 3-nitropyrrole, **1**, and 5-nitroindole, **2**, as well as the 4- and 6-isomers, as universal bases in thermal stability studies of oligonucleotides containing them (7). All four of these compounds are presumed to stabilize DNA duplexes by stacking interactions, rather than by hydrogen bonding. In DNA thermal dissociation (T_m) studies of heptadecamer duplexes these compounds met the main requirement for a universal base by exhibiting little variation when set opposite the four natural bases, with a T_m range of 3°C for each. **1** was found to be more destabilizing than **2**, particularly with multiple substitutions, and **2** was likewise less destabilizing than the 4- or 6-isomers of nitroindole (7). Therefore, we chose **1** and **2** and, for comparison, hypoxanthine (Fig. 1) for further examination.

To be of routine use in recombinant DNA experiments the modified oligonucleotides must be able to prime DNA polymerases. In addition, when used in the polymerase chain reaction the modified base(s) must be able to act as a template for the incorporation of a base for the amplification to proceed. Here we examine the ability of duplexes containing various arrangements of substitutions by **1** or **2** to prime DNA synthesis by *Thermus aquaticus* (*Taq*) DNA polymerase and a modified T7 DNA polymerase (Sequenase™ Version 2.0). This ability is required if oligonucleotides containing such abnormal bases are to be applied to reduction of primer multiplicity, nucleotide sequencing, polymerase chain reaction (PCR) and site-directed mutagenesis.

MATERIALS AND METHODS

Preparation of oligonucleotides

The phosphoramidite monomers of **1** and **2** were synthesized as previously described (7) or purchased from Glen Research and 2′-deoxyinosine monomer was supplied by Cruachem. The 3-nitropyrrole-controlled pore glass support was prepared according to the method of Pon (8), giving material with a loading of 12 μmol/g. Oligonucleotides were synthesized on an Applied Biosystem 380B synthesizer with the normal synthesis cycle. Purification was carried out by HPLC on a Waters system using a Hichrom partisil 10 SAX column and a potassium phosphate (pH 6.3) gradient in aqueous 60% formamide. The required fractions were pooled, dialysed and concentrated. Alternatively, oligomers were purified by electrophoresis using 20% polyacrylamide gels and extracted (0.5 M ammonium acetate, 1 mM EDTA buffer), followed by concentration and desalting using a Sephadex G-25 column (NAP-10; Pharmacia).

Thermal dissociation (T_m) measurements

Ultraviolet spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer fitted with a Peltier cell. Melting transitions were measured at 260 nm in PCR buffer (10 mM Tris, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.001% w/v gelatin, pH 8.3 at 25°C), at an oligomer strand concentration of ~3 μM. Absorbance versus temperature for each duplex was

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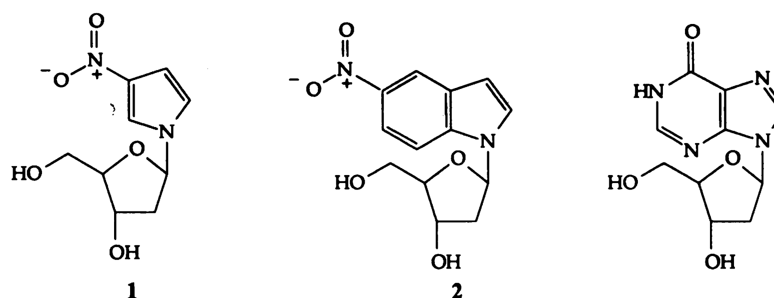

Figure 1. Structure of the deoxynucleosides derived from 3-nitropyrrole, 1, 5-nitroindole, 2 and hypoxanthine.

obtained at a heating and cooling rate of 0.5°C/min and melting temperatures (T_m) were determined as the maxima of the differential curves, with an error of ±1°C.

Template preparation

A plasmid was constructed for use as a template by subcloning a 1288 bp *Eco*47III fragment (blunted with Klenow and dNTPs) to a *Hin*dIII fragment of genomic DNA from the nematode worm *Caenorhabditis elegans* between the *Xho*I (blunted) and *Hin*dIII sites in the polylinker of the plasmid pBluescript II (Stratagene). The nucleotide sequence of the fragment is known (F.Hill and S.Linde, unpublished results). All primers were designed to be complementary to the sequence 5′-GGAGGATTTGGATC-CACGGG (see Table 1 for primer sequences). For sequencing reactions, single-stranded DNA was prepared using the helper phage M13K07. For PCR reactions, double-stranded plasmid DNA was linearized at the unique *Sac*I site in the polylinker.

Sequencing reactions

Sanger dideoxy sequencing reactions were carried out according to the manufacturer's recommendations using a commercially available Sequenase™ Version 2.0 DNA sequencing kit (USB). One picomole of the primer being tested was used to prime synthesis on single-stranded DNA. Reaction products were separated by electrophoresis on a 6% buffer gradient polyacrylamide gel.

Polymerase chain reactions

Each 50 µl reaction contained 100 ng linearized plasmid as template DNA, 200 µM each dNTP, 2.5 U *Taq* polymerase (AmpliTaq), 5 µl 10× buffer (100 mM Tris, 500 mM potassium chloride, 15 mM magnesium chloride, 0.01% w/v gelatin, pH 8.3 at 25°C), 50 pmol T7 primer (5′-GTAATACGACTCACTA-TAGGGC) and 50 pmol primer under test. The T7 primer sequence flanks the polylinker in pBluescript II. The reactions were overlaid with 35 µl paraffin oil. PCR reactions were carried out on a Techne PHC-3 apparatus and thermal cycling conditions were denaturation at 96°C for 5 min (during which *Taq* polymerase was added), followed by 30 cycles of denaturation at 96°C for 5 s, annealing at 50°C for 5 s, extension at 72°C for 30 s. A final extension was performed at 72°C for 10 min. After cooling to room temperature, the reaction products were analysed by electrophoresis on standard 2% agarose gels. The expected size of the correct PCR product was 430 bp.

RESULTS

In order to compare the effect of substituting normal DNA bases with **1** or **2**, a series of oligonucleotide primers were prepared; these were designed to be complementary to the sequence 5′-GGAGGATTTGGATCCACGGG present in the plasmid used as template as shown in Figure 2. The sequences of the primers are shown in Table 1. Thermal dissociation (T_m) studies were performed on duplexes composed of the test oligonucleotide and an oligonucleotide corresponding to the complementary sequence with normal DNA bases at salt concentrations corresponding to those present in the PCR reactions. Consistent with previous results (7), duplexes containing **2** substitutions were more stable than those in which the same residues were replaced by **1** (compare, for example, primer 4 with primer 8).

Table 1. Primers for PCR and sequencing and their melting temperatures in PCR buffer

Primers		T_m in PCR buffer (°C)
1	5′-CCC GTG GAT CCA AAT CCT CC	68[a]
2	--I --I --I --I --I --I --	50
3	--- --5 --- --- --- --- --	65
4	--- --5 --- --5 --- --- --	61
5	--5 --5 --- --5 --- --5 --	56
6	--5 --5 --5 --5 --5 --5 --	32
7	--- --3 --- --- --- --- --	60
8	--- --3 --- --3 --- --- --	51
9	--3 --3 --3 --3 --3 --3 --	not detected
10	--- --- --- --- --- --- -5	56
11	--- --- --- -55 --- --- --	61
12	--- --- --- 555 --- --- --	56
13	--- --- --5 555 --- --- --	54
14	--- --- --- -33 --- --- --	57
15	--- --- --- 333 --- --- --	51
16	--- --- --3 333 --- --- --	48
17	--- --- --- --- --- 555 --	61
18	--- --- --- --- --- 333 --	58

[a]T_m measured against an oligomer complementary to a site on the template (5′-GGA GGA TTT GGA TCC ACG GG). The primer was modified by substituting 5-nitroindole-2′-deoxyriboside (**5**), 3-nitropyrrole-2′-deoxyriboside (**3**) or hypoxanthine (I) residues at various positions as listed.

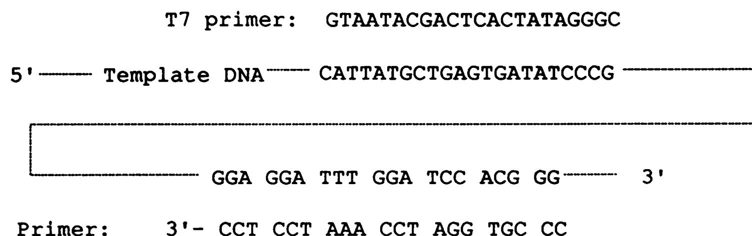

Figure 2. The reverse primers, modified by substituting 3-nitropyrrole, 5-nitroindole or hypoxanthine residues at various positions are shown in Table 1.

Each modified oligonucleotide was tested for its ability to prime DNA synthesis by a modified T7 DNA polymerase (Sequenase™ Version 2.0) in a Sanger sequencing reaction. Single-stranded DNA was prepared from the plasmid template using the helper phage M13K07. The sequencing reaction imposes two important requirements on the modified oligonucleotides; first, a significant proportion of the oligonucleotide must prime or else a weak or no sequence ladder will be produced; second, the oligonucleotide must prime in only one place in order to obtain a unique sequence.

Primers containing six **1** residues (primer 9) and four or six **2** residues (primers 5 and 6) failed to give a sequencing ladder on this template and on two similar, related templates (data not shown). Primers with one or two **1** (primers 7 and 8) or **2** (primers 3 and 4) substitutions produced the correct sequence (Fig. 3), with little difference between them and the unmodified primer (primer 1), demonstrating that they anneal uniquely and prime efficiently (Fig. 3). The primer containing six inosine substitutions (primer 2) also failed to prime on this template (Fig. 3), but gave good sequencing results on two related templates differing only in the bases pairing with inosine (results not shown).

Oligomers containing consecutive **2** substitutions proved to be more effective in priming sequencing reactions than those containing the same number of substitutions distributed at dispersed positions. Primers containing two, three and four (primers 11–13) consecutive **2** substitutions (further contiguous substitutions were not made) each gave the correct sequence (Fig. 4). On the template we have used, the corresponding oligonucleotides containing two to four consecutive **1** substitutions (primers 14–16) worked markedly less well as sequencing primers as the number of substitutions was increased. This difference in performance cannot be explained by the lower T_m values for duplexes containing **1** substitutions rather than equivalent **2** substitutions, as the annealing reactions were allowed to cool to room temperature before the polymerase was added.

Each primer was then tested in the polymerase chain reaction; a primer corresponding to the T7 promoter in the plasmid was used to prime in one direction and the oligonucleotide being assessed was required to prime in the opposite orientation. The size of the expected product was 430 bp. In this situation quantitative priming is less important than in the sequencing reaction, but the modified residues must be recognized by the polymerase to allow amplification to occur. Only primers with one (primer 7) or two (primer 8) dispersed **1** substitutions amplified a product from the template (Fig. 5). With **2** the corresponding primers incorporating one or two substitutions (primers 3 and 4) likewise produced PCR products of the correct length. A primer incorporating **2** at the 3′-terminus (primer 10)

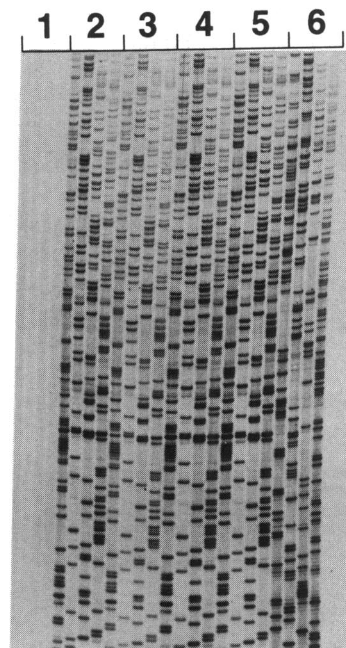

Figure 3. Dideoxy sequencing using primers:

```
1. d(CCI GTI GAI CCI AAI CCI CC)
2. d(--- --5 --- --- --- --- --)
3. d(--- --5 --- --5 --- --- --)
4. d(--- --3 --- --- --- --- --)
5. d(--- --3 --- --3 --- --- --)
6. Reverse primer
```

produced a very small amount of product when used in PCR; the product formed was ~1400 bp long and is therefore clearly the result of incorrect priming. In addition, this primer failed to give a ladder in a sequencing experiment. These findings are in contrast to the earlier results reported for **1**, where it was shown that a primer with nitropyrrole at the 3′-end produced the correct PCR product; this might be explained by the use of both a different template and a different primer (5,6).

With the oligomers containing consecutive substitutions (primers 11–13 for **2** and 14–16 for **1**), the **2** primers each gave the correct PCR product (Fig. 6), though the product using primer 13, with four consecutive **2** residues, was less abundant than that produced with two or three consecutive substitutions. The **1** oligomers also gave the correct product, though the yield was considerably reduced with even three substitutions. With four contiguous substitutions **1** was essentially ineffective, unlike the

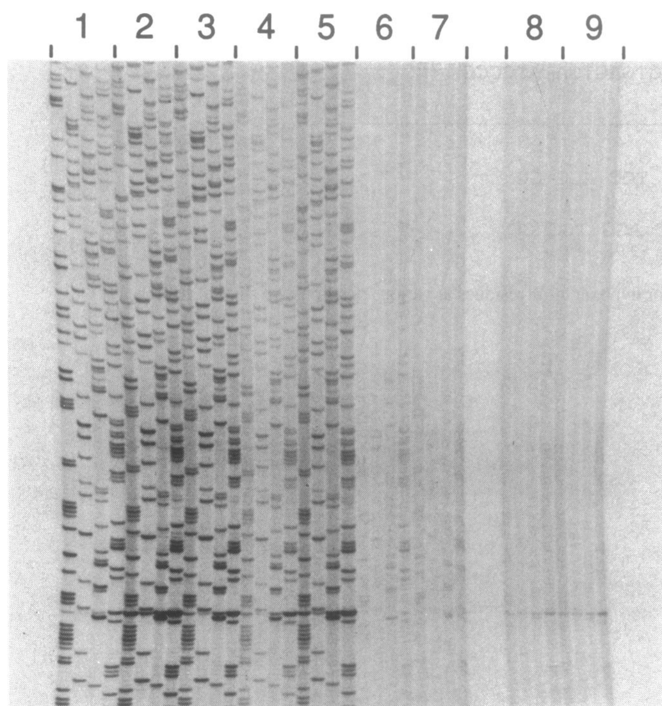

Figure 4. Dideoxy sequencing using primers:

```
1. d(CCC GTG GAT CCA AAT CCT CC)
2. d(--- --- --- -55 --- --- --)
3. d(--- --- --- 555 --- --- --)
4. d(--- --- --5 555 --- --- --)
5. d(--- --- --- -33 --- --- --)
6. d(--- --- --- 333 --- --- --)
7. d(--- --- --3 333 --- --- --)
8. d(--- --- --- --- --- 555 --)
9. d(--- --- --- --- --- 333 --)
```

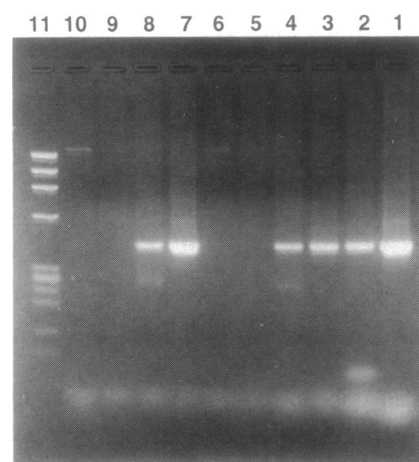

Figure 5. PCR products using primers containing universal bases at codon third positions

```
1.   5' CCC GTG GAT CCA AAT CCT CC
2.      --I --I --I --I --I --I --
3.      --- --5 --- --- --- --- --
4.      --- --5 --- --5 --- --- --
5.      --5 --5 --- --5 --- --5 --
6.      --5 --5 --5 --5 --5 --5 --
7.      --- --3 --- --- --- --- --
8.      --- --3 --- --3 --- --- --
9.      --3 --3 --3 --3 --3 --3 --
10.     --- --- --- --- --- --- -5
11.       ΦX HaeIII
```

corresponding **2** primer. Here the T_m measurements must be considered; the T_m values for the oligonucleotides containing three or four consecutive substitutions with **1** were lower than when an equivalent number of **2** substitutions were used (see Table 1) and were, in fact, close to the temperature used for annealing in the PCR reactions (50°C). Lowering the annealing temperature to 45°C was sufficient to allow the **1**-containing oligonucleotides to produce the correct PCR product in a reasonable yield (data not shown), though the yield was still reduced and some mispriming occurred.

A major advantage proposed for using **1** was that oligomers with up to nine consecutive substitutions near the 3′-end were able to function as primers for sequencing and in PCR in at least one template/primer system (5,6). In our system we found that incorporation of three **2** or three **1** substitutions near to the 3′-end (primers 17 and 18) both failed to prime synthesis by PCR (Fig. 6) and sequencing (Fig. 4). When six codon third positions were modified by **1** (primer 9), no product was observed. The thermal melting curve for this primer, measured in the PCR buffer, against an oligomer corresponding to the template sequence showed no melting behaviour. Even when the annealing temperature in the PCR experiment was reduced to 30°C, no products were observed. When four or six substitutions with **2** were made (primers 5 and 6), a PCR product could be observed, but the amount amplified was very low. A primer incorporating six inosine substitutions, however, was as effective as the unmodified primer (primer 1).

DISCUSSION

We have compared 3-nitropyrrole and 5-nitroindole as universal bases in oligodeoxynucleotide primers used both in sequencing reactions and in PCRs. Nichols *et al.* (5) reported that **1** could be used as a universal base in DNA primers at sites corresponding to the degenerate third positions of codons. In particular, they showed that 17mer primers incorporating four **1** residues or an oligonucleotide with a **1** substitution at the 3′-terminal position were able to prime DNA synthesis in PCR. These were interesting results, as it has been suggested that the three terminal bases at the 3′-end must be perfectly matched with the template for PCR to be successful (9) and that even inosine should not be incorporated into the 3′-end (10). Additionally, they reported that primers incorporating up to nine consecutive **1** residues were able to give ladders in Sanger dideoxy sequencing experiments.

It is clear from our results that **1** is not always effective when used under such demanding conditions, but it can be used opposite each of the natural bases in primers either in PCR or in sequencing reactions. In thermal dissociation studies, however, it consistently gives lower T_m values than when **2** was used in equivalent positions (Table 1) (7). Furthermore, **2** is also effective as a universal base when incorporated into primers for DNA synthesis used for sequencing and PCR; in PCR, substitutions with this base allow higher annealing temperatures to be used, consistent with the higher duplex T_m values. This could be critical

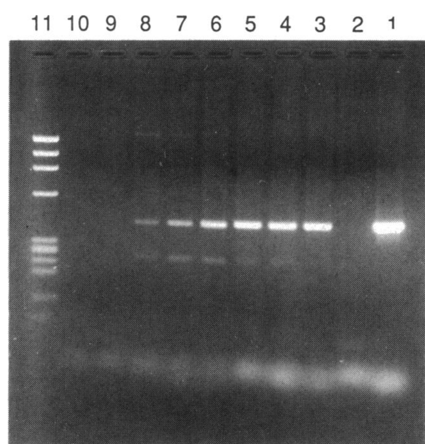

Figure 6. PCR products using primers containing consecutive 5-nitroindole or 3-nitropyrrole residues. Note that no template was added to the reaction for lane 2, thus serving as a negative control.

```
1.   5' CCC GTG GAT CCA AAT CCT CC
2.      --I --I --I --I --I --I --    (no template)
3.      --- --- --- -55 --- --- --
4.      --- --- --- 555 --- --- --
5.      --- --- --5 555 --- --- --
6.      --- --- --- -33 --- --- --
7.      --- --- --- 333 --- --- --
8.      --- --- --3 333 --- --- --
9.      --- --- --- --- --- 555 --
10.     --- --- --- --- --- 333 --
11.        ΦX HaeIII
```

when performing PCR on templates as complex as genomic DNA samples. Perhaps the most interesting feature of this base is that it is particularly effective when consecutive substitutions are made. Further work is being carried out to try to determine the nature of the interaction between **2** and natural bases and also with itself and to determine why **2** is more effective when contiguous substitutions are made. One possible explanation may lie in the fact that both **1** and **2** are hydrophobic and they therefore have a preference to interact with other hydrophobic bases, i.e. with themselves.

There has been a small number of reports recently on hydrophobic base analogue interactions in duplexes, though the authors have given no indication regarding PCR or sequencing reactions using these novel bases. Bischofberger and Matteucci (11) described some polycyclic derivatives of thymidine, e.g. **3** (Fig. 7), in which the bases were shown to stabilize duplexes when incorporated towards the (5′- or 3′-)ends of the oligomers. In these cases the analogues are presumably interacting with the opposite strand by intercalation. In the case of **2** a similar situation could occur, as the presence of the nitro group effectively increases the size of the base by the equivalent of an extra ring; indeed, **1** in this sense can be considered a purine analogue (see Fig. 1).

Schweitzer and Kool (12,13) have studied some hydrophobic isosteres of purines (**4**) and pyrimidines (**5**) when paired with the natural DNA bases or with themselves. These analogues were found to be non-discriminatory towards the natural bases, but higher duplex stability was observed when they were paired with themselves (**4** + **4**) or each other (**4** + **5**). Greater duplex stability was also observed when these analogues were incorporated towards the ends of the oligomers, rather than in the middle. The preference for self-pairing occurs due, presumably, to the hydrophobic nature of the analogues, with greater stability occurring when hydrophobic bases were allowed to interact with each other in preference to the natural bases. Similar results in duplex stability have been observed when the base is replaced by an aliphatic chain (14) and this was also attributed to hydrophobic interactions. However, no enzymology with oligomers containing these residues has been described at present.

For **2** it is possible that a similar situation is occurring, in that each individual residue is able to help stabilize the duplex by bridging the hydrogen bond gap into the opposite strand and, by making consecutive substitutions, the hydrophobic bases are stabilized by stacking together. It can be seen from Table 1 that the primers with consecutive substitutions at the 3′-end (primers 17 and 18) have higher T_m values than for similar substitutions in the centre (primers 12 and 15). However, introducing substitutions near to the 3′-end, we have shown, can result in poor primers of DNA synthesis; this may be due to inefficient recognition of the resulting duplex by the polymerase, as the T_m values of these primers are well within the range of the PCR annealing conditions.

CONCLUSION

We have shown that the performance of **1** when incorporated into oligomers used as DNA primers was disappointing when compared with the template/primer system used by Nichols *et al.* (5), suggesting that their results are not of general validity; their results might be explained by a marked template/primer dependence for the use of this universal base. We have not attempted an experiment as complex as amplifying a specific cDNA sequence

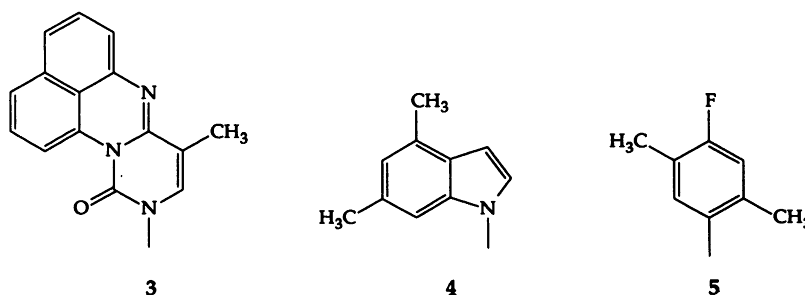

Figure 7.

from reverse transcribed total RNA of *Drosophila melanogaster* heads, as Nichols *et al.* did, using a primer containing **1** residues.

2 consistently gives higher T_m values than **1** and this would account for its effectiveness at higher temperatures in PCR. For primers requiring dispersed residues to be replaced, inosine, of this limited set, is the best choice. However, **2** may be incorporated in a number of consecutive positions and give oligomers which are effective as primers for PCR and sequencing. One possible advantage of this is that an entire codon may be replaced by **2**, as can also be done with inosine (15). It is of considerable interest to know which base or bases are incorporated opposite these universal bases: we have begun experiments in order to elucidate this.

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