

New photonucleases based on tetrapyrrole, polypyridine and diazo-arene chromophores

Bhaskar G. Maiya

School of Chemistry, University of Hyderabad, Hyderabad 500 046, India

Binding and cleavage of DNA by new, rationally-designed photonucleases based on porphyrin, metallo-polypyridyl and diazo-arene chromophores are described in light of their application as photosensitizers in the photodynamic therapy of tumours and as structural/spectroscopic probes for DNA.

CURRENTLY considerable attention is being focused on the design of small molecules that can bind and cleave DNA. These molecules, commonly referred to as artificial nucleases, have numerous biochemical and biomedical applications. While most of the hitherto reported DNA-cleaving agents have been activated thermally, in recent years, there is an increasing emphasis on photoactivated cleavage agents, because this methodology possesses significant practical advantages. In particular, photonucleases can be triggered by exposure to light; light is an attractive 'co-factor' since it is easy to manipulate. This article summarizes some of our recent results on the design, DNA-binding and DNA-cleavage of new, rationally-designed photonucleases based on porphyrin, metallo-polypyridyl and diazo-arene chromophores. Our interest in the DNA-binding and DNA-photocleavage chemistry of these chromophores is mainly related to the use of porphyrins as photosensitizers in the photodynamic therapy of malignant tumours and metallo-intercalators as structural/spectroscopic probes and site-specific cleavage agents for DNA.

DNA photocleavage by 'porphyrin-intercalator' and 'porphyrin-chemotherapeutic drug' conjugates – relevance to PDT

Photodynamic therapy (PDT), involving the action of light on a photosensitizer to generate singlet oxygen ($^1\text{O}_2$, or other reactive oxygen species) that ultimately leads to tumour necrosis, is currently being explored as an alternative modality to radio- and chemotherapy for the treatment of cancer (Figure 1)¹⁻³. DNA is considered to be one among the more susceptible intracellular sites of photodamage during such a photodynamic action.

Ideally, the treatment of tumours by PDT requires a photosensitizer which is a single, nontoxic, stable compound of known chemical structure that can be retained with a high degree of selectivity in malignant tumours.

In addition, from the photochemical point of view, an ideal photosensitizer should be able to absorb light in the tissue-transparent region (ca. 600 to 800 nm) and photo-generate high yields of $^1\text{O}_2$. Porphyrins, by virtue of their robust, 18π -electron system and their rich redox and photophysical activity as well as biocompatibility, fulfill most of these criteria. Indeed, haematoporphyrin derivative (HpD or Photofrin II®) is currently being marketed as an anti-cancer drug⁴. However, the uncertainty about the chemical structure and mechanism of action of HpD and, moreover, its ability to absorb only weakly in the red region of the visible spectrum necessitated the development of other photosensitizers for use in PDT. A plethora of new potential PDT agents based on porphyrin and related macrocycles has been reported recently^{5,6}. Many of these second generation photosensitizers, although absorb strongly in the 600–800 nm region and generate $^1\text{O}_2$ in good-to-moderate yields, suffer severely from their inability to accumulate in tumours.

One possible way to circumvent the problem of low accumulation of a given porphyrin-based drug in the cells is to equip the drug with an intracellular recognition element, e.g. a DNA/membrane-binding agent. This

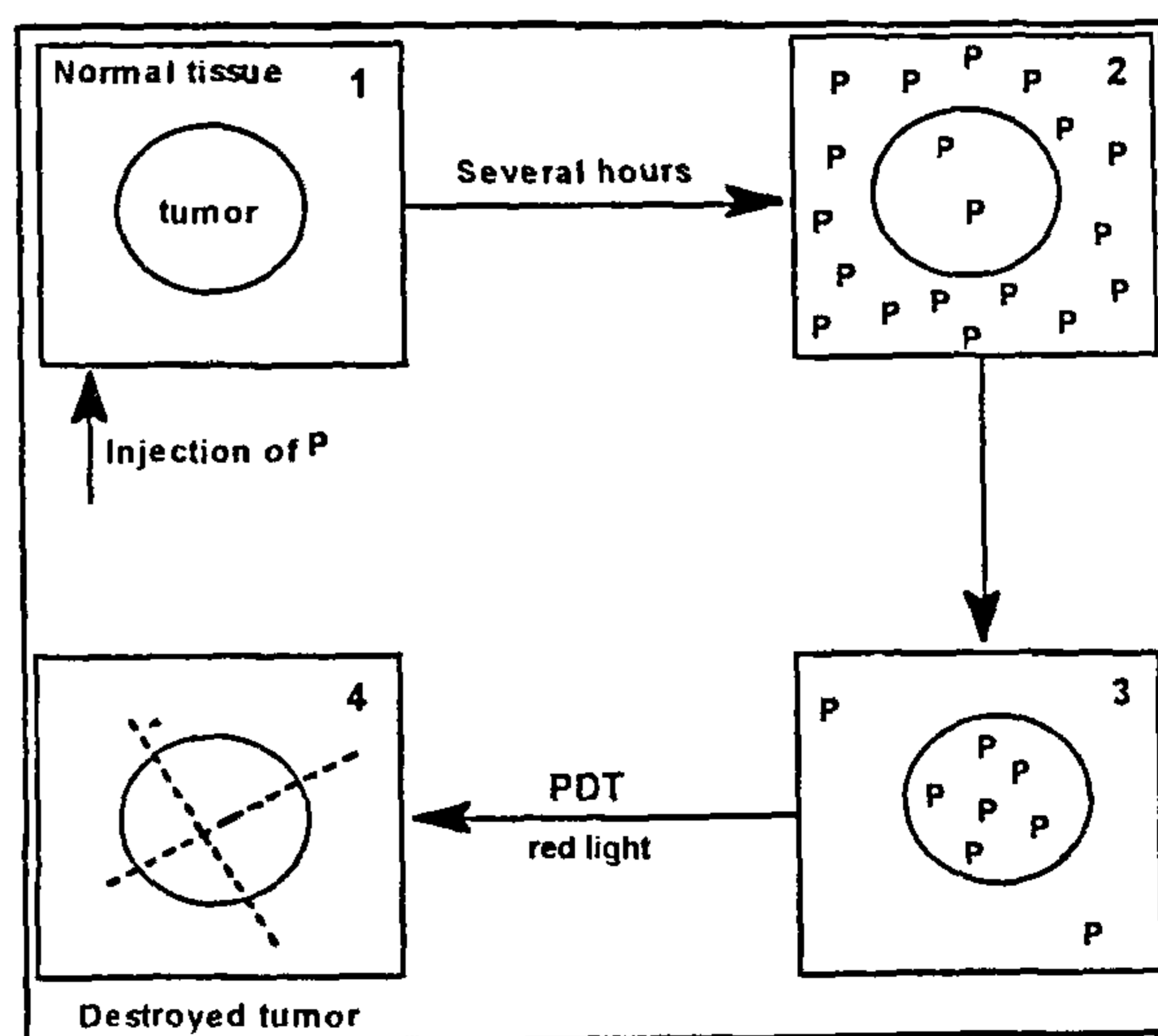


Figure 1. Schematic diagram showing the principles involved in photodynamic therapy.

approach has the potential to deliver a better tumour-targeting device, as the appended intracellular recognition element can, in principle, enhance the cellular uptake of the drug. Porphyrin conjugates endowed with oligonucleotide, monoclonal antibody, ellipticine, or other intracellular recognition elements have been reported, but the majority of these conjugates have been shown to effect the DNA cleavage only via chemical means and, very little effort seems to have been done to induce the photocleavage of DNA utilizing these modified photosensitizers⁷⁻¹⁰. This has prompted us to investigate on the photonuclease activity of rationally-designed 'porphyrin-intercalator' conjugates in light of their potential application to PDT.

In addition to the DNA intercalators, clinically used anti-cancer drugs can also be grafted onto the porphyrin photosensitizers and the so-derived 'combination drugs' are expected to be beneficial in the co-lateral application of chemo- and photo-therapy. Specifically, such 'porphyrin-chemotherapeutic drug' conjugates, while permitting the chemotherapeutic-drug-mediated conventional therapy to be carried on both in the presence (light-on) and absence (light-off) of light, enable the photodynamic therapeutic condition to prevail upon irradiation into the porphyrin absorption bands with the visible light. Synergistic effects toward the tumour eradication are thus expected to predominate during the irradiation period. In addition, these new hybrids can also refine the ability of the porphyrin to home the cancerous cell/tissue by virtue of its conjugation to the chemotherapeutic drug which, on the other hand, would usually possess an inherent ability to localize in the cancerous tissue.

The above concepts have been actualized by us recently and, a few recent examples of 'porphyrin-intercalator' and 'porphyrin-chemotherapeutic drug' conjugates that are capable of photocleaving DNA will be discussed in this article. More than two dozens of new hybrids in which a porphyrin is linked, via, varying lengths and types of covalent bridges (spacers or linkers), to either a DNA intercalator (e.g. acridone, phenothiazine or acridine) or a chemotherapeutic drug (e.g. chlorambucil – a DNA cross-linking 'mustard', currently marketed as an anti-leukaemic drug) were synthesized and their photonuclease activity investigated¹¹⁻¹³. The structures of a few such hybrids are shown in Figure 2.

As seen from Figure 2, the porphyrin unit has been covalently linked to the intercalators or the chemotherapeutic drug *only* at those sites on them that are found to be not absolutely essential for the intercalating/antitumour activity. Similarly, enough care has been taken to ensure that the spectral and photophysical properties of the porphyrins that are necessary for their photodynamic activity remain essentially unchanged even upon linking them to the intercalator/chemotherapeutic drug subunits. Thus, the intrinsic chemical, photochemical

and, consequently, the biological activities of the constituent components were expected to be unaltered in these new hybrids. This is indeed the case as evidenced by the UV-Vis, ¹H NMR and electrochemical data which suggest that there is minimal ground state interaction between the porphyrin and the intercalator/chemotherapeutic drug π -rings in these new conjugates. In addition, the fluorescence and singlet oxygen quantum yields of the porphyrin parts of these new hybrids are also in the same range as those of reference porphyrin **5a**.

While all of the hybrid porphyrins investigated in this study did not affect the DNA (supercoiled pBR 322) cleavage in the absence of irradiation, the reference compounds, which either lack the intracellular recognition element (ca. **5a** or an analogue) or the porphyrin (i.e. individual intercalators or chlorambucil) showed no nuclease activity both in the presence and absence of light. On the other hand, the hybrid compounds **1a-e**, **3a-d** and **4a-f** all affected relaxation of the supercoiled form I to generate open circular form II when irradiated by visible light ($\tau > 400$ nm). Interestingly, the cationic porphyrins **3h-j** displayed DNase activity. 'Inhibitor' studies revealed that all of these photocleavage reactions predominantly proceed through a ¹O₂-based mechanism.

Among the many interesting features noticed during the photocleavage studies with compounds **1-4**, the one that is concerned with the role of 'linker' moiety connecting the photosensitizer and the intracellular recognition element merits further discussion. This aspect can be illustrated by considering the example of porphyrin-phenothiazine conjugates **3a-j** (ref. 13). The observed highest cleavage efficiency for **3a** ($n=3$) compared to the cleavage ability of any other porphyrin in that series and the DNase activity of the dicationic species **3h-j** are revealing in this regard. While the shorter chain length between the porphyrin and the intracellular recognition element in **3a** is responsible for a better cleavage ability of this hybrid photosensitizer, the DNAase activity observed for compounds **3h-j** is possibly due to their hydrophilic nature and additional involvement of Coulombic interactions between the quaternized piperidine and the phosphate backbone of DNA. In this regard, it can be noted that ¹O₂ has to diffuse within its lifetime to effect the DNA cleavage and that this process is more facile in the case of hybrids **3a** and **3h-j** where the distance between the porphyrin moiety (the ¹O₂ generator) and the appended DNA intercalator is short (Figure 3).

Thus, besides a strong DNA-binding by the intercalator and the spacer, a close proximity of the porphyrin moiety and the appended intracellular recognition element play a crucial role in the photocleavage efficiency of these new PDT agents.

The DNA photonic efficacy exhibited by the

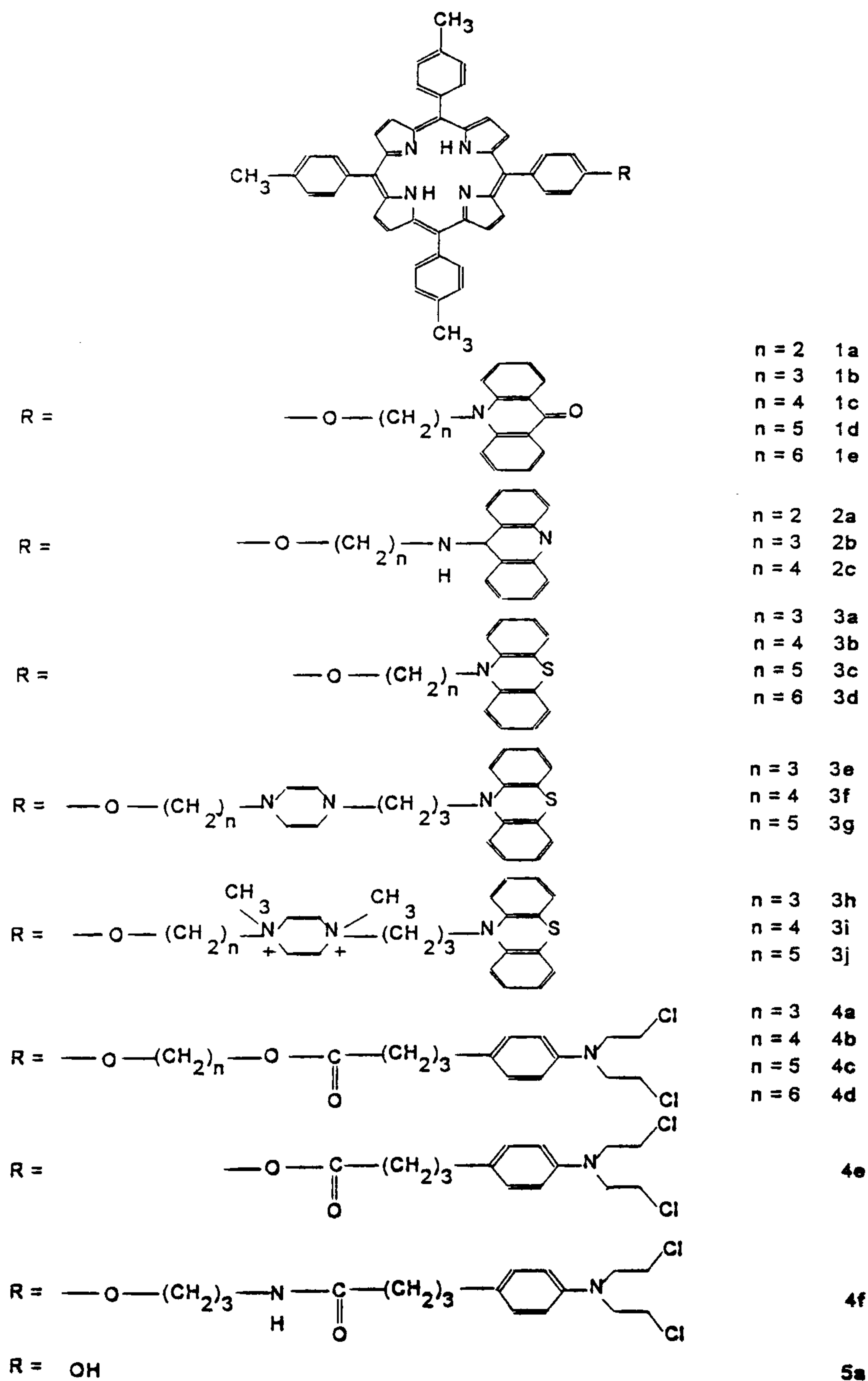


Figure 2. 'Porphyrin-intracellular recognition element' conjugates - new generation photodynamic therapy agents.

porphyrin-chlorambucil (P-Chl) hybrids (4a-f) was observed to be the best among all of hybrids 1-4. This is illustrated in Figure 4 which shows that irradiation of P-Chl hybrid 4a results in an efficient, near quantitative conversion of the supercoiled form I DNA to form II.

In addition to this observation, the fact that P-Chl hybrids can also act as chemotherapeutic drugs even in the absence of light (compounds 4a-f were seen to convert the single-stranded DNA to the double-stranded variety in dark) prompted us to amplify the theme of what can be nick-named as the 'double war-head' approach and to construct new, more potent PDT agents. Accordingly, a new series of PDT agents in which a porphyrin is linked either to one (P-col-Chl) or two (P-col-Chl₂) chlorambucil subunits, via, a cholic acid (col) were designed¹⁴. The cholic acid 'platform' had been specifically employed for the construction of these newer hybrids owing to its ability in recognizing and localizing in the liver cells. As expected, P-col-Chl₂ was found to be much more active in its DNA cleavage ability in comparison with either P-col-Chl or compounds 4a-f.

All these results indicate that the combination of an intercalator/chemotherapeutic drug with a photosensitizer enhances DNA cleavage proclivity under the influence of visible light. The cellular level *in vitro* photodynamic efficiency of these new hybrids also suggests the same; preliminary data indicate that the P-Chl diads and triads effectively photokill the TF-1 leukaemic cancer cells when irradiated by visible light¹⁵. In this regard, it may be noted that, notwithstanding the recent reports which suggest that the current clinical PDT agents target the cell membrane, it is essential to examine the effect of photoirradiation on DNA in the presence of porphyrin conjugates such as those discussed here. These studies are also useful in the development of new and more efficient PDT agents.

DNA-binding and photocleavage by metallo-intercalators

Metal complexes are particularly attractive species for

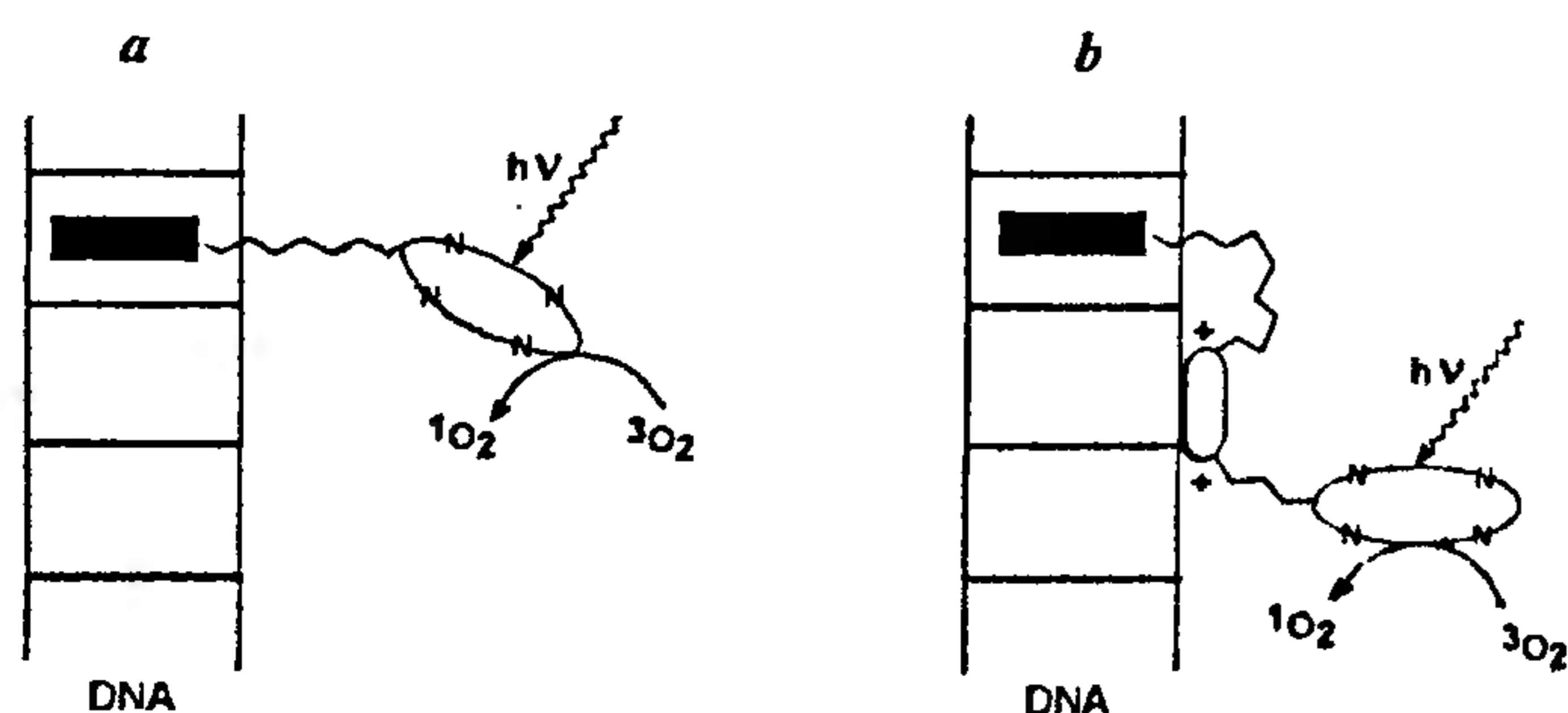


Figure 3. Possible modes of DNA-binding and DNA photocleavage by porphyrin-phenothiazine hybrids (a) 3a and (b) 3b-j.

developing new diagnostic and therapeutic agents that can recognize and cleave DNA^{16,17}. The ligands or the metal in these complexes can be varied in an easily controlled manner to facilitate an individual application. We have been interested to know the effect of variation of the metal ion and also the ligand in complexes containing strongly intercalating and photo-active ligands on their ability to bind and cleave DNA. Dipyrrodo-(3,2-a:2',3'-c)-phenazine (dppz) seemed to be an ideal ligand to start with because (i) it is a near-planar, heteroaromatic entity and hence, can intercalate between the base-pairs on DNA, (ii) its complexes absorb in the visible region and (iii) it can be easily modified to suit various applications^{18,19}. In a separate study that is unrelated to PDT, we have investigated the DNA-binding and photocleavage by a series of new $[M(\text{phen})_2(\text{LL})]^{n+}$ type ($M = \text{Co(III)}$, Ni(II) , or Ru(II) , $\text{phen} = 1,10\text{-phenanthroline}$, $\text{LL} = \text{dppz}$ or modified dppz and $n = 3$ or 2) metallo-intercalators by using absorption and emission

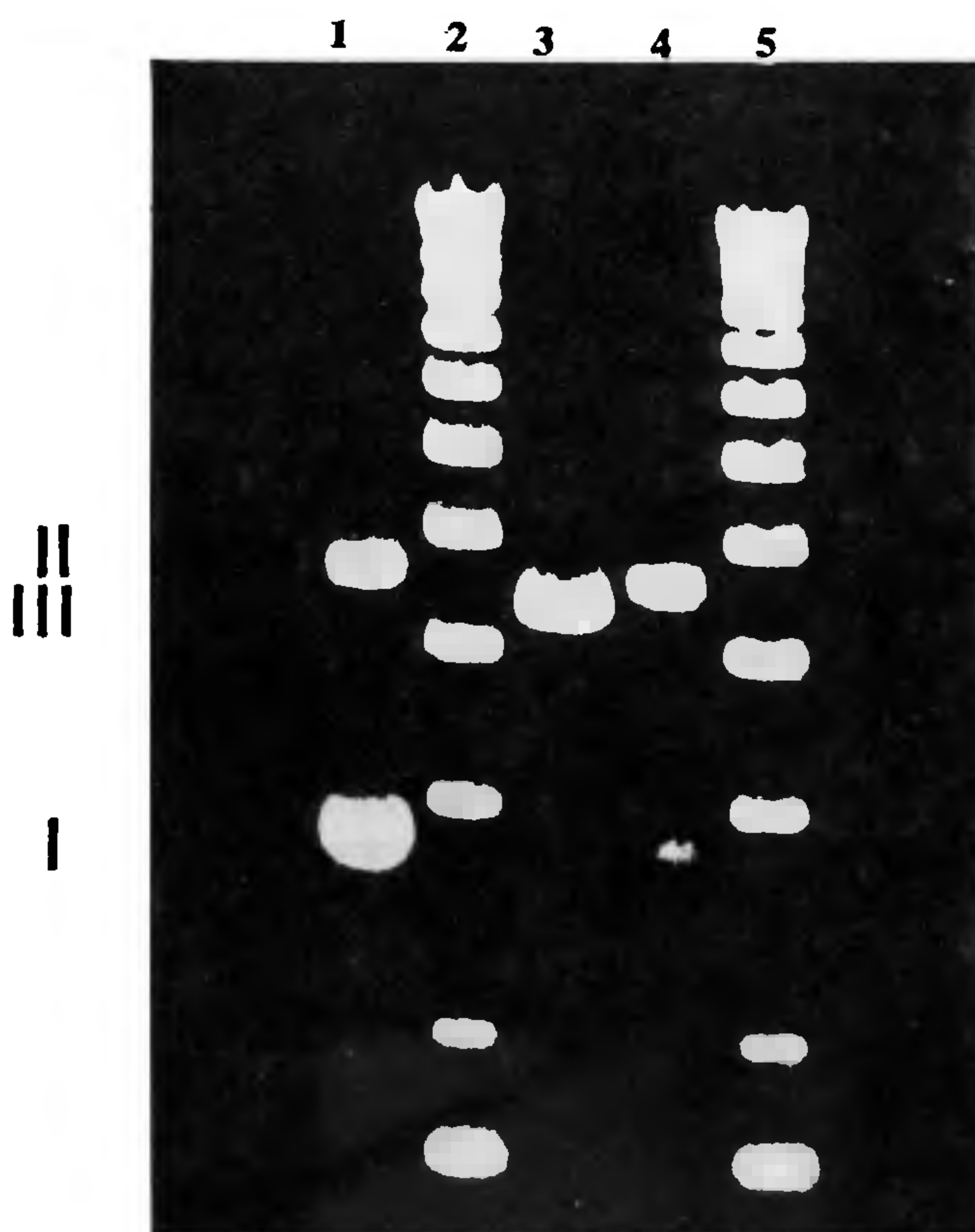


Figure 4. Light-induced nuclease activity of porphyrin-chlorambucil hybrid 4a. (From left to right): Lane 1: untreated pBR 322 DNA; Lane 2: DNA marker; Lane 3: pBR 322 digested with *Hind*III; Lane 4: pBR 322 irradiated in the presence of 4a ($\lambda > 400\text{ nm}$, 150 W Xe-arc lamp, 1 mW/cm^2 , $t = 10\text{ min}$); Lane 5: DNA marker. Supercoiled, relaxed-circular and linearized DNA have been marked as I, II and III, respectively on the gel. Gel electrophoresis experiments were carried out as described in refs 11-14 and 20, 21.

titration, thermal denaturation, gel electrophoresis including the topoisomerase assay and electrochemical methods.

Initially, effects of changing the metal ion in the mixed-ligand dppz complexes on their DNA-binding and photocleavage characteristics were investigated. Two new mixed-ligand complexes, containing either cobalt(III) ($[\text{Co}(\text{phen})_2(\text{dppz})]^{3+}$, **6**) or nickel(II) ($[\text{Ni}(\text{phen})_2(\text{dppz})]^{2+}$, **7**) as the central metal ion, were seen to be avid binders of calf thymus (CT) DNA²⁰. Results of various spectroscopic, electrochemical and biochemical investigations carried out with these complexes revealed that the dipyridophenazine ligand on them is engaged in the intercalative interaction with DNA and that the intrinsic binding constant, K_b , is as high as $(9 \pm 2) \times 10^5 \text{ M}^{-1}$ for both the complexes. Thus, although these two complexes provided a good opportunity to compare directly the binding of isosteric intercalating species of +2 and +3 charge to DNA, such a comparison could not be made. On the other hand, while the cobalt(III) complex was found to affect photocleavage of the supercoiled pBR 322 DNA via a OH^\bullet -based mechanism, as evidenced by 'inhibitor' and 'spin-trapping/ESR' studies, the nickel(II) complex (a d^8 system), being paramagnetic, was ineffective under similar experimental conditions. Thus, these results, while underscoring the importance of dppz in the DNA-binding, also demonstrate that substitution by different metal ions can bring about subtle modulation in the properties and, consequently, in the DNA interaction of this new class of mixed-ligand complexes containing the versatile dipyridophenazine ligand.

During our continued investigations on the dppz-based complexes, it occurred to us that further derivatization of this ligand with suitable electron withdrawing/donating group(s) might not only accentuate DNA-binding and photocleavage efficiencies of the ensuing complexes but also serve to explore other interesting functional aspects associated therein. A series of new, mixed-ligand ruthenium(II) complexes, incorporating modified dppz ligands, have been designed recently. The DNA-binding and photocleavage abilities as well as redox and luminescence properties of two representative complexes will be discussed here.

The mixed-ligand ruthenium(II) complex **8**, incorporating a quinone-fused dipyridophenazine ligand [10,11-(1,4-naphthalenedione) dipyrdo (3,2'-a:2',3'-c)phenazine, qdppz] was synthesized first²¹. The choice of quinone moiety for modifying dppz is owing to the known DNA-binding ability and the rich redox chemistry of this ubiquitous electron-deficient functional group^{22,23}. Chemical or electrochemical reduction of **8** lead to the generation of **9** – a complex containing the hydroquinone form of qdppz (Figure 5).

Complex **8** is an avid binder of CT DNA ($K_b \geq 10^6 \text{ M}^{-1}$) due to a strong intercalation by the ruthenium-bound qdppz whereas, the reduced complex **9** shows only a

moderate binding ($K_b = (2 \pm 0.3) \times 10^4 \text{ M}^{-1}$). The DNA photocleavage efficiencies of these complexes also follow a similar trend in that the MLCT excited state of **8** is more effective than that of **9** in cleaving the supercoiled plasmid pBR 322 DNA ($\lambda_{\text{exc}} = 440 \pm 5 \text{ nm}$). While irradiation into the MLCT band of **8** can generate a species containing oxidized ruthenium and reduced qdppz (semi-quinone), direct excitation of the bound-qdppz is expected to provide the triplet quinone. Both these quinone-based, transient species are known to be potent DNA-cleaving agents capable of reacting with the duplex via various mechanisms, including hydrogen abstraction, electron transfer, etc.²². Finally, by the combined application of exhaustive bulk-electrolysis and steady state fluorescence spectroscopic methods, it was demonstrated that the ' $2e^-/2\text{H}^+$ ' redox couple **8/9** represents a molecular light-switching device, displaying interconversion between the non-luminescent 'quinone' and luminescent 'hydroquinone' states in aqueous CH_3CN solutions (Figure 5)²¹. Thus, these complexes are useful not only in the design of photonucleases but also in the development of molecule-based, electronic devices.

Tris- and mixed-ligand ruthenium(II) complexes, which incorporate another modified dppz ligand dicnq [10,11-dicyanodipyridoquinoline], were investigated next; complex **10** is a typical example (Figure 6)²⁴.

It was observed that these complexes are also avid binders of DNA by the intercalative mode and, in addition, that they photocleave DNA when irradiated into their MLCT absorption envelope. The most interesting aspect of these compounds is, however, their 'light-switching' ability in the presence of DNA. For example, while complex **10** is nearly non-luminescent in aqueous buffered solutions, addition of increasing amounts of DNA to the aqueous buffered solutions, containing this complex, was seen to result in the luminescence enhancement to the tune of ca. 15–20 times (Figure 6). Thus, this complex is an efficient 'luminescence reporter' of DNA.

Carbene-mediated DNA photocleavage

In a majority of earlier studies with photonucleases, photogenerated ion, radical or oxygen-centered reactive species have been proposed to be responsible for cleaving DNA. However, carbenes – a class of unstable and highly reactive organic species, which are known to be generated upon photoirradiation of arene diazo compounds and to be reactive towards a wide variety of organic and bioorganic substrates including proteins – seem to have never been tested for their ability to cleave DNA²⁵. In a more recent study, we have been able to show, for the first time, that photolysis of simple, readily available arene diazo compounds leads to the cleavage of DNA via carbene-mediated pathway²⁶.

9-Diazoanthrone (**11**), a carbene-precursor capable of absorbing the visible light ($\lambda_{\text{max}}(\text{DMF}) = 431 \text{ nm}$), has been chosen here for the demonstration of carbene-mediated DNA photocleavage not only because of its well-known photochemical reactivity but also for its planar, aromatic structure which assists in the intercalative binding with DNA. Irradiation of supercoiled pBR 322 DNA by light of $\lambda > 400 \text{ nm}$ in the presence of this diazo-arene resulted in a facile generation of the relaxed circular form and, careful experiments with various 'inhibitors' established the participation of carbene species in this photoconversion. Specifically, while

D_2O , mannitol, dimethyl sulphoxide and super-oxide dismutase did not affect the DNA-nicking efficiencies of **11**, suggesting that none of the reactive oxygen species ($^1\text{O}_2$, OH^\bullet or $\text{O}_2^{\bullet-}$) play a role in the cleavage mechanism, a 90–60% inhibition exhibited by 2-propanol, triphenyl phosphine and cumene collectively provided evidence for the participation of carbenes in the observed DNA cleavage by this reactive diazo compound.

Concluding remarks

In summary, we have attempted to show here that it is

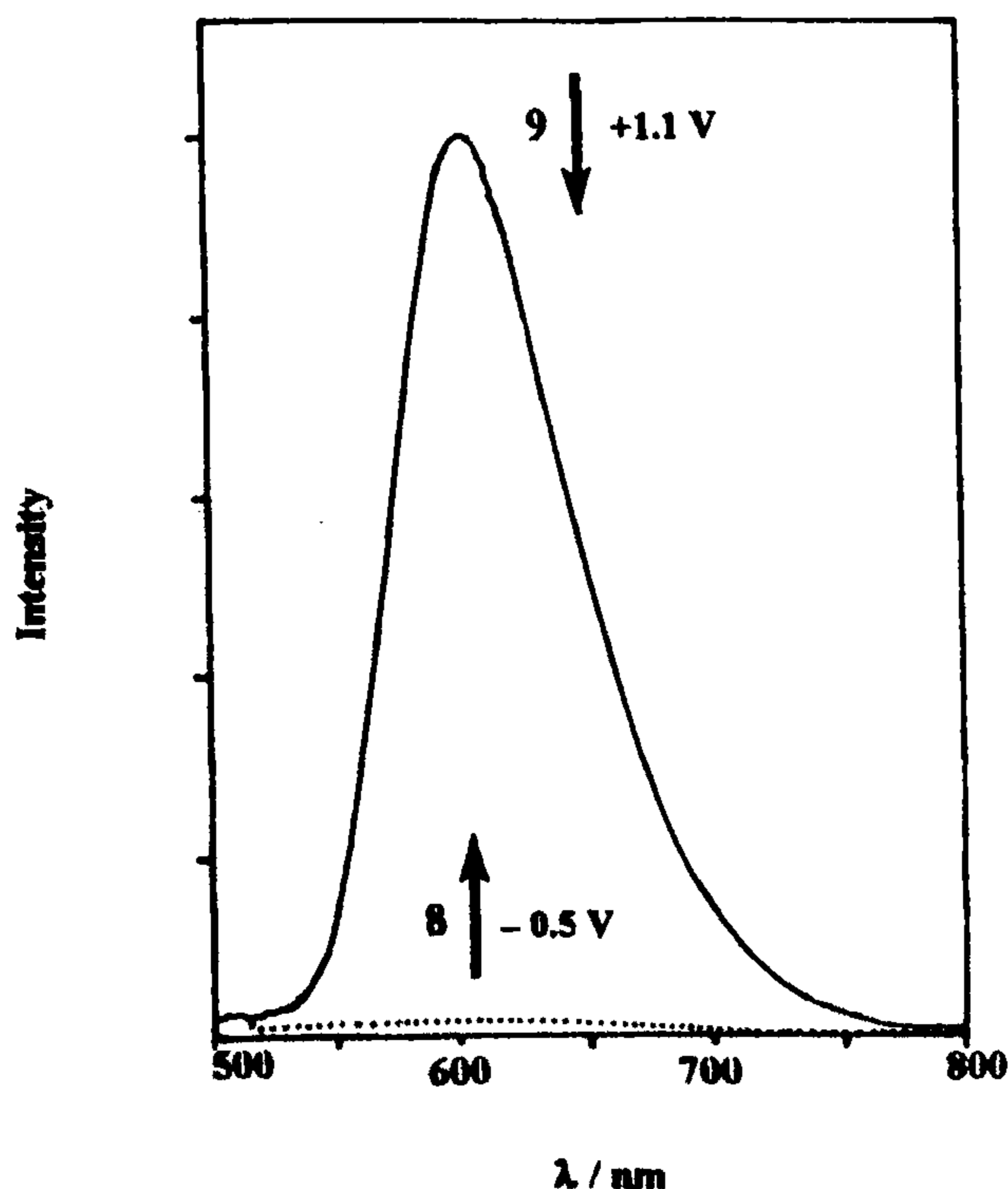
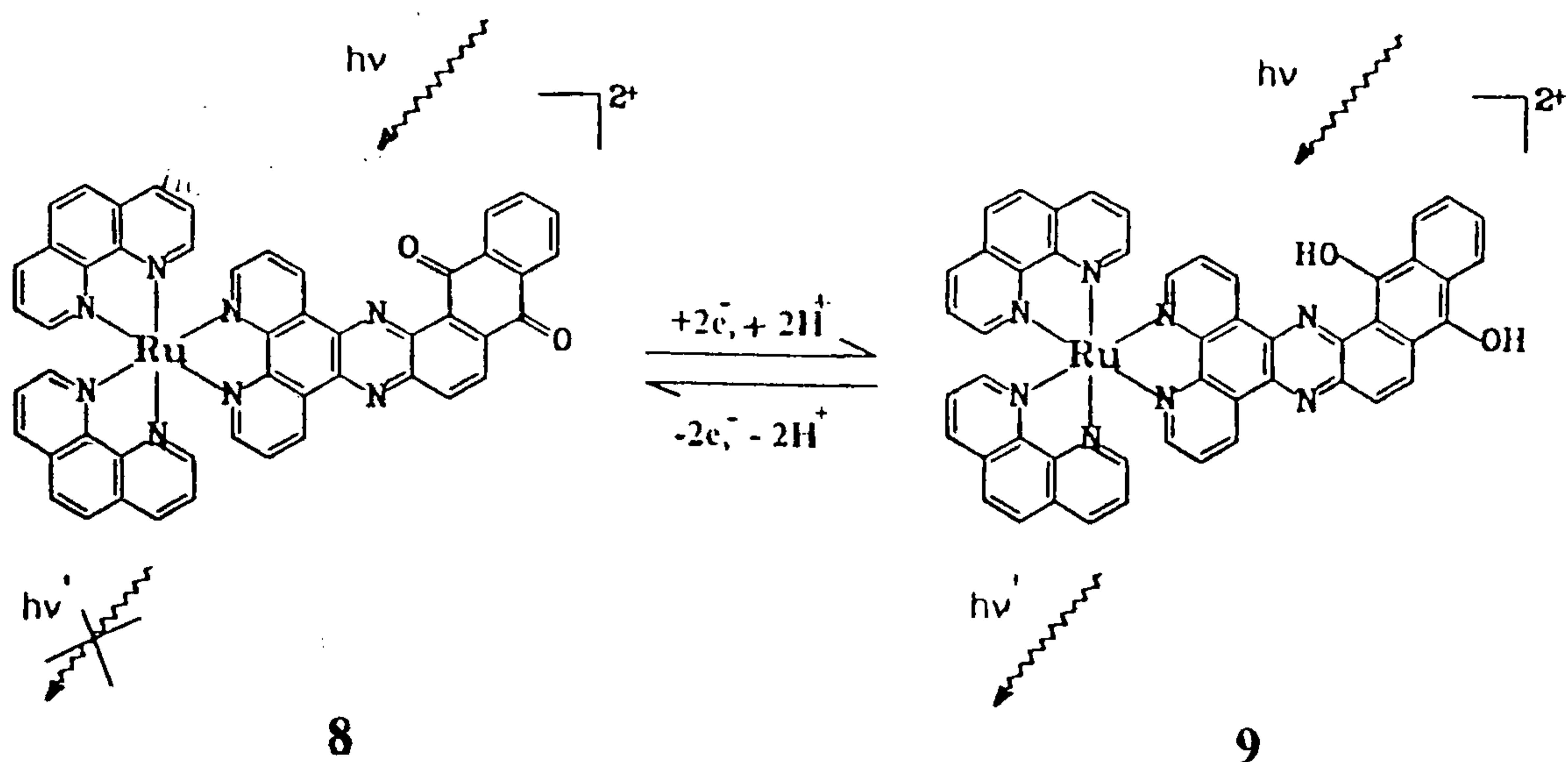


Figure 5. An 'electro-photo switch': Luminescence spectra ($\text{CH}_3\text{CN}/5\% \text{ H}_2\text{O}$, 0.1 M TBAF_6 , $\lambda_{\text{exc}} = 440 \text{ nm}$) of **8** and **9** as obtained by exhaustive electrolyses at the indicated potentials in each case. The arrows refer to the reversible changes observed upon electrochemical interconversion of these complexes.

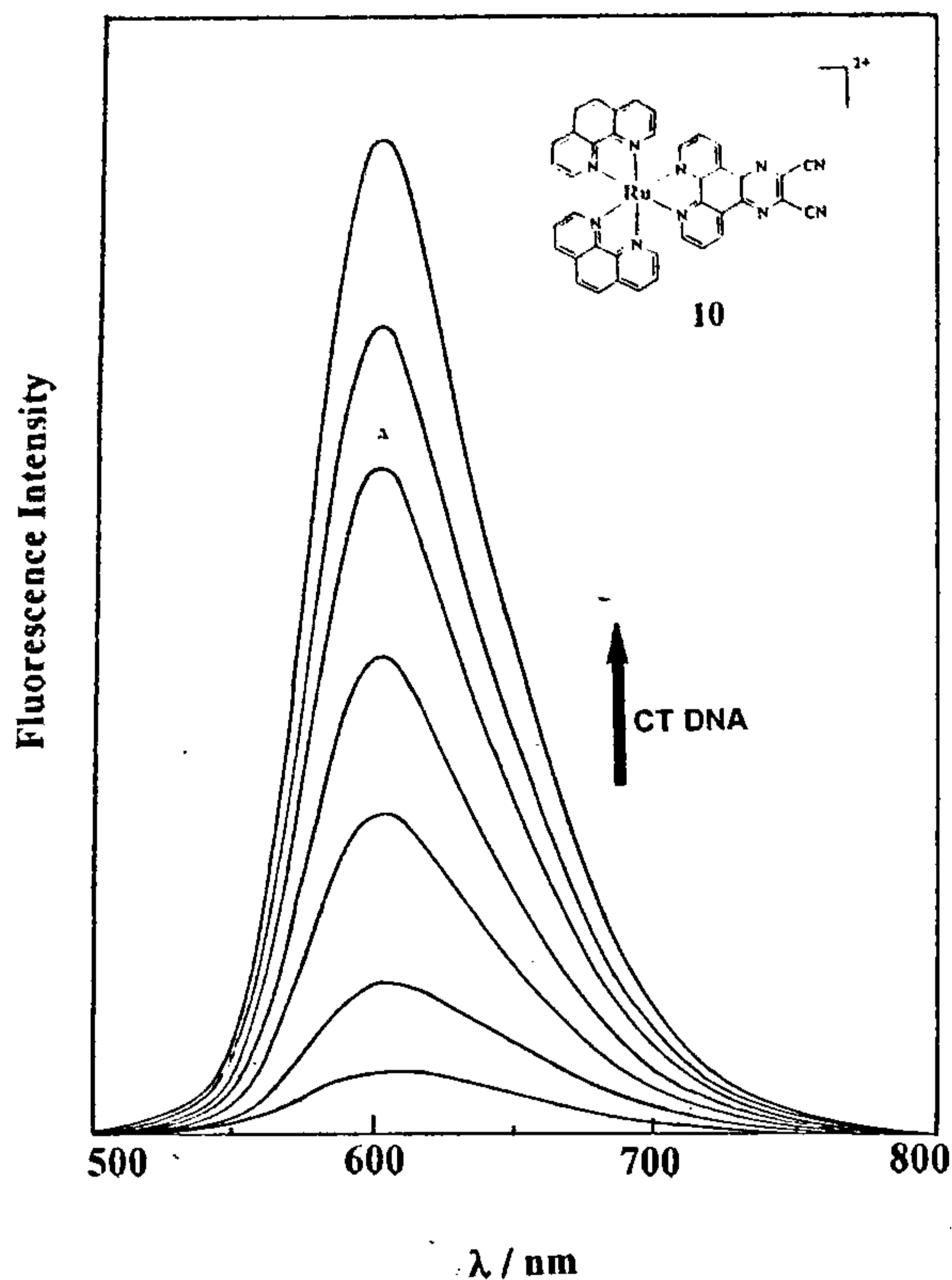


Figure 6. Luminescence enhancement observed during the addition of increasing concentrations (0–600 mM) of CT DNA to a buffered solution containing complex 10 (10 mM) – a ‘luminescence reporter’ of DNA. ($\lambda_{\text{exc}} = 440 \text{ nm}$).

possible to accomplish potent photonucleases by suitably modifying the structures of visible-light-absorbing chromophores. A highlight of this approach is that the chromophores either themselves can intercalate between the base-pairs of DNA (e.g. diazo arenes) or can employ other structural components grafted on them to strongly bind to the duplex by noncovalent (intercalation) and covalent (alkylation) interactions. The principal theme, common to these new photonucleases, is that they all absorb light in the 400–650 nm region, generate cytotoxic species such as reactive oxygen intermediates ($^1\text{O}_2$ or OH^\bullet), radical or carbene species and lead to DNA cleavage. The photonucleases described here also have several practical applications. The ‘dual action’ theme, as exemplified in studies with the porphyrin-chlorambucil conjugates, can be perceived to have immense application in the ‘combination therapy’ (i.e. chemotherapy + phototherapy) against cancer. The ‘light-switching’ ability of complex 10 in the presence of DNA permits the use of this complex as a ‘luminescence reporter’ of DNA.

Finally, DNA-binding and photo-cleavage by complexes 8 and 9 together with the finding that this redox couple represents an ‘electro-photoswitch’ testify to the suitability of these complexes in the design of both photonucleases and molecule-based, opto-electronic devices. Currently, we are engaged in examining the site-specificity involved in the DNA-binding and DNA photocleavage of these novel photonucleases.

1. Morgan, A. R., *Curr. Med. Chem.*, 1995, 2, 604–610.
2. Levy, J. G., *Trends Biotechnol.*, 1995, 13, 14–17.
3. Henderson, R. W. and Dougherty, T. J. (eds), *Photodynamic Therapy: Basic Principles and Clinical Applications*, Marcel Dekker, New York, 1992.
4. Dougherty, T. J., *Photochem. Photobiol.*, 1987, 45, 879–889.
5. Bonnett, R., *Chem. Soc. Rev.*, 1995, 19–33.
6. Dolphin, D., *Can. J. Chem.*, 1994, 72, 1005–1013.
7. Bigey, P., Pratviel, G. and Meunier, B., *J. Chem. Soc., Chem. Commun.*, 1995, 181–182.
8. Milgrom, L. R. and O'Neill, F., *Tetrahedron*, 1995, 51, 2137–2144.
9. Harada, A., Shiotsuki, K., Fukushima, H., Yamaguchi, H. and Kamachi, M., *Inorg. Chem.*, 1995, 34, 1070–1076.
10. Magda, D., Wright, M., Miller, R. A., Sessler, J. L. and Sansom, P. I., *J. Am. Chem. Soc.*, 1995, 117, 3629–3630.
11. Mehta, G., Sambaiah, T., Maiya, B. G., Sirish, M. and Chatterjea, D., *J. Chem. Soc. Perkin Trans. 1*, 1993, 2667–2669.
12. Mehta, G., Sambaiah, T., Maiya, B. G., Sirish, M. and Dattagupta, A., *Tetrahedron Lett.*, 1994, 35, 4201–4204.
13. Mehta, G., Sambaiah, T., Maiya, B. G., Sirish, M. and Dattagupta, A., *J. Chem. Soc. Perkin Trans. 1*, 1995, 295–297.
14. Mehta, G., Muthusamy, S., Maiya, B. G. and Sirish, M., *J. Chem. Soc. Perkin. Trans. 1*, 1996, 2421–2423.
15. Viola, A., Mannoni, P., Chanon, M. et al., *J. Photochem. Photobiol. B: Biology*, (submitted for publication).
16. Mesmaeker, A. K. -D., Lecomte, J. -P. and Kelly, J. M., *Topics Curr. Chem.*, 1996, 177, 25–76.
17. Pyle, A. M. and Barton, J. K., *Prog. Inorg. Chem.*, 1990, 38, 413–475.
18. Lopez, R. B., Loeb, B. L., Boussie, T. and Meyer, T. J., *Tetrahedron Lett.*, 1996, 37, 5437–5440.
19. Hartshorn, R. M. and Barton, J. K., *J. Am. Chem. Soc.*, 1992, 114, 5919–5925.
20. Arounagiri, S. and Maiya, B. G., *Inorg. Chem.*, 1996, 35, 4267–4270.
21. Arounagiri, S., Dattagupta, A. and Maiya, B. G., *Proc. Indian Acad. Sci. (Chem. Sci.)*, 1997, 109, 155–158.
22. Ly, D., Kan, Y., Armitage, B. and Schuster, G. B., *J. Am. Chem. Soc.*, 1996, 118, 8747–8748.
23. Bhattacharya, S. and Mandal, S. S., *J. Chem. Soc. Chem. Commun.*, 1996, 1515–1516.
24. Arounagiri, S. and Maiya, B. G., (unpublished results).
25. Jones, M. Jr. and Moss, R. A., *Carbenes*, Wiley-Interscience, vol. 1, 1973.
26. Maiya, B. G., Ramana, C. V., Arounagiri, S. and Nagarajan, M., (unpublished results).

ACKNOWLEDGEMENTS. I thank Prof. G. Mehta for his interest in this work, for encouragement and collaboration in research related to PDT and Prof. M. Nagarajan for collaboration in carbene chemistry. I also thank all the coworkers whose names appear in the listed references. I am grateful to the Indo-French Centre for the Promotion of Advanced Research (Centre Franco-Indien pour la Promotion de la Recherche Avancee), New Delhi, for financial support.