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Novel Heterocyclic Dyes as DNA Markers. Part II. Structure and Biological Activity

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Abstract: Management and control of malaria allows epidemiological studies. Microscopic examination of blood films is actually the most rapid and accurately method for diagnosis malaria. Malaria parasites can be stained with many dyes, while Romanowsky-type stains are usually used for morphological studies, fluorescent cyanine dyes are used for rapid diagnosis and quantification. Quantum mechanical methods were used for structural characterisation. The results have allowed the structure activity correlations to be established. © 1997 Elsevier Science Ltd.

INTRODUCTION

The management and control of malaria requires early and sensitive detection of the infection. Currently the most rapid and practical diagnostic method for malaria remains the microscopic examination of blood films. The presence of malaria parasites in the blood is a sign of infection but not necessarily a cause of the disease.[1] The invasion of one red blood cell which follows the pre-erythrocytic phase is the basic pathological process in malaria infections and therefore the stage which should be used for diagnosis. Of the 140 species of Plasmodia there are four generally recognised species of malaria parasites infecting man. They are *Plasmodium falciparum* (Welch, 1897), *Plasmodium malariae* (Laveran, 1881), *Plasmodium vivax* (Grassi and Feletti, 1890) and *Plasmodium ovale* (Stephens, 1922). Of the four species of human plasmodium, *P. falciparum* causes malignant malaria and is the most pathogenic in non-immune subjects. The degree of parasitaemia produced by the different species of plasmodia varies considerably.

Malaria parasites in blood films can be stained with many dyes. In general these stains can be subdivided into Romanowsky type stains, such us Giemsa, Field, and fluorescent-type stains each of them providing different information. While Romanowsky-type stains are usually used for morphological studies, fluorescent-type stains are used for rapid diagnosis and quantification purposes. In particular PUR-1[b] is a fluorescent cyanine dye, the ability of which to stain parasites is attributed to its ability to penetrate the cell, enter all subcellular compartments and bind to the DNA in these. Despite the large number of cyanine dyes, few have been characterised by modern methods.

In this study structure activity correlations have been established using quantum mechanical calculations which are consistent with spectroscopic data.

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[b] The synthesis of PUR-1 and its analogues are described in "Novel Heterocyclic Dyes as DNA markers. Part I. Synthesis and Characterization, from the same authors.

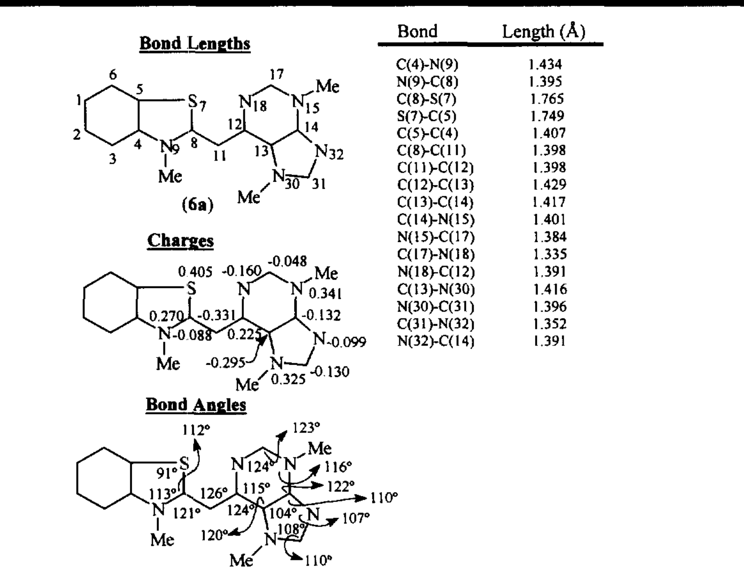
RESULTS AND DISCUSSION

Molecular Structure of Cyanine Dyes

The characteristic features of the electronic structure of cyanine dyes are: (i) maximum bond-order equalisation,[2] and (ii) alternation of electron densities at the carbon atoms of the conjugated systems of the dyes.[3,4] These criteria imply a maximum equalisation of the C—C bond lengths and an alternation in the C—C—C bond angles.

Molecular structural data can be obtained by application of quantum mechanical methods using geometry optimisation procedures. The quality of the calculated bond lengths and bond angles depends on the MO method used. The optimised molecular geometries obtained by MNDO[5] (Modified Neglect of Diatomic Differential Overlap) and NDDO[6] (Neglect of Diatomic Differential Overlap) agree well with the experimental values.

Table 1 Structural data for the quantum chemically optimised structure of the cyanine dye (**6a**).



Bond	Length (Å)
C(4)-N(9)	1.434
N(9)-C(8)	1.395
C(8)-S(7)	1.765
S(7)-C(5)	1.749
C(5)-C(4)	1.407
C(8)-C(11)	1.398
C(11)-C(12)	1.398
C(12)-C(13)	1.429
C(13)-C(14)	1.417
C(14)-N(15)	1.401
N(15)-C(17)	1.384
C(17)-N(18)	1.335
N(18)-C(12)	1.391
C(13)-N(30)	1.416
N(30)-C(31)	1.396
C(31)-N(32)	1.352
N(32)-C(14)	1.391

Table 2 Dihedral angle between planes of heterocyclic rings of cyanine dyes.

Compound	Dihedral Angle
(6a)	0.05°
(7)	50.0°
(8)	1.2°
(9a)	0.87°
(10)	22.5°
(12)	6.0°

From **Table 1** it can be seen, that the molecular structure of (**6a**) is characterised by the nearly complete equalisation of bond lengths, the alternation of bond angles and charges in the conjugated system of the cyanine. The two heterocyclic nuclei are nearly co-planar (the dihedral angle between the planes being 0.05°).

In contrast the cyanine (7) does not have bond equalisation because of steric effects; the dihedral angle between the two heterocyclic systems is about 50° implying a loss of planarity in the molecule. **Table 2** shows the dihedral angles for these and other cyanine dyes. Molecular data for other cyanine dyes are shown in **Table 3**.

Table 3 Quantum chemically optimised (MNDO/PM3) structural data for some cyanine dyes

Compound	Bond	Length(Å)	Atom	Charge	Atoms	Angle
(7)	C(4)-N(9)	1.424	N(9)	0.302	C(4)-N(9)-C(8)	109°
	N(9)-C(8)	1.394	C(8)	-0.268	N(9)-C(8)-N(7)	108°
X = N-Me	C(8)-N(7)	1.385	N(7)	0.354	C(8)-N(7)-C(5)	109°
	N(7)-C(5)	1.427	C(11)	0.011	N(9)-C(8)-C(11)	123°
	C(8)-C(11)	1.433	C(12)	-0.156	C(8)-C(11)-C(12)	125°
	C(11)-C(12)	1.364	N(18)	-0.007	C(11)-C(12)-C(13)	125°
	C(12)-N(18)	1.414	C(17)	-0.200	N(18)-C(12)-C(13)	115°
	N(18)-C(17)	1.326	N(15)	0.391	C(12)-N(18)-C(17)	123°
	C(17)-N(15)	1.390	C(14)	-0.200	N(18)-C(17)-N(15)	124°
	N(15)-C(14)	1.407	C(13)	-0.129	C(17)-N(15)-C(14)	116°
	C(14)-C(13)	1.410	N(30)	0.301	N(15)-C(14)-C(13)	122°
	C(13)-C(12)	1.437	C(31)	-0.173	C(14)-C(13)-C(12)	120°
	C(13)-N(30)	1.415	N(32)	-0.076	C(13)-N(30)-C(31)	108°
	N(30)-C(31)	1.395			N(30)-C(31)-N(32)	110°
	C(31)-N(32)	1.353			C(31)-N(32)-C(14)	107°
	N(32)-C(14)	1.391				
(8)	C(4)-N(9)	1.439	N(9)	0.293	C(4)-N(9)-C(8)	113°
	N(9)-C(8)	1.401	C(8)	-0.118	N(9)-C(8)-S(7)	112°
X = S	C(8)-S(7)	1.748	S(7)	0.438	C(8)-S(7)-C(5)	90.5°
	S(7)-C(5)	1.739	C(11)	-0.332	N(9)-C(8)-C(11)	121°
	C(8)-C(11)	1.402	C(12)	0.220	C(8)-C(11)-C(12)	126°
	C(11)-C(12)	1.395	N(18)	-0.165	C(11)-C(12)-C(13)	124°
	C(12)-N(18)	1.394	C(17)	-0.046	N(18)-C(12)-C(13)	115°
	N(18)-C(17)	1.334	N(15)	0.332	C(12)-N(18)-C(17)	124°
	C(17)-N(15)	1.385	C(14)	-0.131	N(18)-C(17)-N(15)	123°
	N(15)-C(14)	1.401	C(13)	-0.296	C(17)-N(15)-C(14)	116°
	C(14)-C(13)	1.416	N(30)	0.324	N(15)-C(14)-C(13)	122°
	C(13)-C(12)	1.430	C(31)	-0.134	C(14)-C(13)-C(12)	120°
	C(13)-N(30)	1.416	N(32)	-0.099	C(13)-N(30)-C(31)	108°
	N(30)-C(31)	1.396			N(30)-C(31)-N(32)	110°
	C(31)-N(32)	1.352			C(31)-N(32)-C(14)	107°
	N(32)-C(14)	1.391				

Stereoisomerism of Cyanine Dyes[7]

For unsubstituted cyanine dyes the all-*trans*(E) form is the most stable and is present in solid state and in solution. However, the molecules may have a certain structural mobility, the stereochemistry being dependent on the constitution, and the type of end groups of the conjugated chain.

The shapes of cyanine dyes are of three types based on the molecular structure:[7]

a) "Planar but loose"

b) "Compact"

c) "Overcrowded"

In some cases the cyanine dyes are essentially coplanar but the substituents in the end groups are distorted out of the plane by up to 90°.[8] The cyanine dye (**12**) is such an example for which the most stable configuration has a heat of formation of 1023.6 kJ mol^{-1}.

Conformational analysis of the cyanine dye (**8**) shows that the Z conformer (i.e. conformer A) shown in **Figure 1** is the more stable. This was confirmed by NMR measurements in solution over a range of temperatures.

Figure 1 **Figure 2**

The solid state spectra also confirm the Z stereoisomeric conformation. The alternative, conformer B, shows steric hindrance between the methyl group of the naphthothiazole ring and the nitrogen in the purine ring (**Figure 2**).

Structure-Activity Relationships

The biological activity[9,10] of cyanine dyes is attributed to their ability to penetrate living cells, enter all subcellular compartments, and bind to numerous macromolecules.[11] Nucleic acids, which carry genetic information in living systems, are possible targets for such interactions[12] and it is of interest to investigate the interaction of cyanine dyes with nucleic acids, and also with the nucleic acid bases.

(**8**) (**13**)

Figure 3

SCFMO-calculations (using MNDO/PM3) of the charges on the dye cation and on thymine (**13**) are shown in **Figure 3**. They indicate that the purine part of the cyanine dye (**8**) is a good acceptor for the excess of charge over the nucleic acid base. The exact geometry of such a complex cannot be determined but a structure where the plane of the pyrimidine ring of the thymine is parallel to that of the dye cation seems likely[13] because the complex involves an n-electron donor thymine and the dye cation as acceptor. The overlap of the π orbitals of the thymine with π orbitals of the cation would give rise to 1:1 parallel stacking such as that shown in **Figure 4**. Other complexes of dye-cations and related bases, such as adenine, appear to behave in similar fashion. Adenine (**14**) possesses more π-electron donor sites, making complexation easier. **Figure 5** shows the charge on the atoms involved in the complexation for adenine base (**14**). The suggested complex for the dye cation (**8**) and the adenine base (**14**) is shown in **Figure 5**.

Figure 4

(**14**)

Figure 5

Dye-Nucleic Acid Interactions

Dye-nucleic acid interactions are dependent on three components of the nucleic acids: the purine and pyrimidine bases, deoxyribose and ribose, and orthophosphate groups.[14] Each dye cation when combined with a phosphate group must lie in the space between the planes of the pyrimidine or purine rings. Two energetically different modes of binding occur, the first being a strong binding of the monomeric dye with the nucleic acid bases. After the first binding site has been saturated, with one dye molecule per four or five nucleotides,[15] a weaker complexation occurs outside the DNA helix between further molecules and those already bound.

The intercalation proceeds in two steps. In the first step the dye binds to the phosphate groups at the outside of the helix, by electrostatic attraction. This needs little activation energy and is almost completely diffusion controlled. The second step, the insertion into the helix, proceeds far less rapidly, the reaction rate depending on the steric configuration of the intercalating dye. The dye moves from a state of external binding into the space between neighbouring base pairs. The base pairs probably separate without breaking of the hydrogen bonds, a process described as "accordion-like longitudinal flexing of the DNA molecule, with intercalation occurring at the same time". The weaker external binding involves the electrostatic attraction of the dye[16] to the outside of the helix by the pairing of the cations to the negatively charged phosphate residues of the chain. The activation energy of formation of this complex is much smaller than that for the strong internal complex. There are two possible sites of binding: either in an isolated phosphate group or in the neighbourhood of other dye molecules. In the latter case, besides the electrostatic forces between the ligand and DNA, there are also forces which, depending on the strength of the aggregation tendency, lead to further stabilisation. These forces are van der Waal's interactions between the ligands. In one case the planes of the dye molecule are oriented parallel to the planes of the base pairs of the DNA and in the other the dye molecules stack so that their planes lie parallel to the axis of the DNA. A possible DNA-PUR-1 complex is shown in **Figure 6**. The black formula represents the adenine-thymine paired bases in a doubled stranded DNA, and the red formula represents the PUR-1 derivative. The properties of the dyes, either structural (planarity) and/or electronic (fluorescence), influence the ease of the dye-nucleic acid interaction in the intercalation model and in the efficiency of the localisation of the stained DNA.

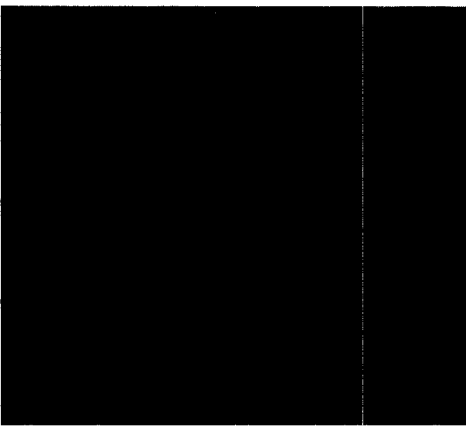
Figure 6

Staining Procedures in Malaria Diagnosis

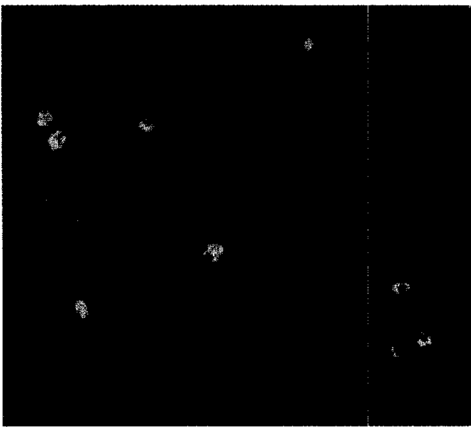
Two main types of stains are used for the diagnosis of malaria, the Romanowsky- type (Field's)[17] and the fluorescent-type (PUR-1 and analogues). The former is composed of two compounds, one basic and one acidic both of which have staining properties. The basic stain is methylene blue and the acid stain is eosin. This mixture stains the cytoplasm of the malaria parasite pale purplish blue and the chromatin deep red. The use of a slightly alkaline buffer (pH 7.2) is important for a clear differentiation of nuclear and cytoplasmic material.

The fluorescent stain PUR-1 and its analogues are cationic cyanine dyes which stain the chromatin a fluorescent bright apple-green and the cytoplasm yellow.

Photograph 1 shows a thick blood smear with a 7.5% parasitaemia stained with Field's stain; the very small deep red spots represent the *P. falciparum* chromatin while the large spot is the leucocyte chromatin. **Photograph 2** shows a thin blood smear stained with PUR-1. The parasitaemia was 7.5%, the bright apple-green spots corresponds to the parasite chromatin, and the uninfected erythrocytes can just be seen in the background.



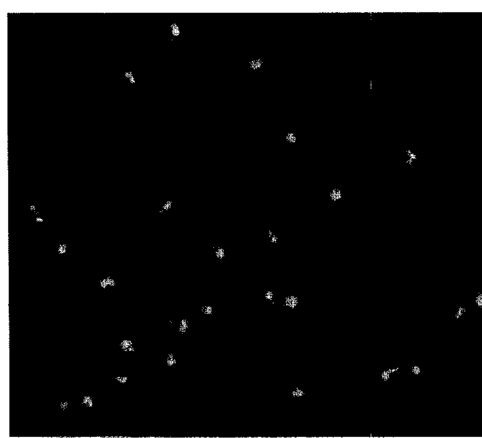
Photograph 1



Photograph 2

Effectiveness of PUR-1 and its Analogues

PUR-1 and its analogues were tested under the same conditions against Field's stained blood smears, the results being assessed by subjective visualisation by four independent members of staff of the London Hospital for Tropical Diseases (4 St Pancras Way, London NW1 0PE). From a comparison of the two photographs (**1** and **2**) it is clear that the diagnosis with the PUR-1 stain is much easier than with Field's stain. The fact that the PUR-1 is fluorescent permits easy detection of the chromatin of the parasite even by unskilled personnel. This, together with the speed of diagnosis are its major advantages. A comparison of the effectiveness in the diagnosis by PUR-1 and related compounds in terms of ease of staining and localisation of DNA of malaria parasites is given in **Table 4**. The "size" refers to apparent area and relative intensity of fluorescence of the stained chromatin spots.



Photograph 3

Photograph 4

Table 4 Comparison of the effectiveness of PUR-1 and its analogues in the diagnosis of malaria parasites.

PUR-1 (**6a**)	R = Benzo-	X = S
(**7**)	R = Benzo-	X = N
(**8**)	R = Naphtho-	X = S
(**9a**)	R = Benzo-	X = O
(**10**)	R = 4,5-Dihydro-	X = S
(**11**)	R = Benzo-	X = Se
(**12**)	R = 4,5-Diphenyl-	X = O
(**6b**)	*p*-Toluenesulphonate salt of (**6a**)	

Compound	**"Size" of Chromatin**	**Fluorescence**	**Colour**	**Staining of Cytoplasm**	**Staining of Leucocytes**
(**6a**)	tiny	moderate	green	±	+ +
(**8**)	large	good	yellow-green	+	+ + +
(**11**)	moderate	moderate	green	±	+ +
(**6b**)	tiny	moderate	green	-ve	+ +
(**9**)	tiny	very poor	green	-ve	+ +
(**10**)	-ve	-ve	-ve	-ve	-ve
(**12**)	-ve	-ve	-ve	-ve	-ve
(**7**)	-ve	-ve	-ve	-ve	-ve

PUR-1 (**6a**) shows a tiny area of stained chromatin and a moderate fluorescence. The PUR-1 molecule consists of two parts, the purine portion which is mainly involved in the complexation with the DNA and the benzothiazole part which provides not only the anchoring to the phosphate of the DNA, but also extends the π-electrons system which characterise the molecule in terms of electronic properties. PUR-1 (**6a**) is almost planar (dihedral angle 0.05°).

The extensive π-electron delocalisation is possible throughout the molecule which allows the π-π complexation with the purine rich areas of the DNA and which enhances the fluorescence efficiency. In summary, both parts of the molecule play important roles in the staining and visualisation of the parasites (see **Photograph 2**). The second compound listed in **Table 4**, cyanine dye (**8**), showed a large chromatin area with good fluorescence. Like PUR-1 the molecule is planar as shown in **Table 2**. This probably results in the enhancement of the structural and electronic properties of the compound in ways which provide a better dye-nucleic acid interaction. The presence of three fused rings in the heterocyclic part of the molecule provides more π-electrons resulting in enhancement both in the DNA-binding ability and in the fluorescence efficiency. The large area of chromatin revealed and the increase in the fluorescence are shown in **Photograph 3**.

The selenium analogue of PUR-1 (**11**), as might be expected the effectiveness of this dye in the diagnosis of malaria is very similar to that shown by PUR-1. There is a small increase in the area of the chromatin probably because the change of the heteroatom from sulphur to selenium.

The cyanine dye (**6b**) is the *p*-toluenesulphonate salt corresponding to PUR-1 and as expected its behaviour is identical to that of PUR-1. It was synthesised in order to improve the solubility and thus the applicability of the dye.

The oxygen analogue of PUR-1 (**9a**), is planar (see **Table 2**). However its effectiveness as a malaria diagnostic is very much reduced due to the electronic properties of the oxygen in the benzoxazole part of the molecule. The substitution of oxygen for sulphur changes the delocalisation of π-electrons through the molecule and therefore alters both the binding properties of the purine part and also the efficiency of fluorescence. The cyanine dye (**10**) differs from PUR-1 in having a dihydrothiazole system in place of a benzothiazole. It is completely ineffective as a diagnostic stain. The dye is nearly planar, the dihedral angle between the two ring systems being only 6°, but its fluorescence is negligible because of the reduction in the size of the π-electron system. The remaining dyes listed in **Table 4**, (**12**) and (**7**), also proved ineffective as malaria diagnostics in both cases because of the non-coplanarity of the molecules (dihedral angles being 20° and 50° respectively). This affects the degree of π-electron delocalisation throughout the molecules which reduces the binding properties to the DNA and also the fluorescence efficiency. The non-coplanarity will also hinder the intercalation of the molecules between the base pairs in the DNA.

Range of Applications of PUR-1 and its Analogues in DNA Recognition

Although Romanowsky-type stains are more suitable for morphology and species characterisation, fluorescent stains such as PUR-1 and its analogues, in particular dye (**8**), offer a sensitive and rapid method for the examination of both thick and thin blood films in malaria diagnosis.

The new dye (**8**) has several advantages over PUR-1. In the diagnosis of low parasitaemias (down to 0.001%) thin film morphology was sharper (see **Photograph 4**) and thick films were more sensitive. Leucocytes were not confused with malaria parasites and there was no obvious interference by non-parasitic material such as precipitated stain at the dilution employed. Distinctive features of asexual and sexual stages of *Plasmodium* species other than *P. falciparum* were discernible.

The use of the dye (**8**) with dried thick blood films allows the collection of samples in the field and their examination at a base laboratory, so eliminating some of the problems inherent in other fluorescent methods which require more rapid inspection of samples or suffer from precipitation of the fluorescent dye at high temperature or high humidity. Thick blood films which have been stored in Parafilm® at -20° C for one year also showed good fluorescence. The stability of stained slides was good and fluorescence remained visible in preparations kept for more than 48 hours at 25° C. The glycerol/methanol dye solution has been stable at room temperature (22° C) and at 37° C for at least 5 months.

The staining of other parasites present in blood has been examined in order to check the applicability of the dye (**8**). Thin films of amoebae-infected blood showed the great affinity of the dye for the parasitic . This illustrates the broad extent of application of such dyes not only in parasitic diseases such as malaria, but also in other applications such as flow cytometry, DNA structural characterisation, chromosome fingerprints, ...etc.

EXPERIMENTAL

Methodology

The samples used were thick or thin blood smears of different parasitaemias and species which had been stored, but smears were also prepared, from fresh "peripheral" blood samples.

Thin films were produced by placing a small drop of blood on a grease-free microscope slide and using a second slide, held at 45°, to spread the blood smoothly and rapidly; the film was then air dried. A thick blood smear was prepared by spreading approximately 5 μl of blood over a 1 cm^2 area on a grease-free microscope slide and allowing the sample to dry in the air.

When Field's stain was used the blood smear was fixed by treatment with methanol for 1 minute, and the slide was dipped into undiluted Field's stain "A" for 3 seconds and rinsed with tap water. The slide was then dipped into undiluted Field's stain "B" for 3 seconds, washed gently in tap water to remove the excess of stain, drained vertically and allowed to dry for about 5 minutes, and finally examined under the microscope using an oil-immersion objective. When searching for malarial parasites the slides should be examined for at least 10 minutes before deciding whether they are negative or positive. For the second type of stain, PUR-1, the method uses a solution of the stain in methanol/glycerol (1:1) at a concentration of 500 μg/ml, equilibrated using a Spiramix® mixer for 5 min.. The stain (10 μl) was added to a thick or a thin smear covered with a cover slip (22 x 22 mm) and allowed to develop for two minutes.

Both types of stained films were examined in a Carl Zeiss Axiophot (with a 450-490 nm filter for the fluorescent stains) equipped with 10 x eyepiece and a 40 x objective.

Methods of Calculation in the Present Work

The calculations were performed using the quantum chemical program Hyperchem. The molecular mechanics method used was MM+[18] with the Polak-Ribiere algorithm for gradient minimisation. The semiempirical calculations were made with MNDO/PM3[19] in the restricted Hartree-Fock condition, again using the Polak-Ribiere algorithm.

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REFERENCES

1. Bruce-Chwatt, L. J. "Essential Malariology", second edition, Wlliam Heinemann Medical Books Ltd., 1980.
2. Dyadyusha, G. G.;Repyakh, I. V. and Kachkouskii, A. D. *Teor. Eksp. Khim.*, 1984, **20**, 398; 1985, **21**, 138; 1986, **22**, 347.
3. Radeglia, R. *J. Prakt. Chem.*, 1973, **315**, 1121.
4. Kleinpeter, E.; Borsdorf, R.; Bach, G. and Grossmann, J. V. *J. Prakt. Chem.*, 1973, **315**, 587.
5. Dewar, M. J. S. and Thiel, W. *J. Am. Chem. Soc.*, 1977, **99**, 4899.
6. Pople, J. A. and Beveridge, D. L. "Approximate Molecular Orbital Theory", McGraw-Hill, New York, 1970.
7. Kolesnikov, A. M. and Mikhailenko, F. A. *Usp. Khim.*, 1987, **56**, 466.
8. Nakao, K.; Yakeno, K.; Yoshioko, H. and Nakatsu, K. *Acta Cryst.*, 1979, **B35**, 415.
9. El-Maghraby, M. A.; Koraiem, A. J. M.; Kalil, Z. H. and Abu El-Hamed, R. M. *Indian J. Chem.*, 1987, **26B**, 52.
10. Oseroff, D. R.; Ohuoha, D.; Ara, G.; McAuliffe, D.; Foley, J. and Cincotta, L. *Proc. Natl. Acad. Sci. (USA)*, 1986, **83**, 9729.

11. Zigman, S. and Gilman, P. *SCI*, 1980, **208**, 188.
12. Watson, J. "Molecular Biology of Gene", Benjamin, California, 1970, 327, 355.
13. Girgis, M. M.; Yanni, A. S. and Khalil, Z. H. *Indian J. Chem.*, 1990, **29A**, 138.
14. Venkataraman, K. "The Chemistry of Synthetic Dyes", Vol. VII, Academic Press, New York, 1974, 336.
15. Bradley, D. F. and Wolf, M. K. *Proc. Natl. Acad. Sci. (U.S.A.)*, 1959, **45**, 944.
16. Duchesne, L. "Physico-Chemical Properties of Nucleic Acids", Vol. I, Academic Press, New York, 1973, 231.
17. Granharm, P. C. C. "Malaria Parasites and other Haemasporidia", Blackwell, Oxford, 1966, 1029.
18. Allinger, N. L. *J. Am. Chem. Soc.*, 1977, **99**, 8127.
19. Stewart, J. J. P. *J. Comp. Chem.*, 1989, **10**, 209; 1989, **10**, 221.

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