RESEARCH REPORT 1989

DEVELOPMENT OF A NEW STRATEGY FOR ANTI-MALARIAL DRUGS.

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A: CHEMICAL WORK.

This is the first report, presenting the results produced by the two partners in Thailand and Israel during the last year, within the joint project on new anti-malarials based on pyridoxal. The chemical research endeavour on behalf of the Thai partner is presented in two forms. One, in a concise manner, assembling the essential chemical data in table forms presented in tables 1-3; and two, in a more extended form submitted by Dr. Nipon Wongvisetsirikul, containing some 48 pages *(Appendix A). During the period of this report, the above concerned partners aimed at studying the synthesis, characterization, and structure determination of two new series of lipophilic chelators based on pyridoxal. The Thai partner engaged itself in the synthesis of 13 new ligands of the "acyl-hydrazone" type (I and III), along with some 7 iron(III) complexes of general formula IV, and two iron complexes of the "aryl-hydrazone" type represented by formula V and VI. The Israeli partner, on the other hand, addressed itself to synthesis, characterization, and crystal structure of the "aryl-hydrazone" type of chelators represented by general formulae VII, IX, and X, along with some metal complexes represented by formula VIII. Both partners utilized essentially the same synthetic approach for the formation of the two type of chelators as outlined in the research project, namely, condensation between pyridoxal hydrochloride with the appropriate hydrazine derivatives. The latter, in turn were either purchased or synthesized according to the literature.

The assignment of structure by the Thai partner followed from: (i) elementary analysis; (ii) spectral measurements in the ultraviolet range of the spectrum. The Israeli partner, in addition, addressed itself to ^{1}H- and ^{13}C-NMR spectroscopic measurements. When amenable, the crystals were subjected to single X-ray analysis, the results of which will be presented in a special forthcoming report.

Almost none of the compounds prepared by the Thai partner have as yet been tested biologically. However, all compounds included in the biological research on anti-malarial activities originating from Prof. Spira's laboratory in Jerusalem were produced by the Israeli partner at Prof. Sarel's laboratory. These comprise compounds No. 1,4,5,6,7,8,12,13,14,15, listed in pages 4-6. Testings on compound 5, bearing the code SAG-9 have already been reported.

B: BIOLOGICAL TESTING

In our previous report, we have shown that a group of pyridoxal based iron chelators were able to inhibit *in vitro* the growth of a chloroquine resistant malaria pasrasites *Plasmodium falciparum* (FCR-3) with an IC-50 of 10μM. In a 2h pulsing experiment, 20μM of one of the chelators - (SAG-9) achieved up to 80% growth inhibition. This and other related results have now been published in the Transactions of the Royal Society of Tropical Medicine and Hygiene *(Appendix B).

In the spectrophotometric analysis of SAG-9-Fe complex, we showed that under anoxic conditions, SAG-9 is capable of one electron reduction which is could lead to the generation of free radicals. In an electron spin resonance (ESR) analysis, we showed that free radicals could be monitored, presumably arising from the formation of a complex between SAG-9 and iron, followed by an internal one electron transfer to yield carbon cenyered free radicals. In a fluorimetric analysis of SAG-9-DNA complex, we found that the intrinsic fluorescence of SAG-9 was quenched by its interaction with DNA.

From our previous reports therefore, we deduced that SAG-9 was inhibiting the growth of parasites not by a simple iron withdrawal process but probably through the generation of highly reactive ligand- iron complexes (Carbon centered free radicals). This could account for its highly effective inhibition of parasite growth in 2 hours of pulsing.

To further confirm that our chelator wa not inhibiting the growth of parasites by a simple iron withdrawal mechanism, we investigated the effect of another iron chelator desferrioxamine (DFO) on the inhibitory potential of SAG-9. The results showed that the inhibitory effect of SAG-9 could be reversed by an equimolar concentration of DFO. This reversal action was observed only when the parasites were first suspended in DFO medium for 15 minutes followed by the addition of the chelator (ie SAG-9). If a mixture containing the pyridoxal based chelator and DFO was used in the incubation of parasites, no reversal activity of DFO was manifested or observed. This effect of DFO on the inhibitory action of SAG-9 gives further proof that SAG-9 is acting via a free radical mediated pathway. DFO inhibits the formation of reactive oxygen intermediates such as hydroxyl and superoxide radicals by chelating the available iron. Since DFO binds iron more avidly than SAG-9, it therefore protected the parasite against SAG-9 induced damage, indicating the requirement for iron.

To further understand the mode of action of our chelators, we checked their effects on two major sites of free radical mediated damage - the DNA and the membrane. To check the effect on the parasite DNA, we used both the Nick translation method and Electrophoretic analysis.

a). <u>Nick Translation:</u> The method for radiolabelling DNA using the Nick translation reaction is based on a study by Kelly et al. (1970). These investigators

reported that before chain copying can occur using purified polymerase 1, scissionsor nicks have to be introduced into a single strand. Nicks can be introduced into a DNA molecule via a free radical mediated reaction.

100μg calf thymus DNA (CT DNA) was suspended in a freshly prepared solution of SAG-9-Fe(II) complex (200μl), and left to stand for 16 hours. Thereafter, the DNA was re-extracted and dissolved in double distilled water to give a final DNA concentration of 0.25μg/ml. The control was an equal amount of DNA suspended in buffer for the same lenght of time and re-extracted in the same way.

When the Nick translation wa performed, the results showed that SAG-9-Fe(II) introduced nicks in the DNA which resulted in the incorporation of dCTP into the chelator treated DNA three fold more than in the untreated. Another portion of the CT DNA treated with restriction enzymes incorporated dCTP only twice as much as the chelator treated.

Electrophoretic Analysis: Due to the large size and the complex problems involved in dealing with eukaryotic DNA, smaller and simpler DNAs were used for this analysis. To study SAG-9 induced radical effect on DNA, we used lambda phage DNA (λ DNA) and a plasmid - pBR322.

i) Lambda DNA was incubated in a freshly prepared chelator - iron complex for 2 hours. The treated DNA was then applied to an agarose gel and electrophoresed (final concentration of DNA was 0.015μg). The result showed that the chelator - iron complex caused breaks in the DNA molecule.

Using plasmid DNA: A single nick introduced in a supercoiled plasmid relaxes it. This is a sharp indicator of single strand breaks in a DNA molecule which could otherwise not be seen when a larger DNA molecule is used. We incubated supercoiled plasmids in 40μM chelator Fe(II) or Fe(III) complexes for 2 hours. Thereafter, electrophoretic analysis was performed. The results showed that only the chelator - Fe(II) treated plasmid was relaxed, indicating the intrduction of nicks. Chelator - Fe(III) treated plasmid was not relaxed.

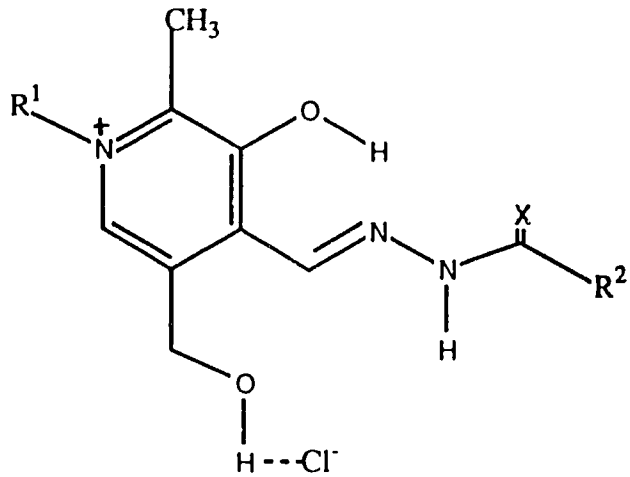
These results showed that chelator - Fe(II) complex induces a reaction in DNA which results in nicks or breaks in the molecule. These nicks or breaks can not be induced by chelator - Fe(III) complex. This further corroborates our findings using the ESR, where we showed that a six line ESR spectrum indicative of a carbon centered spin adduct was obtained only when chelator - Fe(II) but not chelator - Fe(III) complex was used.

We have investigated the effect of our chelators on the membrane proteins of parasitized erythrocytes using the more sensitive silver staining procedure. Highly synchronized cultures at the late trophozoic or schizont stages were suspended in 40μM chelator for 2 hours. Thereafter, the cells were washed with RPMI washing solution and

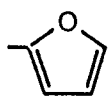
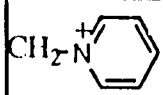
concentrated by gelatin sedimentation. The membranes were then extracted and the membrane proteins analyzed electrophoretically using the silver staining method.

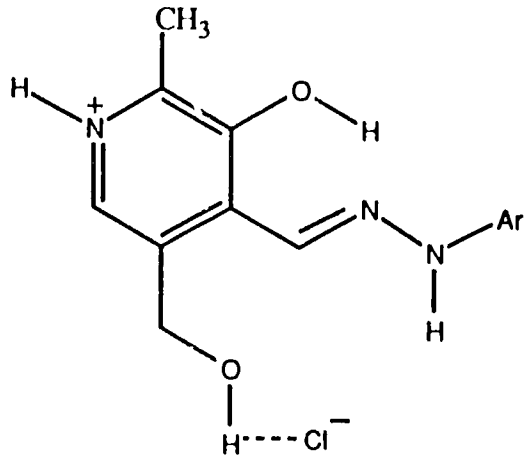
The result showed that 2 hours of pulsing of parasites caused no net change in the membrane proteins of the parasitized erythrocytes. Free radical mediated lipid peroxidation has been shown by many workers. We intend to perform analysis of the membrane lipids of our chelator treated parasitized erythrocytes. That would indeed show us if in addition to the effect on the parasite DNA, our chelator is affecting the parasitized erythrocyte membrane.

COMPOUNDS PREPARED BY THE THAI PARTNER: DR. NIPON WONGVISETSIRIKUI

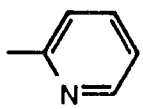
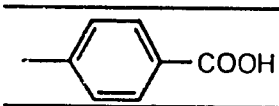
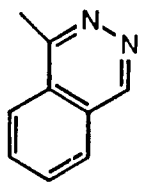


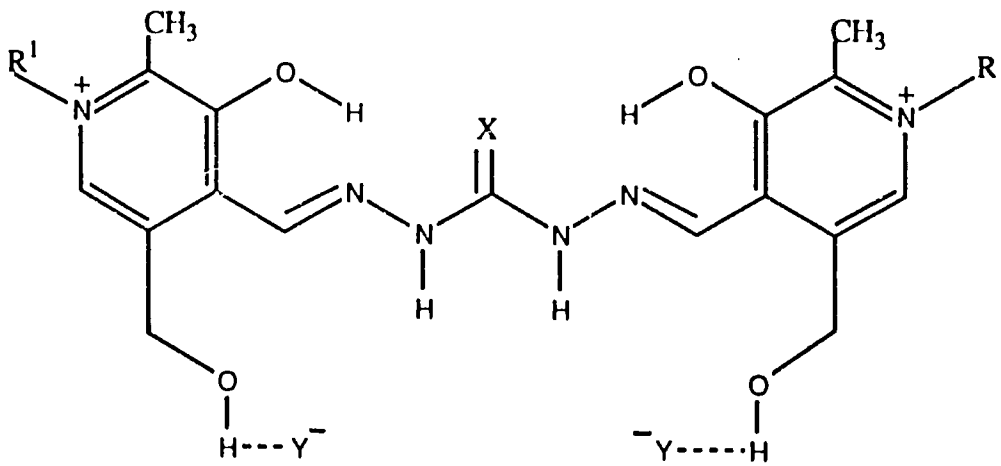
(I)

No	R^1	X	R^2	mp °C	λmax nm	Ir (KBr) cm^{-1}
1	H	O	NH_2	220-2	320, 285	3460, 3410, 3350, 3280, 3130, 3000, 2900, 2840, 1700.
2	H	S	NH_2	225-6	333	3420, 3340, 3280, 3220.
3	H	O	$NHNH_2$	207-9	335, 310	3480, 3250, 3140, 2600-2800, 1740, 1615.
4	H	S	$NHNH_2$	230-2	440, 350 318	3400, 3320, 3150, 2700-2900.
5	H	O		219-21	340, 314 304	3450, 3250, 3150, 1680, 1660
6	H	O		206-8	334, 288	3050, 3100, 3250, 2650-2850, 1700.
7	CH_2 COONa	O	NH_2	220	-	3460, 3375, 3280, 3200-3150 2830-2900, 1680, 1690.



II

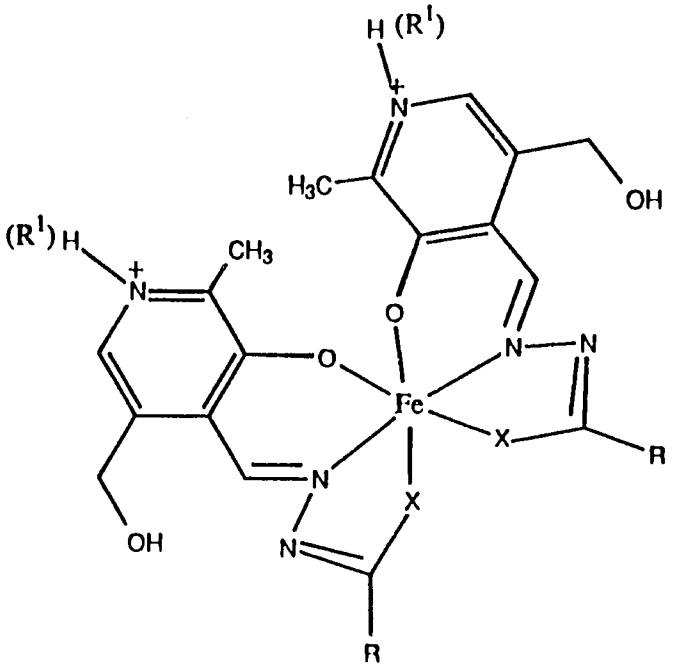
	Aryl	mp°C	λmax nm	IR (KBr) cm^{-1}
8		255-7	404, 358, 334, 310.	3320, 3220, 3140, 3050, 2650-2750, 1610, 1600.
9		275-7	375.	3430-3380, 3200, 3050, 3100, 2700, 1685, 1640.
10		245-7	420, 401, 386, 301, 283.	3350, 3100, 2850-2700, 1640, 1620, 1600.

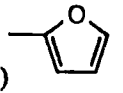
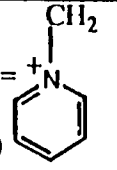


III

	R^1	X	Y	mp°C	λmax	IR (KBr) cm^{-1}
11	H	O	Cl	215-217	340, 312	3490, 3400-3200. 3120, 2600-2650 1950, 1850, 1730.
12	CH_3	O	I	215-217	-	3400-3220, 2700-2850, 1730.
13	H	S	Cl	225-227	440, 371 352, 323	3380, 3280, 2700-2880, 1620, 1600.

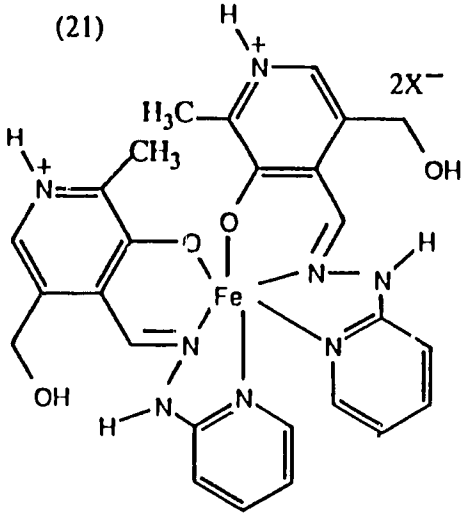
FORMULA IV: IRON COMPLEXES



14	X = O; R = NH_2 (λmax 442nm, 334, 298).	15	X = S; R = NH_2 (303nm)	16	X = O; R = $NHNH_2$ (346, 312, 297.)
17	X = S; R = $NHNH_2$ (386, 309nm)	18	X = O; R =  (304nm)	19	X = O; R =  (452, 356, 298, 258)

20. X = O; R= NH_2; R' = CH_2COOH
(438, 334, 290nm.)

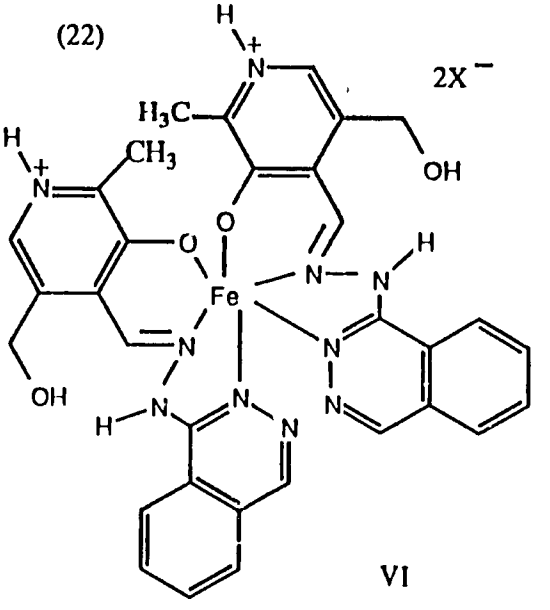
(21)



V

λmax 464, 350, 240nm

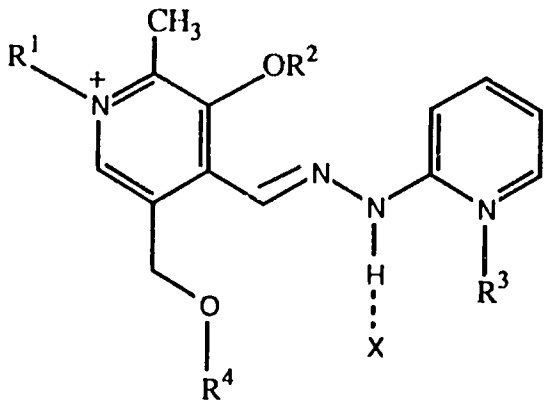
(22)



VI

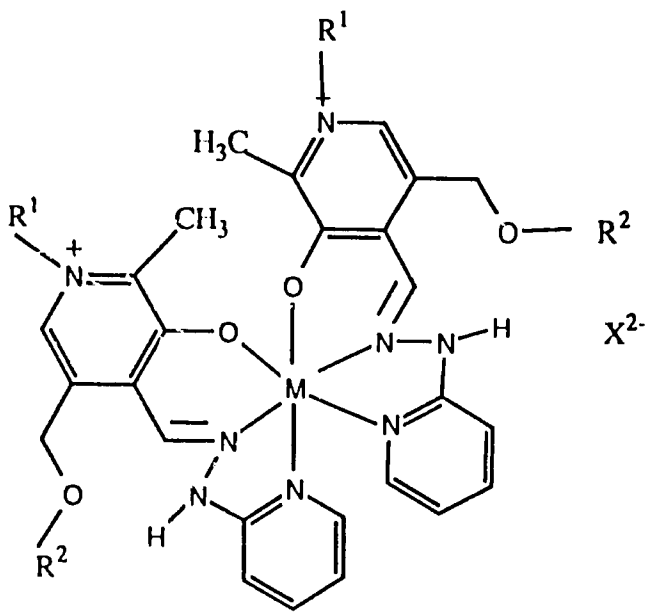
λmax 421, 376, 358nm

COMPOUNDS SYNTHESIZED IN JERUSALEM BY DRS. S. GRISARU AND S. SAREL



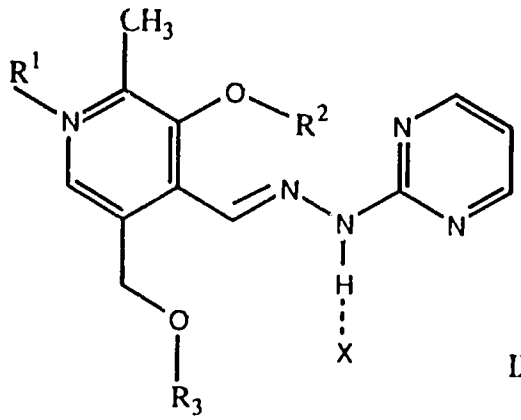
VII

No. ISR	R^1	R^2	R^3	R^4	X	mp °C	λmax nm	IR (KBr) cm^{-1}
1	H	H	H	H	Cl	298	376, 339, 240, 213.	3360, 3040, 2960, 2650.
2	CH_3	H	H	H	I	235-6	399, 339.	3300, 3186, 2895, 2600, 3411, 3403, 3390, 3323.
2a	CH_3	H	H	H	$OClO_3$	215		
3	CH_3	H	CH_3	H	I	276-7	467, 345, 339, 268	
3a	CH_3	H	CH_3	H	$OClO_3$	258-9	468, 341, 339, 268	
4	H	$COCH_3$	H	$COCH_3$	$OCOCH_3$	137-8	-	3060, 3015, 2930, 2850 1755, 1740.
5	$CH_2COOC_2H_5$	H	H	H	Br	202	407, 301, 242, 380	3290, 3150, 3015, 2990
5a	$CH_2COOC_2H_5$	H	H	H	$OClO_3$	253	390, 339, 299	



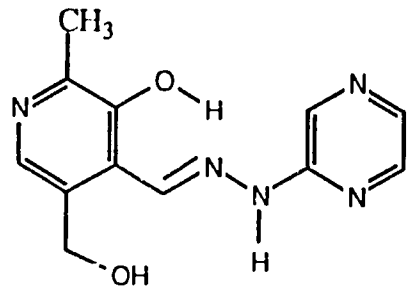
VIII

No. ISR	R^1	R^2	M	X	λmax,nm MeOH	
6	H	H	Cu	SO_4	467, 410, 338, 239	
7	H	$COCH_3$	Cu	SO_4		
8	$CH_2COOC_2H_5$	H	Cu	SO_4	478, 412, 344, 339, 242	
9	$CH_2COOC_2H_5$	H	Fe	SO_4	461, 344, 339, 225	
10	H	H	Fe	SO_4	454, 417, 342, 339	



IX

No	R^1	R^2	R^3	X	mp°C		
11		H	H		298	342, 340, 307, 232	3140-2800, 2300
12	$CH_2COOC_2H_5$	H	H	Br		385, 378, 339, 240	
13		H	$COCH_3$		270		3140, 2530, 2040, 1995, 1750
14	H	H	$COCH_3$	$OCOCH_3$	170		3210, 3160, 3000, 2990, 2940, 1735



X

15					288-90		