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Design, Synthesis, and Biological Evaluation of a Series of Simple and Novel Potential Antimalarial Compounds

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Abstract—A series of compounds bearing an endocyclic –N–O– moiety with potential antimalarial activity based on simple derivatives of the tropolone purpurogallin was prepared by means of a hetero Diels–Alder reaction using nitrosobenzene as a dienophile. The rationale behind the design of these compounds is presented, together with the synthetic route to derivatives bearing aromatic and aliphatic esters of the C4′-position hydroxyl group of the purpurogallin framework, as well as biological data obtained from in vitro assays against *Plasmodium falciparum* and *Trypanosoma cruzi*. Several of the new compounds have activities in the 3–9 μM range, and provide leads for the development of a novel class of antiparasitic drugs with improved biological and pharmacological properties.

Due to its high morbidity and mortality, human malaria is an infectious disease of enormous importance in tropical countries. Despite the eradication programs that started over 80 years ago, malaria is still a threat to over 2 billion people living in areas of high incidence. Although the statistics vary widely, it is estimated that there are 200 million infected humans, along with 150 million new cases every year. *Plasmodium falciparum*, the causative agent of the most malignant forms of malaria, is a particularly resistant parasite which is known to have high adaptability by mutation. This mutability makes quite likely the development of resistance to vaccines and chemotherapies currently being introduced.[1] Even for drugs with novel modes of action, such as artemisinin (Fig. 1a) and other natural and synthetic endoperoxides, there is a high likelihood that resistant Plasmodia strains will evolve. This would be especially problematic, because there is high probability that the mechanism of resistance will involve the inactivation of the endoperoxide moiety, thus rendering these strains resistant to drugs with similar chemical structure.

The trademark of artemisinin action has been proposed to be the interaction between its reactive endoperoxide bond with active iron(II) species present in the protozoan food vacuole, which catalyses the generation of highly cytotoxic oxygen- and carbon-centered radicals.[2] Although this has not been decisively proven to occur in the parasite, the hypothesis is strongly supported by results obtained from biomimetic studies.[3] We note that while the standard bond dissociation energy (BDE) of the peroxo linkage (–O–O–) is 33.2 kcal/mol, the corresponding BDE of the –N–O– linkage is 35.8 kcal/mol.[4] Although the BDEs for these bonds are likely to change when forming part of larger molecules, both moieties will remain highly labile and prone to undergo homolitic cleavage. Therefore, oxazines could follow a reaction pattern (i.e., –N–O– bond opening and radical generation), and thus biological activity, related to that of endoperoxides. However, the differences in chemical structure could make these compounds unaffected by the potential *Plasmodium* variants capable of inactivating endoperoxides which are likely to appear. In this communication we report the design, preparation, and biological evaluation of a series of such compounds (Fig. 1b).

A facile route for the preparation of oxazines involves a hetero Diels–Alder reaction between an appropriate diene and nitrosobenzene derivatives. The procedure has been extensively documented in the literature and can be applied to a large range of diene-containing frameworks,[5] and was therefore ideal for our purposes.

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For our exploratory series of compounds, we chose nitrosobenzene as the source for the –N–O– functionality, and derivatives of the benzotropolone purpurogallin as dienes. These compounds are simple to prepare and purify,[6] and have been previously employed as Diels–Alder dienes in several studies.[7] Starting from trimethylpurpurogallin (**1**), 10 aliphatic and aromatic esters of the C4′-position hydroxyl group were prepared in good yield by treatment of a CH_2Cl_2 solution of the benzotropolone with the appropriate acyl chloride, triethylamine, and catalytic DMAP (**2a**–**2j**, Fig. 2).

The resulting esters were then treated with a slight excess of nitrosobenzene at room temperature using benzene as solvent. The Diels–Alder reaction proceeded cleanly, with the products crystallizing out of solution (**3a**–**3j**, Fig. 2).[8] In all cases, the starting dienes were undetectable after 16 h. The Diels–Alder adducts of **1** and of the simple ether tetramethylpurpurogallin (**2k**) were also included in the series (**3k** and **3l**), and prepared following the same protocol employed for compounds **3a**–**3j**. The regioselectivity of the hetero Diels–Alder reaction was first established to correspond to that shown in Figure 2 from the ^1H and ^{13}C NMR spectra of the products, and this was later confirmed by X-ray diffraction studies performed on oxazine **3l**.[9]

The in vitro biological activity of the new compounds was assayed against the protozoan parasites *P. falciparum* and *Trypanasoma cruzi*, and their toxicity evaluated against KB cells.[10] The results are summarized in Table 1. Despite the fact that all the compounds are at least three orders of magnitude less active than chloroquine in the *P. falciparum* assay, most of them still have activities in the micromolar concentration range. On the other hand, oxazines **3a**, **3f**, **3j**, and **3k** exhibited activities comparable to benznidazole, one of the current chemotherapies against *T. cruzi*.

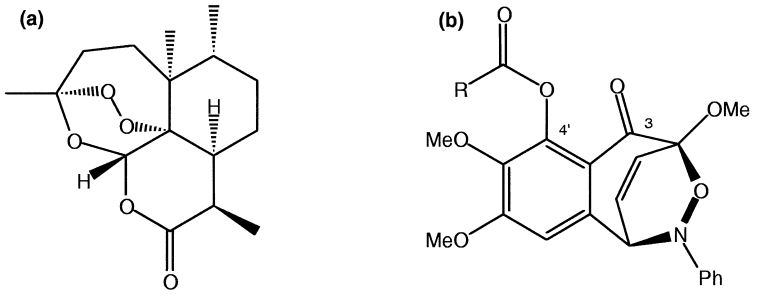

Figure 1. Chemical structures of artemisinin (a) and oxazines with antiprotozoal activity (b).

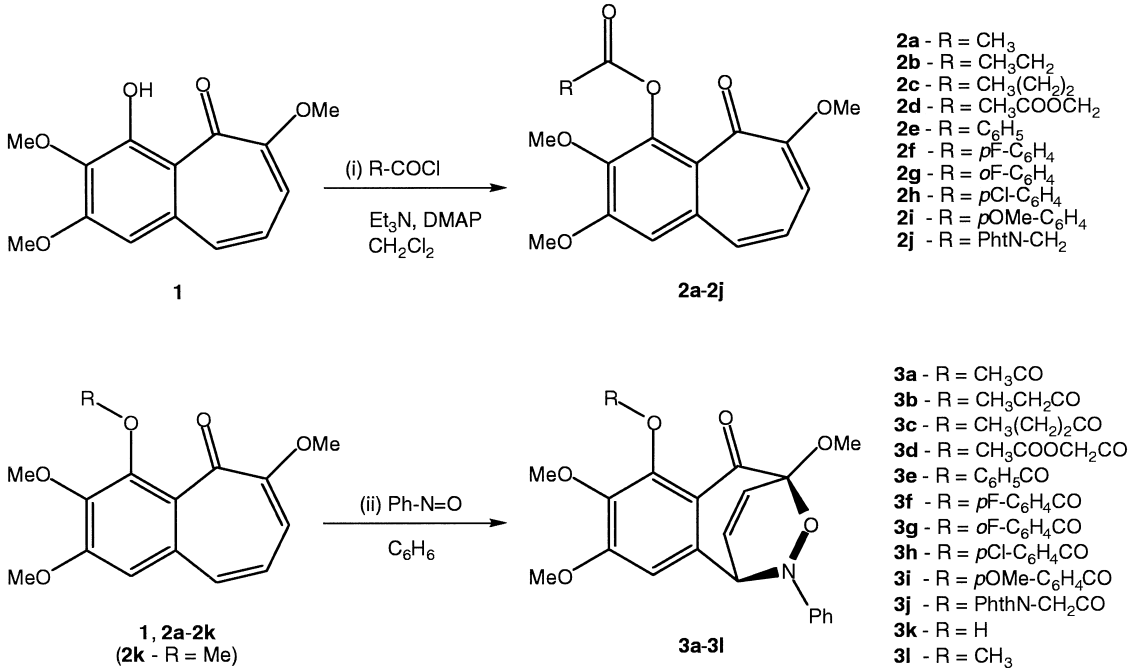

Figure 2. Route to oxazines **3a**–**3l**. (i) R-COCl (1.5 equiv), Et_3N (1.5 equiv), DMAP (0.5 equiv), CH_2Cl_2, rt, 4 to 8 h; (ii) Ph–N=O (1.5 equiv), benzene, 16 h. PhthN represents the phthalimide group (**3j**).

Table 1. In vitro antiprotozoal activity of oxazines **3a**–**3l** against *P. falciparum* (PF) and *T. cruzi* (TC)

Compound	PF (μM)	TC (μM)	Toxicity (μM)
3a	5.3	23.8	6.3
3b	6.6	>70.5	<0.7
3c	3.9	>68.3	3.2
3d	3.2	>63.9	128.2
3e	3.4	>63.4	11.8
3f	7.5	9.2	6.5
3g	6.3	>61.0	7.7
3h	>59.0	>59.0	29.1
3i	6.6	>59.6	13.3
3j	6.1	11.9	89.8
3k	5.4	4.3	<0.8
3l	8.9	65.3	<0.8

Chloroquine (IC_{50} 3.0 nM) and benznidazole (ED_{50} 7.1 μM) were used as positives for *P. falciparum* and *T. cruzi*, respectively. Toxicity was assayed against KB cells, using podophyllotoxin (ED_{50} 0.2 nM) as a standard. All determinations were performed in triplicate.

Several of the compounds display relatively high toxicities in the KB cell assay. This can be associated with their somewhat low thermal stability and propensity to liberate highly cytotoxic nitrosobenzene upon undergoing a retro Diels–Alder reaction. The kinetics of the decomposition process have been studied in detail for oxazine **3l**, for which a half-life of approximately 10 h at 30 °C in chloroform solution has been determined.[9] This could explain why compound **3k**, for example, which has high activity against both *P. falciparum* and *T. cruzi* also shows one of the highest toxicities of the series. However, several of the compounds with the highest activities have low or moderate toxicities. This is especially true for compounds **3d** and **3j**, and seems to indicate that activity is independent from toxicity. Of these, oxazine **3d** is particularly interesting. This compound has the lowest toxicity of the series, one of the poorest activities against *T. cruzi*, and the highest activity against *P. falciparum*, indicating specificity against this parasite. It is tempting to speculate that this compound behaves as a prodrug in which the acetoxy group initially confers high permeability through the membrane, and is later hydrolyzed in the acidic food vacuole to a free hydroxyl group that prevents its escape out of the parasite. A similar absorption mechanism has been postulated for chloroquine and other aminoquinoline derivatives.[11] The poor activity in all assays and relatively low toxicity of the *p*Cl-benzoate derivative **3h** can also be rationalized in terms of its poor bioavailability. This compound has very low solubility in both polar and nonpolar solvents, which can be extrapolated to low membrane permeability.

Additional studies to explain in more detail the observations described above are currently in progress. First, kinetic studies of the decomposition rates of all the compounds in different media are being performed, and these will be correlated with their biological properties. Second, since one of the principal driving forces of the retro Diels–Alder reaction responsible for the release of nitrosobenzene is the return to stable pseudo-aromatic starting materials, tropolone derivatives with reduced or absent ketones at the C3-position will be employed with the goal of reducing the toxicity of the series. Finally, a CoMFA study correlating electrostatic and steric contributions to the activity and toxicity of the compounds in the series is also underway. We are hopeful that these additional studies will allow us to identify the structural traits responsible for activity against different parasites, and will ultimately lead us to the development of effective antiparasitic chemotherapeutic agents with novel chemical structures. The results from these investigations will be reported in due course.

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8. Compounds **3a**–**3l** were fully characterized by IR, MS, and ^{1}H and ^{13}C NMR. For example: Oxazine **3a**: mp 140–142 °C (dec.). ^{1}H NMR δ 2.41 (s, 3H), 3.64 (s, 3H), 3.81 (s, 3H), 3.90 (s, 3H), 5.31 (dd, J=1.0, 6.8 Hz, 1H), 6.12 (dd, J=1.0, 9.6 Hz, 1H), 6.72 (dd, J=6.8, 9.6 Hz, 1H), 6.78 (s, 1H), 7.17 (m, 5H); ^{13}C NMR δ 21.3, 53.5, 56.9, 61.8, 69.3, 103.1, 111.7, 118.9, 122.2, 124.1, 127.4, 129.9, 136.5, 138.6, 143.4, 147.5, 151.3, 157.4, 170.4, 189.9. ESI-MS: 413.3 (M+H). **Oxazine 3d**: mp 154–155 °C (dec.). ^{1}H NMR δ 2.19 (s, 3H), 3.64 (s, 3H), 3.82 (s, 3H), 3.90 (s, 3H), 5.06 (s, 2H), 5.34 (dd, J=0.9, 6.6 Hz, 1H), 6.10 (dd, J=0.9, 9.3 Hz, 1H), 6.73 (dd, J=6.6, 9.3 Hz, 1H), 6.80 (s, 1H), 7.21 (m, 5H); ^{13}C NMR δ 20.0, 52.6, 56.0, 60.7, 61.1, 68.2, 102.1, 111.3, 118.1, 120.8, 123.2, 126.5, 129.0, 135.8, 138.0, 142.6, 146.1, 150.3, 156.8, 166.8, 170.6, 189.1. ESI-MS: 470.5 (M+H). **Oxazine 3k**: mp 129–131 °C (dec.). ^{1}H NMR δ 3.73 (s, 3H), 3.87 (s, 3H), 3.93 (s, 3H), 5.32 (dd, J=1.0, 6.7 Hz, 1H), 6.19 (dd, J=1.0, 9.5 Hz, 1H), 6.77 (dd, J=6.7, 9.5 Hz, 1H), 6.54 (s, 1H), 7.17 (m, 5H), 12.29 (s, 1H); ^{13}C NMR δ 52.6, 56.0, 60.5, 68.3, 101.1, 105.8, 109.4, 118.1, 123.4, 126.6, 129.1, 134.4, 135.7, 138.1, 150.3, 157.5, 159.8, 196.3. ESI-MS: 369.9 (M+H). **Oxazine 3l**: mp 105–107 °C

(dec.). ^{1}H NMR δ 3.69 (s, 3H), 3.86 (s, 3H), 3.91 (s, 3H), 3.93 (s, 3H), 5.35 (dd, $J = 0.8$, 6.7 Hz, 1H), 6.17 (dd, $J = 0.8$, 9.3 Hz, 1H), 6.64 (dd, $J = 6.7$, 9.3 Hz, 1H), 6.77 (s, 1H), 7.14 (m, 5H); ^{13}C NMR δ 52.7, 55.9, 60.8, 61.5, 68.7, 102.1, 108.7, 117.6, 120.6, 122.7, 126.9, 128.6, 135.2, 136.2, 143.4, 150.2, 155.8, 156.1, 189.6. ESI-MS: 384.2 (M + H). All NMR spectra were recorded in $CDCl_3$. Chemical shifts (δ) are in ppm and coupling constants (J) in hertz.

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