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Synthesis of three classes of rhodacyanine dyes and evaluation of their in vitro and in vivo antimalarial activity

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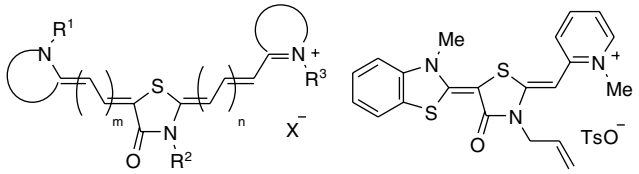
Abstract—Selected members of three classes of rhodacyanine dyes, [0,0]-, [1,0]-, and [0,0,0]-rhodacyanines, were synthesized and their in vitro antimalarial activities against *Plasmodium falciparum* K1 (chloroquine-resistant strain) as well as their in vivo activities against *P. berghei* in mice were determined. The novel [0,0,0]-rhodacynines, **3e** and **3h**, possessing a benzothiazole moiety, were shown to have highly promising antimalarial activities in vivo. Moreover, the [0,0,0]-rhodacyanines were found to be orally bioavailable.

1. Introduction

Malaria is one of the most serious infectious diseases in tropical and subtropical regions. At least 300 million people are afflicted and 1–3 million people die from this disease annually. Another serious issue is that malaria parasites develop resistance to clinically used chemotherapeutic agents such as chloroquine, mefloquine, and pyrimethamine.[1–3] Therefore, a very urgent need exists to develop new classes of antimalarial drugs that operate by novel mechanism of actions.[4–7]

Recently, we reported that rhodacyanine dyes **1**, designed by using the DLC (π-delocalized lipophilic cation) hypothesis,[8] exhibit potent in vitro antimalarial activity against *Plasmodium falciparum* FCR-3 strain (chloroquine-sensitive).[9,10] We also reported that some DLCs in the rhodacyanine family display antimalarial[11–13] and antileishmanial activities.[13] An in vitro structure–activity relationship (SAR) investigation showed that the rhodacyanine MKH-57 (**1a**) possesses high antimalarial activity (EC_{50} = 12 nM) and significantly selective toxicity (1000).[11] We also demonstrated that the tricyclic skeleton of the rhodacyanine is required for activity and a balance between molecular hydrophilicity and hydrophobicity is important for efficacy.

The concept of DLC was originally proposed by Chen in conjunction with the anticancer research efforts.[8] Subsequently, it was reported that several DLC compounds exhibit selective antitumor activity connected with their selective accumulation in mitochondria of carcinoma cells.[15–18] The structures of members of the rhodacyanine family, shown in Figure 1, are comprised of three, linearly linked heterocycles, in which the two end



general structure of rhodacyanine MKH-57 (**1a**)

Figure 1. General structures of [*m*,*n*]-rhodacyanines and MKH-57 (**1a**).

Keywords: Malaria; Rhodacyanines; DLC hypothesis; Drug resistance.
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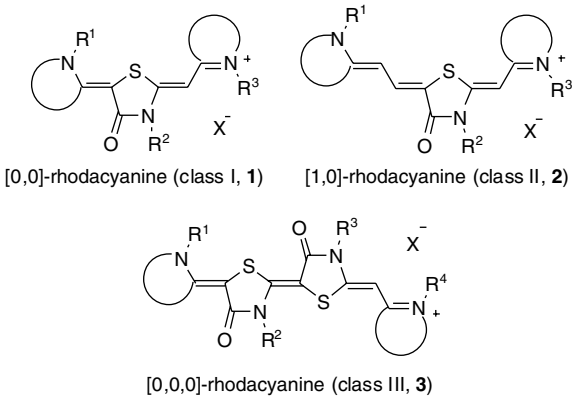
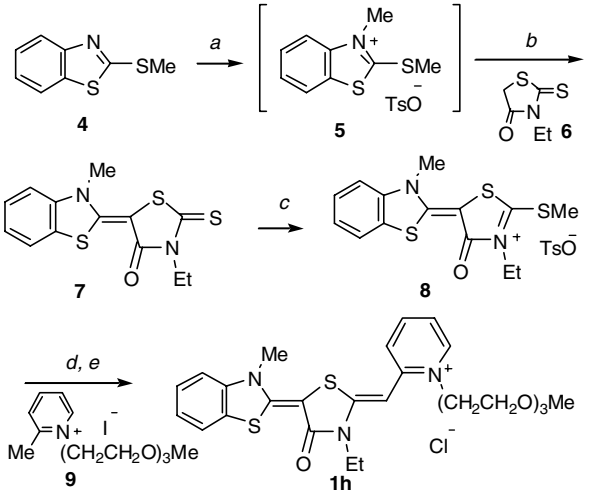


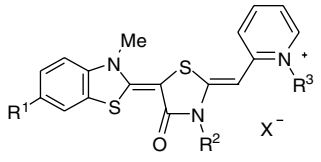
Figure 2. Rhodacyanines probed in this study include [0,0]-rhodacyanine (class I, **1**), [1,0]-rhodacyanine (class II, **2**), and [0,0,0]-rhodacyanine (class III, **3**).

heteroaromatic rings (**A** and **C**) are fused to a central rhodanine (4-oxothiazolidine) ring (**B**). These dyes are conjugates of two different dye units, a neutral merocyanine and a cationic cyanine moiety. Consequently, rhodacyanine compounds can be classified as π-delocalized lipophilic cations (DLCs).

Prior to our efforts in this area, there was no reports describing the in vivo antimalarial activity of rhodacyanines.[19] Thus, the novel and outstanding pharmacological profiles displayed by rhodacyanine MKH-57 (**1a**) prompted us to conduct a broad screen of the in vivo activity of a series of structurally related rhodacyanine dyes. Below, we describe the synthesis of selected members of the three rhodacyanine classes [0,0]-, [1,0]-, and [0,0,0]-rhodacyanines (Fig. 2), and present the results of an evaluation of their in vitro antimalarial activity against chloroquine-resistant strains of *P. falciparum* K1 and in vivo antimalarial activity against *P. berghei* in mice.



Scheme 1. Synthetic route for preparation of [0,0]-rhodacyanines **1** (class I). Reagents and conditions: (a) methyl *p*-toluenesulfonate, anisole, 125 °C; (b) NEt_3, **6**, MeCN, 0 °C to rt; (c) methyl *p*-toluenesulfonate, DMF, 130 °C; (d) NEt_3, **9**, MeCN, 70 °C; (e) Amberlyte® IRA-400(Cl^-).



	R^1	R^2	R^3	X
1a	H	$CH_2CH{=}CH_2$	Me	TsO
1b	H	Et	Et	Cl
1c	H	CH_2CO_2Me	Me	Cl
1d	H	CH_2CO_2H	Me	Cl
1e	H	Et	CH_2CH_2OH	Br
1f	H	$CH_2CH{=}CH_2$	CH_2CH_2OH	Br
1g	H	Et	$(CH_2CH_2O)_3H$	Cl
1h	H	Et	$(CH_2CH_2O)_3Me$	Cl
1i	H	Et	$CH_2C_6H_4$-*p*-CO_2H	Cl
1j	$NHCO(CH_2)_4CO_2Me$	Et	Me	Cl

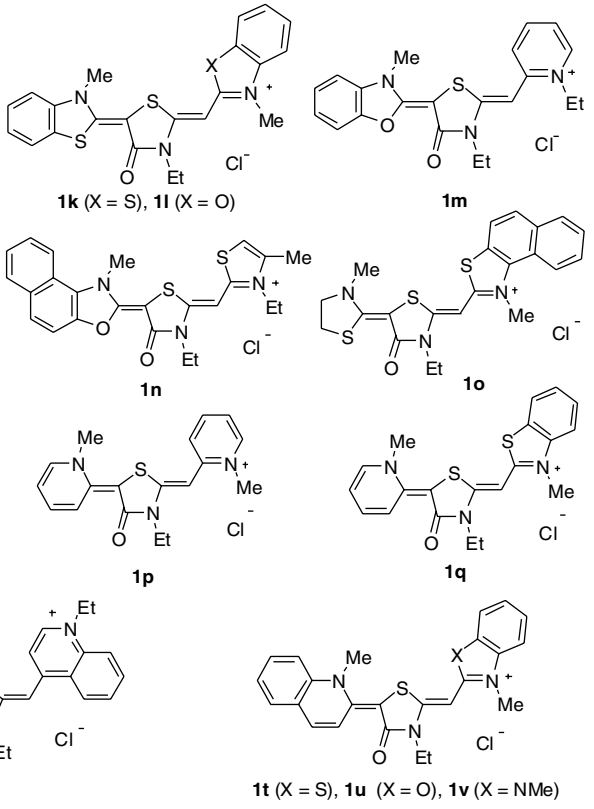
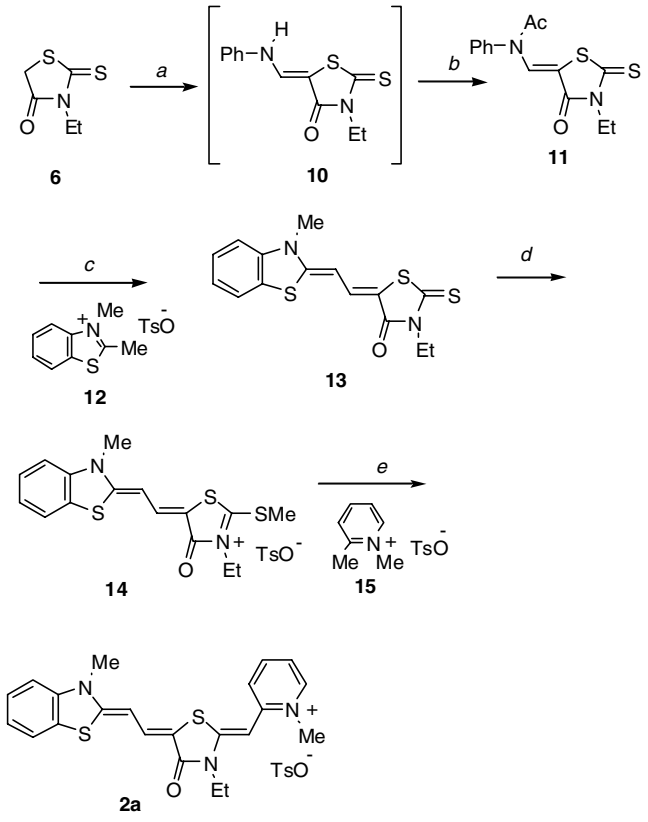


Chart 1. [0,0]-Rhodacyanines **1a**–**1v**.



Scheme 2. Synthetic route for preparation of [1,0]-rhodacyanines **2** (class II). Reagents and conditions: (a) PhN=CHNHPh, MeCN, 70 °C; (b) Ac_2O, NEt_3, 110 °C; (c) NEt_3, **12**, MeCN, 60 °C; (d) methyl *p*-toluenesulfonate, DMF, 110 °C; (e) NEt_3, **15**, MeCN, 75 °C.

2. Results and discussion

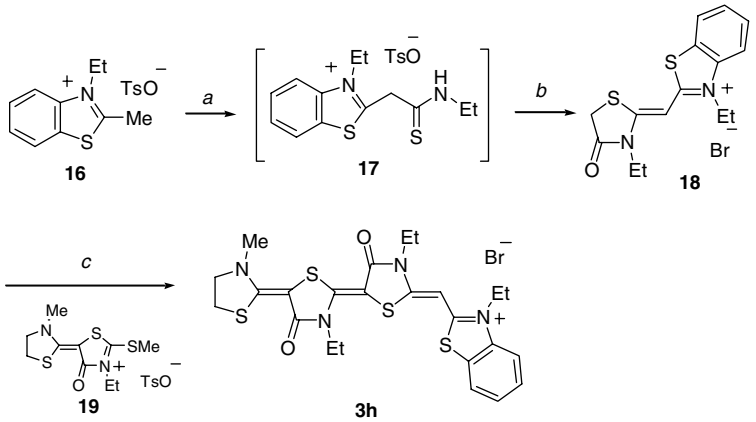
2.1. Synthesis of the rhodacyanines

The rhodacyanine dyes probed in this effort are categorized into three classes in the following manner, [0,0]-rhodacyanine (class I), [1,0]-rhodacyanine (class II), and [0,0,0]-rhodacyanines (class III) (Fig. 2). Dyes in classes I and II are distinguished by the length of the methine linkage between the heterocyclic A-rings and rhodanine B-rings. In class I, the A-ring is directly conjugated to the rhodanine by double bond and, in class II, the A-ring is tethered to the rhodanine by two methine carbons. The class III [0,0,0]-rhodacyanines are unique in that they possess two linked rhodanine moieties in the center of the skeleton.

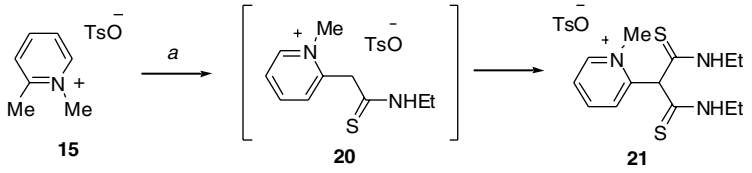
More than 100 different [0,0]-rhodacyanines, including **1a**–**1v** shown in Chart 1, were prepared by using the previously reported procedure.[11,16] The routes used are exemplified in Scheme 1 for the synthesis of **1h**. Methylthiobenzothiazolium salt (**5**), obtained by N-methylation of methylthiobenzothiazole (**4**) with methyl *p*-toluenesulfonate, was condensed with ethylrhodanine (**6**) to afford merocyanine **7**. In order to enhance the electrophilicity of thiocarbonyl group, S-methylation of **7** was carried out with methyl *p*-toluenesulfonate to afford thioiminium salt **8**. Treatment of **8** with picolinium salt **9** in the presence of triethylamine at 70 °C followed by ion exchange using Amberlyte® IRA-400 (Cl^-) gives the desired rhodacyanine dye **1h**.[20]

The class II [1,0]-rhodacyanines **2a**–**2g** were synthesized by using a modification of the general procedure.[15] An example of the routes used is shown in Scheme 2 for the synthesis of **2a**. Rhodanine **6** is reacted with *N*,*N*′-diphenylformamidine to give **10**, which without purification is treated with acetic anhydride to produce acetamide **11**. Subsequent reaction of **11** with *N*-methyl methylbenzothiazolium salt (**12**) furnishes merocyanine **13**, a vinylog of merocyanine **7**. Cation **14**, obtained by S-methylation of **13** with methyl *p*-toluenesulfonate, is condensed with picolinium salt **15** to afford the desired [1,0]-rhodacyanine **2a**.

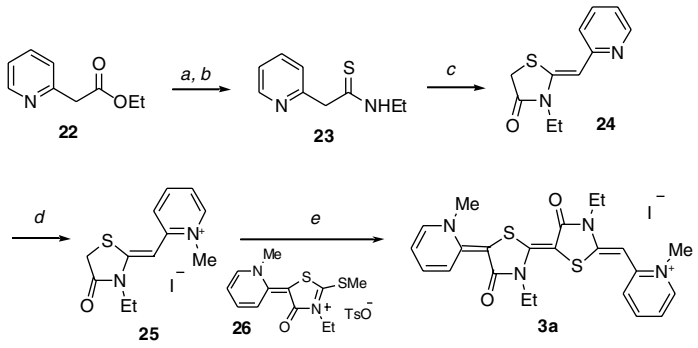
A possible method for synthesis of the class III [0,0,0]-rhodacyanines involves condensation of a cyanine derivative, as a right half segment consisting of two heterocycles (Fig. 2, class III), with a merocyanine derivative (as a left half segment). By using this strategy, the synthesis of cyanine **18**[15] was accomplished by a route involving reaction of benzothiazolium salt **16** with ethyl isothiocyanate, followed by treatment with bromoacetic acid. Following the same procedure used for the synthesis of class I and II rhodacyanines, **18** is condensed with **19** to furnish **3h** (Scheme 3). However, the above method



Scheme 3. Synthetic route for preparation of [0,0,0]-rhodacyanines bearing benzothiazolium moiety at the right edge. Reagents and conditions: (a) ethyl isothiocyanate, NEt_3, pyridine, 110 °C; (b) bromoacetic acid, AcOH, 90 °C; (c) NEt_3, **19**, MeCN, 70 °C.



Scheme 4. Formation of bis(thioamide) **21**. Reagents and conditions: (a) ethyl isothiocyanate, NEt_3, pyridine, 110 °C.



Scheme 5. Synthetic route for preparation of [0,0,0]-rhodacyanines bearing a pyridinium moiety at the right edge. Reagents and conditions: (a) $EtNH_2$, MeOH, −78 °C to rt; (b) Lawesson's reagent, toluene, 115 °C; (c) bromoacetyl chloride, CH_2Cl_2, rt; (d) MeI, acetone, 60 °C; (e) NEt_3, **26**, MeCN, 70 °C.

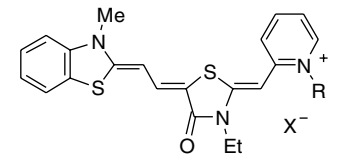
cannot be employed to prepare [0,0,0]-rhodacyanines that possess a pyridinium moiety at the right edge. This is due to the fact that reaction of picolinium cations, such as **15**, with isothiocyanate affords only bis-thioamides (e.g., **21**) by secondary reaction of the initially formed and more highly acidic thioamides **20** with isothiocyanate (Scheme 4). To reduce the acidity of the methylene protons, the non-ionic intermediate **23** was synthesized from ethyl pyridylacetate (**22**) by transamidation[21] and successive thioamidation using Lawesson's reagent.[22] Thioamide **23** was then treated with bromoacetyl chloride followed by quaternarization of the resulting product **24** to provide the target cyanine **25**. Finally, condensation of **25** with **26** yields the [0,0,0]-rhodacyanine **3a** (Scheme 5).

2.2. Biological results

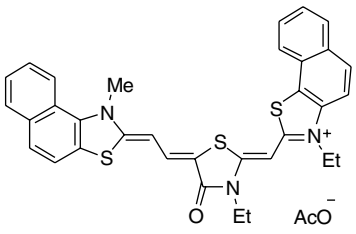
The in vitro antimalarial activities of the rhodacyanines against *P. falciparum* K1 (chloroquine-resistant strain) were evaluated by using the procedures described by Desjardins and his co-workers.[23] The cytotoxicities of these compounds to rat skeletal myoblast L-6 cells were also measured and selective toxicities, defined by the ratio EC_{50} (L-6)/EC_{50} (*P. falciparum*), were determined. The synthetic rhodacyanines were also subjected to in vivo antimalarial screening against *P. berghei* NK-65 (drug-sensitive strain) in mice. The in vivo assays were performed by using the Peters' 4-day suppressive test protocol.[24] The suppression (expressed as a percentage) of malaria protozoa is determined by comparing parasitemia of infected mice that are administered the compounds by injection for 4 days with those of untreated control mice (Chart 1–3).

2.2.1. Class I [0,0]-rhodacyanines. The antimalarial potencies of the class I [0,0]-rhodacyanines are summarized in Table 1. All compounds in this group show promising in vitro antimalarial activities, having EC_{50} values <100 nM and low cytotoxicity levels (with the exception of compounds **1l** and **1s**). As a result, the selectivity indices of these substances are estimated to be quite high. The inhibitory effects of the class I [0,0]-rhodacyanines against K1 strain of *P. falciparum* (chloroquine-resistant) parallel those against the chloroquine-sensitive FCR-3 strain, as described in our previous reports.[11,12] For example, the respective EC_{50} values for **1a** (MKH-57) against FCR-3 strain and K1 strain are 12 and 19 nM. In contrast, chloroquine shows a 80-fold lower activity against *P. falciparum* K1 (EC_{50} = 1500 nM, entry 27) than FCR-3[11] (EC_{50} = 18 nM). The results indicate that no chloroquine-resistance appears in the class I [0,0]-rhodacyanines. Similar effects of hydrophilic groups on in vitro antimalarial activity, observed in our previous work,[11] were noted with the class I rhodacyanines. Specifically, compounds **1e**–**1i**, bearing hydrophilic substituents such as hydroxyl groups (**1e**–**1g**), polyether chains (**1h**), and carboxylic acids (**1i**) on the central rhodanine ring, have slightly lower antimalarial activities (entries 6–13).

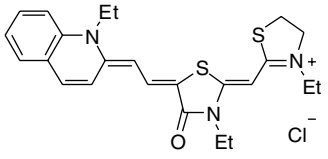
Owing to their high antimalarial activities and low cell cytotoxicities, several class I [0,0]-rhodacyanines were subjected to in vivo testing. The results clearly show that these substances suppress the growth of malaria parasites (Table 1). However, despite having good in vitro activities (EC_{50} = 19 and 21 nM), the parasite growth suppression levels of **1a** and **1b** (10 mg/kg/d, ip) are only 27% and 30%, respectively (entries 1 and 2). In addition, intravenous administration of **1b** (5 mg/kg/d) does not result in an improvement in parasitemia suppression (entry 3), and mice death before the end of the drug-treatment protocol was observed when higher doses of **1b** are administered. Among the tested compounds,



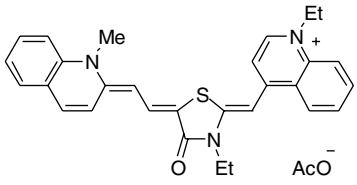
	R	X
2a	Me	TsO
2b	CH_2CH_2OH	Cl
2c	$(CH_2CH_2O)_3Me$	Cl
2d	$(CH_2)_4CO_2H$	Cl



2e

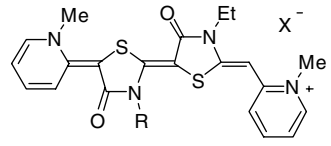


2f

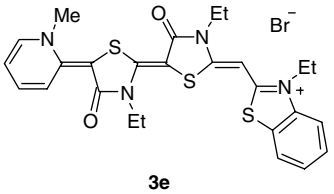


2g

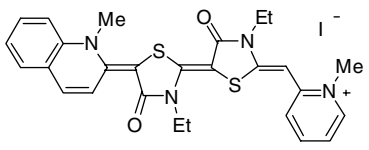
Chart 2. Prepared [1,0]-rhodacyanines **2a**–**2g**.



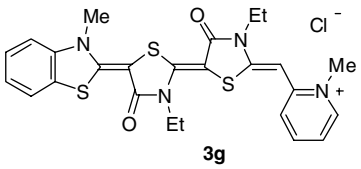
	R	X
3a	Et	I
3b	Ph	Cl
3c	Bn	Cl
3d	CH_2CHCH_2	I



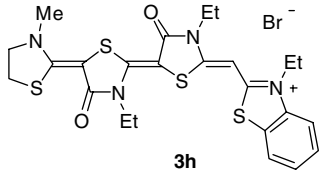
3e



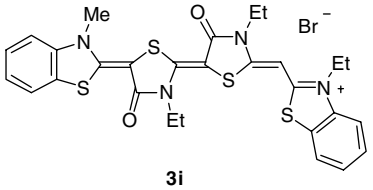
3f



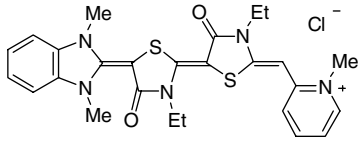
3g



3h



3i



3j

Chart 3. [0,0,0]-Rhodacyanines **3a**–**3j**.

[0,0]-rhodacyanines **1d**, **1g**, **1i**, **1p**, and **1q** (entries 8, 9, 12, 13, 20, and 21) show moderate parasitemia suppression (50–60%) on intraperitoneal administration (5–10 mg/kg/d).

The results of antimalarial screening of the class I rhodacyanines have led to several important conclusions. First, a good correlation does not exist between the in vitro and in vivo activities of these substances. For example, compound **1n** shows the highest antimalarial activity in vitro (EC_{50} = 1.0 nM) but it exhibits only 29% parasitemia suppression (10 mg/kg/d, ip) in vivo (entry 18 in Table 1). On the other hand, compounds **1g** and **1i**, which display much lower in vitro activities (EC_{50} = 37 and 83 nM), have markedly higher in vivo potencies (63% and 53% suppression, respectively) at the same dosages (entries 9 and 13). Second, class I rhodacyanines bearing hydrophilic substituents, such as **1d**, **1e** and **1g**–**1i**, have high in vivo antimalarial activities in comparison to analogous substances having hydrophobic substituents (entries 5, 6, and 8–13). This trend is opposite to that seen in the in vitro testing results. The difference could be a consequence of several factors which can influence in vivo activities including absorption, metabolism, distribution, and excretion (ADME). Third, a linear dependence of antimalarial activities on dosage levels was not observed in the in vivo testing results (entries 8, 9, and 11–13). This suggests that ADME factors influence the in vivo antimalarial potencies of the [0,0]-rhodacyanines. Finally, [0,0]-rhodacyanines that

Table 1. In vitro and in vivo antimalarial activities of [0,0]-rhodacyanines

Entry	Compound	EC_{50} (M), in vitro		Selectivity[c]	4 day suppressive test, in vivo[d]	
		P. falciparum[a]	L-6[b]		Dose, method	% suppression
1	**1a** (MKH57)	1.9×10^{-8}	1.1×10^{-4}	5.4×10^{3}	10 mg/kg/d, ip	27.0
2	**1b** (MKT077)	2.1×10^{-8}	1.1×10^{-4}	5.5×10^{3}	10 mg/kg/d, ip	30.0
3	**1b**				5 mg/kg/d, iv	32.1
4	**1c**	NT[e]	NT[e]	—	10 mg/kg/d, ip	32.2
5	**1d**	NT[e]	NT[e]	—	5 mg/kg/d, ip	51.9
6	**1e**	8.2×10^{-8}	1.8×10^{-4}	2.2×10^{3}	5 mg/kg/d, iv	65.5
7	**1f**	4.0×10^{-8}	1.0×10^{-4}	2.5×10^{3}	NT[e]	—
8	**1g**	3.7×10^{-8}	6.2×10^{-5}	1.7×10^{3}	5 mg/kg/d, ip	60.9
9	**1g**				10 mg/kg/d, ip	62.6
10	**1h**	5.3×10^{-8}	1.3×10^{-4}	2.4×10^{3}	5 mg/kg/d, iv	58.4
11	**1i**	8.3×10^{-8}	5.3×10^{-5}	6.4×10^{2}	1 mg/kg/d, ip	12.4
12	**1i**				5 mg/kg/d, ip	52.3
13	**1i**				10 mg/kg/d, ip	53.0
14	**1j**	1.5×10^{-8}	$>2.0\times10^{-4}$	$>1.3\times10^{4}$	5 mg/kg/d, ip	33.4
15	**1k**	NT[e]	NT[e]	—	10 mg/kg/d, ip	37.3
16	**1l**	NT[e]	NT[e]	—	10 mg/kg/d, ip	38.4
17	**1m**	NT[e]	NT[e]	—	5 mg/kg/d, iv	51.9
18	**1n**	1.0×10^{-9}	1.4×10^{-5}	1.4×10^{4}	10 mg/kg/d, ip	28.6
19	**1o**	3.8×10^{-8}	1.9×10^{-4}	5.1×10^{3}	NT[e]	—
20	**1p**	NT[e]	NT[e]	—	10 mg/kg/d, ip	53.7
21	**1q**	NT[e]	NT[e]	—	10 mg/kg/d, ip	57.3
22	**1r**	1.1×10^{-8}	2.5×10^{-5}	2.2×10^{3}	NT[e]	—
23	**1s**	5.5×10^{-8}	6.0×10^{-6}	1.1×10^{2}	NT[e]	—
24	**1t**	NT[e]	NT[e]	—	5 mg/kg/d, iv	50.2
25	**1u**	NT[e]	NT[e]	—	5 mg/kg/d, iv	51.1
26	**1v**	NT[e]	NT[e]	—	5 mg/kg/d, iv	41.4
27	Chloroquine	1.5×10^{-6}	4.7×10^{-5}	3.2×10	10 mg/kg/d, ip	90.6
28	Quinine	NT[e]	NT[e]	—	10 mg/kg/d, ip	18.6

[a] Chloroquine-resistant strain (K1).
[b] Rat skeletal myoblast L-6 cells representing a model of host.
[c] Selective toxicity, EC_{50} value for L-6/EC_{50} for *P. falciparum*.
[d] Rodent malaria parasites, *P. berghei* (NK65 strain).
[e] NT means 'not tested'.

have pyridinyl **A** and/or **C** ring (e.g., **1m**, **1p**, and **1q**) have contrastingly better in vivo parasitemia suppression properties.

2.2.2. [1,0]-Rhodacyanines (class II). In vitro and in vivo antimalarial activities of members of the [1,0]-rhodacyanine series (class II) are listed in Table 2. All of the substances showed strong in vitro antimalarial activity against the chloroquine-resistant strain with EC_{50} values ranging 12–350 nM. However, the cytotoxicities of these compounds against L-6 cells are almost 10-fold higher than those of [0,0]-rhodacyanines. In vivo evaluation of [1,0]-rhodacyanines showed that **2a**–**2d** have low activities against *P. berghei* (<25% parasitemia suppression at a dosage of 10 mg/kg/d (ip), entries 1–4). It was found that administration of higher dosages (~50 mg/kg/d) results in acute toxicity (animal death during the treatment program).

2.2.3. Class III [0,0,0]-rhodacyanines. The results of in vitro and in vivo antimalarial testing of the class III [0,0,0]-rhodacyanines are summarized in Table 3. In a manner similar to results from studies with the [0,0]-rhodacyanines, no correlation exists between the in vitro and in vivo activities of the class III compounds. Compound **3i**, containing two side edged benzothiazole rings, has strong in vitro antimalarial activity (EC_{50} value of 9.7 nM) and an extremely low cytotoxicity (entry 13 in Table 3). However, this substance is only weakly active in vivo, with a suppression level of 29%, even at a high dosage of 20 mg/kg/d (ip). On the other hand, **3a**, which has pyridine moieties on both edges, is 100-fold less active in the in vitro screen but displays high levels of in vivo activity (64% suppression at half dosage, entry 1). It is noteworthy that compound **3a** is not acutely toxic even when administered at the high dosage of 50 mg/kg/d (entry 2) but the suppression level of **3a** is not greatly improved at higher dosages.

In vivo screening of the class III rhodacyanines was carried out at the highest dosage levels where no mice toxicity was observed. In contrast to the class I and II rhodacyanines, nearly all of the [0,0,0]-rhodacyanines could be safely injected at dosages of >50 mg/kg/d (ip). Several compounds in this series display >50% parasitemia suppression at dosages that do not lead to animal death. Noteworthy are [0,0,0]-rhodacyanines **3e** and **3h** that possess a benzothiazole ring at the right side. These substances have high activities, providing 89% suppression at dosages of 25 mg/kg/d (entries 8 and 12). These activities are comparable to those of chloroquine (entry 30 in Table 1). Moreover, in vivo tests show that **3e** and **3h** have good dose–activity relationships (entries 7, 8, 11, and 12). Unfortunately, mice treated with

Table 2. In vitro and in vivo antimalarial activities of [1,0]-rhodacyanines

Entry	Compound	EC_{50} (M), in vitro		Selectivity[c]	4 day suppressive test, in vivo[d]	
		P. falciparum[a]	L-6[b]		Dose, method	% suppression
1	**2a**	1.3×10^{-7}	7.6×10^{-5}	5.9×10^{2}	10 mg/kg/d, ip	12.6
2	**2b**	3.5×10^{-7}	4.2×10^{-5}	1.2×10^{2}	10 mg/kg/d, ip	11.4
3	**2c**	1.7×10^{-7}	4.4×10^{-5}	2.6×10^{2}	10 mg/kg/d, ip	16.1
4	**2d**	1.7×10^{-7}	7.7×10^{-5}	1.0×10^{2}	10 mg/kg/d, ip	23.3
5	**2e**	1.2×10^{-8}	3.0×10^{-5}	2.5×10^{3}	NT[e]	—
6	**2f**	1.2×10^{-8}	2.0×10^{-5}	1.7×10^{3}	NT[e]	—
7	**2g**	1.4×10^{-7}	1.0×10^{-5}	7.2×10	NT[e]	—

[a] Chloroquine-resistant strain (K1).
[b] Rat skeletal myoblast L-6 cells representing a model of host.
[c] Selective toxicity, EC_{50} value for L-6/ EC_{50} for *P. falciparum*.
[d] Rodent malaria parasites, *P. berghei* (NK65 strain).
[e] NT means 'not tested'.

Table 3. In vitro and in vivo antimalarial activities of [0,0,0]-rhodacyanines

Entry	Compound	EC_{50} (M), in vitro		Selectivity[c]	4 day suppressive test, in vivo[d]	
		P. falciparum[a]	L-6[b]		Dose, method	% suppression
1	**3a**	7.1×10^{-7}	7.8×10^{-5}	1.1×10^{2}	10 mg/kg/d, ip	64.0
2	**3a**				50 mg/kg/d, ip	68.9
3	**3a**				100 mg/kg/d, po	55.0
4	**3b**	NT[e]	NT[e]	—	50 mg/kg/d, ip	56.3
5	**3c**	NT[e]	NT[e]	—	50 mg/kg/d, ip	23.3
6	**3d**	NT[e]	NT[e]	—	50 mg/kg/d, ip	25.9
7	**3e**	NT[e]	NT[e]	—	10 mg/kg/d, ip	46.9
8	**3e**				25 mg/kg/d, ip	88.7
9	**3f**	NT[e]	NT[e]	—	50 mg/kg/d, ip	69.1
10	**3g**	NT[e]	NT[e]	—	50 mg/kg/d, ip	52.8
11	**3h**	NT[e]	NT[e]	—	10 mg/kg/d, ip	56.4
12	**3h**				25 mg/kg/d, ip	89.0
13	**3i**	9.7×10^{-9}	$>2.0 \times 10^{-4}$	$>2.0 \times 10^{4}$	20 mg/kg/d, ip	28.7
14	**3j**	NT[e]	NT[e]	—	50 mg/kg/d, ip	53.4

[a] Chloroquine-resistant strain (K1).
[b] Rat skeletal myoblast L-6 cells representing a model of host.
[c] Selective toxicity, EC_{50} value for L-6/ EC_{50} for *P. falciparum*.
[d] Rodent malaria parasites, *P. berghei* (NK65 strain).
[e] NT means 'not tested'.

3e and **3h** (25 mg/kg/d, ip, 4 days) gradually lose body weight and survive only 1–2 days longer than untreated controls. These results point out that **3e** and **3h** are toxic, but the origin of the toxicity is not known.

Finally, the oral bioavailability of class III rhodacyanine **3a** was determined. Significantly, **3a** has a 55% suppression level at a dosage of 100 mg/kg/d (po) and it does not have a significant toxicity (entry 3).

3. Conclusions

Members of three classes of rhodacyanines were prepared and evaluated in vitro against *P. falciparum* (chloroquine-resistant K1) and cytotoxicity (mammalian L-6 cells) screens. Almost all of the substances were found to possess promisingly high in vitro activities and several showed both strong activities (EC_{50} values ranging from 1 to 20 nM) and excellent selective toxicities (up to 10,000). Finally, the in vivo activities of several of rhodacyanines were examined by using *P. berghei* in an animal model. The results clearly demonstrated that no obvious correlation exists between in vitro and in vivo activities of these substances. In vivo antimalarial screening revealed that members of the [0,0,0]-rhodacyanine class, which possess two central rhodanine moieties, exhibit comparably weak acute toxicities and potent suppressive effects against malaria parasites. This is especially true for **3e** and **3h** that have benzothiazole moieties at the right edge. These substances are highly active in vivo with suppressions of parasitemia that approach 89% at dosages of 25 mg/kg/d (ip). However, further improvement of toxicological properties of these compounds is still required. Finally, we found that [0,0,0]-rhodacyanines can be orally administered. Observations made in this study serve as the rationale for continuing work on the synthesis and biological evaluation of novel antimalarial rhodacyanine dyes.

4. Experimental

4.1. General methods

Unless otherwise described, the materials and the solvent were obtained from commercial suppliers and used with-

out further purification. Melting points were determined using an AS ONE ATM-01 apparatus and have not been corrected. IR spectra were recorded with a SHIMADZU FTIR-8300. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (300 MHz) and a JEOL JNM-AL400 (400 MHz) with tetramethylsilane as internal standard. Chemical shifts are given in ppm, coupling constants are in hertz, and splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. Fast atom bombardment (FAB) mass spectra were determined with a JEOL JMS DX-303 mass spectrometer. Electron ionization (EI) mass spectra were recorded on a JEOL JMS AX-500 instrument. Elemental analyses were performed on Yanagimoto MT-3, and the results (C, H, N) were within ±0.4% of theoretical values. Because of deliquescence and hygroscopicity, correct elemental analyses for most of the compounds could only be obtained by factoring in partial hydration of these organic salts. Compounds **1a**, **1b**, **1e**, **1m–o**, **1q–s**, **1u**, and **2e–g** were known compounds.[10,11,13,15,16]

4.2. Typical procedure for the synthesis of [0,0]-rhodacyanines (class I)

4.2.1. 5-(3-Methyl-(3*H*)-benzothiazol-2-ylidene)-3-(2-propenyl)-2-thioxo-4-thiazolidinone (7)[10]. A mixture of 2-(methylthio)benzothiazole (**4**; 10.0 g, 55.2 mmol) and methyl *p*-toluenesulfonate (15.4 g, 81.7 mmol) in anisole (14 mL) was stirred at 120 °C for 4 h. After the reaction mixture was cooled to ambient temperature, to the resulting mixture was added a solution of 3-ethyl-4-oxothiazolidine-2-thione (**6**; 8.90 g, 55.2 mmol) in acetonitrile (200 mL). To this mixture was added triethylamine (12.6 mL, 90.5 mmol) dropwise under 0 °C with constant stirring and cooling, and the resulting mixture was stirred at ambient temperature for 1 h. The yellow precipitate was collected and washed with acetonitrile to give **7** (13.6 g, 80% yield) as yellow crystals, whose spectral data were identical with reported ones.[10]

4.2.2. 3-Ethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-2-methylsulfanyl-4-oxo-4,5-dihydrothiazol-3-ium *p*-toluenesulfonate (8)[10]. A mixture of **7** (14.6 g, 47.4 mmol) and methyl *p*-toluenesulfonate (26.5 g, 142 mmol) in *N*,*N*-dimethylformamide (15 mL) was stirred at 130 °C for 3 h. After the mixture was cooled to 95 °C, acetone (125 mL) was added. The resulting mixture was further cooled to ambient temperature with constant stirring. The precipitate formed was collected and washed with acetone to yield **8** (21.8 g, 93% yield) as orange crystals, whose spectral data were identical with reported ones.[10]

4.2.3. 2-[3-Ethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-1-{2-[2-(2-methoxyethoxy)ethoxy]ethyl}pyridinium chloride (1h). To a mixture of **8** (672 mg, 1.36 mmol) and **9** (500 mg, 1.36 mmol) in acetonitrile (6.8 mL) was dropwise added triethylamine (0.54 mL, 9.08 mmol) at 70 °C, and the mixture was stirred for 16 h at the same temperature. After the mixture was cooled to ambient temperature, EtOAc was poured onto the resulting mixture. The orange precipitate was collected and washed with ethyl acetate to give the iodide salt as orange crystals. Then, a solution of the iodide in methanol/dichloromethane (1/1, v/v) was passed through the basic anion-exchange resin (Amberlite® IRA-400, chloride form), and the resin was washed with methanol. After the concentration of the eluent chloride, the residue was crystallized from ethyl acetate to give **1h** (389 mg, 52% yield) as orange solids. Mp 124–127 °C (decomp.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.61 (1H, d, J = 6.6 Hz), 8.28 (1H, dd, J = 8.0, 7.6 Hz), 8.08 (1H, d, J = 8.6 Hz), 7.86 (1H, d, J = 8.0 Hz), 7.59 (1H, d, J = 8.4 Hz), 7.50–7.45 (2H, m), 7.29 (1H, dd, J = 7.6, 7.6 Hz), 6.11 (1H, s), 4.79 (2H, t, J = 4.8 Hz), 4.08 (2H, q, J = 6.8 Hz), 4.02 (3H, s), 3.90 (2H, t, J = 4.6 Hz), 3.55 (2H, t, J = 3.9 Hz), 3.46–3.40 (6H, m), 3.20 (3H, s), 1.23 (3H, t, J = 6.8 Hz); MS (FAB) m/z 514 (M^+); Anal. $C_{26}H_{32}ClN_3O_4S_2\cdot0.2H_2O$; C, 56.39; H, 5.90; N, 7.59. Found: C, 56.64; H, 5.80; N, 7.74.

4.2.4. 2-[3-Methoxycarbonylmethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-1-methylpyridinium chloride (1c). Orange solids; mp 275–277 °C (decomp.); IR (KBr) 2943, 1745, 1656, 1548, 682 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.69 (d, 1H, J = 6.3 Hz), 8.27 (m, 1H), 8.06 (d, 1H, J = 8.1 Hz), 7.83 (d, 1H, J = 7.7 Hz), 7.58 (d, 1H, J = 8.1 Hz), 7.45–7.42 (m, 2H), 7.31–7.26 (m, 1H), 5.90 (s, 1H), 4.96 (s, 2H), 4.07 (s, 3H), 4.04 (s, 3H), 3.73 (s, 3H), 2.27 (s, 3H); MS (FAB) m/z 426 (M^+); Anal. $C_{21}H_{20}ClN_3O_3S_2$; C, 54.60; H, 4.36; N, 10.39. Found: C, 54.79; H, 4.59; N, 10.23.

4.2.5. 2-[3-Carboxymethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-1-methylpyridinium chloride (1d). Orange solids; mp 257–258 °C (decomp.); IR (KBr) 3621–3200, 1651, 1627, 1506, 956 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.65 (d, 1H, J = 6.3 Hz), 8.31–8.25 (m, 1H), 8.06 (d, 1H, J = 8.5 Hz), 7.86 (d, 1H, J = 7.7 Hz), 7.63 (d, 1H, J = 8.8 Hz), 7.53–7.42 (m, 2H) 7.31–7.26 (m, 1H), 5.90 (s, 1H), 4.85 (s, 2H), 4.07 (s, 3H), 4.04 (s, 3H); MS (FAB) m/z 412 (M^+); Anal. $C_{20}H_{18}ClN_3O_3S_2\cdot1.75H_2O$; C, 50.10; H, 4.52; N, 8.76. Found: C, 50.32; H, 4.89; N, 8.53.

4.2.6. 2-[3-Allyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-1-(2-hydroxyethyl)pyridinium bromide (1f). Orange solids; mp 294–295 °C (decomp.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.54 (1H, d, J = 6.3 Hz), 8.29 (1H, dd, J = 8.5, 7.6 Hz), 8.05 (1H, d, J = 8.5 Hz), 7.86 (1H, d, J = 7.8 Hz), 7.61 (1H, d, J = 8.5 Hz), 7.52–7.44 (2H, m), 7.29 (1H, dd, J = 7.6, 6.3 Hz), 5.99 (1H, s), 5.93–5.83 (1H, m), 5.31–5.19 (2H, m), 4.69 (2H, m), 4.60 (2H, m), 4.05 (3H, s), 3.79 (2H, m); MS (FAB) m/z 424 (M^+); Anal. $C_{22}H_{22}BrN_3O_2S_2\cdot0.36H_2O$; C, 51.71; H, 4.48; N, 8.22. Found: C, 51.63; H, 4.12; N, 8.56.

4.2.7. 2-[3-Ethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-1-{2-[2-(2-hydroxyethoxy)ethoxy]ethyl}pyridinium chloride (1g). Orange solids; mp 216–217 °C (decomp.); IR (KBr) 3423, 1628, 1506, 1445, 1387, 1169 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.62 (1H, d, J = 7.8 Hz), 8.27

(1H, dd, J = 8.5, 8.5 Hz), 8.07 (1H, d, J = 7.8 Hz), 7.84 (1H, d, J = 7.8 Hz), 7.59 (1H, d, J = 8.5 Hz), 7.50–7.43 (2H, m), 7.27 (1H, dd, J = 8.5, 7.8 Hz), 6.10 (1H, s), 4.79 (2H, t, J = 4.9 Hz), 4.59 (1H, s), 4.07 (2H, q, J = 7.1 Hz), 4.02 (3H, s), 3.90 (2H, t, J = 4.9 Hz), 3.58–3.53 (2H, m), 3.48–3.41 (4H, m), 3.40–3.25 (2H, m), 1.23 (3H, t, J = 7.1 Hz); MS (FAB) m/z 500 (M^+); Anal. $C_{25}H_{30}ClN_3O_4S_2{\cdot}0.4H_2O$; C, 55.27; H, 5.71; N, 7.73. Found: C, 55.21; H, 5.74; N, 7.69.

4.2.8. 1-(4-Carboxybenzyl)-2-[3-ethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]pyridinium chloride (1i). Orange solids; mp 210–212 °C (decomp.); IR (KBr) 3412, 1713, 1624, 1497, 1381, 1159 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.85 (1H, d, J = 6.0 Hz), 8.34 (1H, dd, J = 8.0, 8.0 Hz), 8.06 (1H, d, J = 8.5 Hz), 7.97 (2H, d, J = 8.2 Hz), 7.87 (1H, d, J = 7.5 Hz), 7.63 (1H, d, J = 8.2 Hz), 7.55–7.45 (2H, m), 7.34–7.29 (2H, m), 5.96 (2H, s), 5.74 (1H, s), 4.04 (3H, s), 3.88 (2H, q, J = 7.2 Hz), 0.78 (3H, t, J = 7.2 Hz); MS (FAB) m/z 502 (M^+); Anal. $C_{27}H_{34}ClN_3O_3S_2{\cdot}0.68H_2O$; C, 57.87; H, 6.36; N, 7.50. Found: C, 58.09; H, 6.03; N, 7.71.

4.2.9. 2-{5-[6-Ethoxycarbonylpentanoylamino]-3-methyl-(3*H*)-benzothiazol-2-ylidene}-3-ethyl-4-oxothiazolidin-2-ylidenemethyl}-1-ethylpyridinium chloride (1j). Orange solids; mp 241–243 °C (decomp.); IR (KBr) 1732, 1697, 1647, 1541, 866, 761 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 10.17 (1H, s), 8.65 (1H, d, J = 6.3 Hz), 8.24 (1H, dd, J = 8.1, 8.1 Hz), 8.15 (1H, s), 8.00 (1H, d, J = 8.3 Hz), 7.60–7.50 (2H, m), 7.40 (1H, dd, J = 6.6, 6.3 Hz), 5.93 (1H, s), 4.13 (3H, s), 4.09 (2H, q, J = 7.1 Hz), 4.01 (3H, s), 3.59 (3H, s), 2.34 (4H, m), 1.59 (4H, m), 1.26 (3H, t, J = 7.1 Hz); MS (FAB) m/z 539 (M^+); Anal. $C_{28}H_{35}ClN_4O_4S_2{\cdot}1.2H_2O$; C, 54.88; H, 6.15; N, 9.14. Found: C, 54.59; H, 6.01; N, 9.17.

4.2.10. 2-[3-Ethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-3-methylbenzothiazol-3-ium chloride (1k). Red amorphous; ^1H NMR (300 MHz, DMSO-d_6) δ 8.23 (1H, d, J = 8.0 Hz), 7.97–7.91 (2H, m), 7.76–7.67 (2H, m), 7.54–7.49 (2H, m), 7.35 (1H, dd, J = 8.0, 7.1 Hz), 6.67 (1H, s), 4.26 (2H, q, J = 7.1 Hz), 4.20 (3H, s), 4.04 (3H, s), 1.28 (3H, t, J = 7.1 Hz); MS (FAB) m/z 438 (M^+); Anal. $C_{22}H_{20}ClN_3O_2S_3{\cdot}0.9H_2O$; C, 52.19; H, 4.34; N, 8.30. Found: C, 52.35; H, 4.21; N, 8.68.

4.2.11. 2-[3-Ethyl-5-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-3-methylbenzooxazol-3-ium chloride (1l). Red amorphous; ^1H NMR (300 MHz, DMSO-d_6) δ 7.97 (1H, d, J = 8.5 Hz), 7.94 (1H, d, J = 8.7 Hz), 7.73 (1H, d, J = 8.0 Hz), 7.71 (1H, d, J = 8.2 Hz), 7.57–7.45 (3H, m), 7.32 (1H, dd, J = 7.6, 7.5 Hz), 6.28 (1H, s), 4.20 (2H, q, J = 6.9 Hz), 4.15 (3H, s), 3.86 (3H, s), 1.26 (3H, t, J = 6.9 Hz); MS (FAB) m/z 422 (M^+); Anal. $C_{25}H_{27}ClN_3O_4S_2{\cdot}0.3H_2O$; C, 55.93; H, 4.61; N, 8.89. Found: C, 55.76; H, 4.82; N, 8.91.

4.2.12. 2-[3-Ethyl-{5-(1-methyl-(1*H*)-pyridin-2-ylidene)-4-oxothiazolidin-2-ylidene}methyl]-1-methylpyridinium chloride (1p). Orange solids; mp 226–229 °C (decomp.); IR (KBr) 3430, 1629, 1163 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.56 (1H, d, J = 8.8 Hz), 8.46 (1H, d, J = 6.3 Hz), 8.08–8.03 (2H, m), 7.66 (1H, dd, J = 8.8, 7.1 Hz), 7.14–7.16 (1H, m), 6.88 (1H, dd, J = 7.1, 6.6 Hz), 5.74 (1H, s), 4.12 (3H, s), 4.07 (2H, q, J = 7.0 Hz), 4.02 (3H, s), 1.23 (3H, t, J = 7.0 Hz); MS (FAB) m/z 326 (M^+); Anal. $C_{25}H_{27}ClN_3O_4S_2{\cdot}0.3H_2O$; C, 55.76; H, 5.17; N, 7.80. Found: C, 55.38; H, 4.89; N, 8.11.

4.2.13. 2-[3-Ethyl-5-(1-methyl-(1*H*)-quinolin-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-3-methylbenzothiazolium chloride (1t). Purple needles; mp 277–280 °C (decomp.); ^1H NMR (300 MHz, DMSO-d_6) δ 8.29–8.25 (1H, m), 8.21 (2H, dd, J = 8.8, 6.8 Hz), 7.99–7.85 (4H, m), 7.72 (1H, dd, J = 9.4, 6.8 Hz), 7.61–7.50 (2H, m), 6.71 (1H, s), 4.26 (2H, q, J = 6.6 Hz), 4.13 (3H, s), 4.05 (3H, s), 1.29 (3H, t, J = 7.0 Hz); MS (FAB) m/z 432 (M^+); Anal. $C_{24}H_{22}ClN_3OS_2{\cdot}0.6H_2O$; C, 60.20; H, 4.88; N, 8.78. Found: C, 60.52; H, 4.91; N, 8.60.

4.2.14. 2-[3-Ethyl-5-(1-methyl-(1*H*)-quinolin-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]-1,3-dimethyl-(3H)-benzoimidazol-1-ium chloride (1v). Purple amorphous; ^1H NMR (300 MHz, DMSO-d_6) δ 8.75 (1H, m), 7.99–7.89 (3H, m), 7.63–7.30 (6H, m), 6.02 (1H, s), 3.97 (2H, q, J = 7.0 Hz), 3.95 (6H, s), 3.78 (3H, s), 1.28 (3H, t, J = 6.6 Hz); MS (FAB) m/z 429 (M^+); Anal. $C_{25}H_{25}ClN_4OS{\cdot}2.1H_2O$; C, 59.71; H, 5.85; N,11.14. Found: C, 59.60; H, 5.79; N, 11.52.

4.3. Typical procedure for the synthesis of [1,0]-rhodacyanines (class II)

4.3.1. *N*-(3-Ethyl-4-oxo-2-thioxothiazolidin-5-ylidenemethyl)-*N*-phenylacetamide (11). A mixture of 3-ethylrhodanine (**6**; 9.98 g, 62.0 mmol) and *N*,*N*′-diphenylformamidin (12.1 g, 62.0 mmol) in acetonitrile (58 mL) was stirred at 70 °C for 1 h and then cooled to ambient temperature. The precipitate formed was collected and washed with acetone to give 3-ethyl-5-phenylaminomethylene-2-thioxothiazolidin-4-one (**10**) as a crude product (9.96 g). The crude product was mixed with acetic anhydride (40.8 mL, 434 mmol) and triethylamine (0.16 mL, 1.28 mmol), and then the resulting mixture was stirred at 100 °C for 30 min. The reaction mixture was concentrated to about one-half volume under reduced pressure. The residue was added MeOH (184 mL) and stirred at ambient temperature for 1 h. The precipitate formed was collected and washed with MeOH to give **11** (9.46 g, 50% yield) as yellow crystals, whose spectral data were identical with reported ones.[15]

4.3.2. 3-Ethyl-5-[2-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-ethylidene]-2-thioxothiazolidin-4-one (13). A mixture of **11** (1.00 g, 3.3 mmol), 2,3-dimethylbenzothiazol-3-ium *p*-toluenesulfonate (**12**; 1.09 g, 3.3 mmol), and acetic anhydride (0.43 mL, 4.6 mmol) in acetonitrile (33 mL) was stirred at 50 °C for 1 h. After triethylamine (16.1 mL, 12.1 mmol) was added to the mixture, the resulting mixture was stirred for an additional 4 h at 60 °C and, then, cooled to ambient temperature. The precipitate formed was collected and washed with acetonitrile to give **13** (0.93 g, 85% yield)

as yellow amorphous. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.81 (1H, d, J = 7.8 Hz), 7.52–7.41 (3H, m), 7.26 (1H, dd, J = 7.8, 7.8 Hz), 5.74 (1H, d, J = 13.2 Hz), 4.01 (2H, q, J = 7.1 Hz), 3.66 (3H, s), 1.15 (3H, t, J = 7.1 Hz); MS (EI) m/z 334; Anal. $C_{15}H_{14}N_2OS_3$; C, 53.86; H, 4.22; N, 8.38. Found: C, 53.92; H, 4.36; N, 8.16.

4.3.3. 3-Ethyl-5-[2-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-ethylidene]-2-methylsulfanyl-4-oxo-4,5-dihydrothiazol-3-ium *p*-toluenesulfonate (14). A mixture of **13** (1.50 g, 4.47 mmol) and methyl *p*-toluenesulfonate (2.50 g, 13.4 mmol) in DMF (6 mL) was stirred at 135 °C for 3 h. After being cooled to ambient temperature to the mixture was added acetone. The resulting mixture was stirred for 30 min. The precipitate formed was collected and washed with acetone to yield **14** (2.12 g, 91% yield) as green solids. This compound was used for next reaction without further purification.

4.3.4. 2-{3-Ethyl-5-[2-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-ethylidene]-4-oxothiazolidin-2-ylidenemethyl}-1-methylpyridinium *p*-toluenesulfonate (2a). To a mixture of **14** (100 mg, 0.19 mmol) and 1,2-dimethylpyridinium *p*-toluenesulfonate (**15**; 53.5 mg, 0.19 mmol) in acetonitrile (5.8 mL) was added triethylamine (80 μL, 0.58 mmol) dropwise. The reaction mixture was stirred at 75 °C for 2.5 h and, then, cooled to ambient temperature. The precipitate formed was collected and washed with acetonitrile to give **2a** (33.0 mg, 29% yield) as green solids. Mp 245–248 °C (decomp.); IR (KBr) 1674, 1539, 1371, 1199 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (1H, d, J = 6.3 Hz), 8.35 (1H, dd, J = 8.4, 8.4 Hz), 8.13 (1H, d, J = 8.4 Hz), 7.74 (1H, d, J = 7.7 Hz), 7.58 (1H, dd, J = 7.5, 7.5 Hz), 7.47 (2H, d, J = 8.0 Hz), 7.44 (1H, s), 7.41–7.39 (2H, m), 7.21–7.09 (1H, m), 7.10 (2H, d, J = 8.0 Hz), 6.16 (1H, s), 5.63 (1H, d, J = 12.5 Hz), 4.19 (3H, s), 4.02 (2H, q, J = 7.0 Hz), 3.58 (3H, s), 2.28 (3H, s), 1.23 (3H, t, J = 7.0 Hz); MS (FAB) m/z 408 (M^+); Anal. $C_{29}H_{29}N_3O_4S_3{\cdot}1H_2O$; C, 58.27; H, 5.23; N, 7.03. Found: C, 58.19; H, 5.08; N, 6.85.

4.3.5. 2-{3-Ethyl-5-[2-(3-methyl-(3*H*)-benzothizol-2-ylidene)ethylidene]-4-oxothiazolidin-2-ylidenemethyl}-1-(2-hydroxyethyl)pyridinium chloride (2b). Green solids; mp 276–279 °C (decomp.); IR (KBr) 3566, 1652, 1521, 1369 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (1H, d, J = 5.8 Hz), 8.38 (1H, dd, J = 8.5, 8.5 Hz), 8.19 (1H, d, J = 8.0 Hz), 7.73 (1H, d, J = 7.8 Hz), 7.66 (1H, dd, J = 6.3, 6.3 Hz), 7.42–7.39 (2H, m), 7.20–7.16 (1H, m), 6.35 (1H, s), 5.59 (1H, d, J = 12.6 Hz), 5.32 (1H, t, J = 4.8 Hz), 4.70 (2H, t, J = 4.8 Hz), 4.00 (2H, q, J = 7.0 Hz), 3.85 (2H, t, J = 4.8 Hz), 3.57 (3H, s), 1.20 (3H, t, J = 7.0 Hz); MS (FAB) m/z 438 (M^+); Anal. $C_{23}H_{24}ClN_3O_2S_2{\cdot}1H_2O$; C, 56.14; H, 5.33; N, 8.54. Found: C, 55.92; H, 5.27; N, 8.77.

4.3.6. 2-{3-Ethyl-5-[2-(3-methyl-(3*H*)-benzothizol-2-ylidene)-ethylidene]-4-oxothiazolidin-2-ylidenemethyl}-1-{2-[2-(2-methoxyethoxy)ethoxy]ethyl}pyridinium chloride (2c). Green amorphous; mp 80–86 °C (decomp.); IR (KBr) 1541, 1386, 1199, 1147 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (1H, d, J = 5.8 Hz), 8.38 (1H, dd, J = 7.5, 7.5 Hz), 8.19 (1H, d, J = 8.2 Hz), 7.73 (1H, d, J = 7.8 Hz), 7.65 (1H, m), 7.42–7.39 (3H, m), 7.18 (1H, m), 6.34 (1H, s), 5.58 (1H, d, J = 12.6 Hz), 4.83 (2H, m), 3.99 (2H, d, J = 6.8 Hz), 3.90 (2H, m), 3.57 (3H, s), 3.53 (2H, m), 3.42 (2H, q, J = 7.0 Hz), 3.36 (2H, m), 3.20 (3H, s), 1.21 (3H, t, J = 7.0 Hz); MS (FAB) m/z 540 (M^+); Anal. $C_{28}H_{34}ClN_3O_4S_2$; C, 58.37; H, 5.95; N, 7.29. Found: C, 58.24; H, 6.95; N, 7.28.

4.3.7. 1-(4-Carboxy-butyl)-2-{3-ethyl-5-[2-(3-methyl-(3*H*)-benzothiazol-2-ylidene)-ethylidene]-4-oxothiazolidin-2-ylidenemethyl}pyridinium chloride (2d). Green amorphous; mp 189–201 °C (decomp.); IR (KBr) 1652, 1521, 1369 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.82 (1H, m), 8.43–8.17 (2H, m), 7.73 (1H, d, J = 8.1 Hz), 7.53–7.10 (6H, m), 5.46 (1H, d, J = 12.5 Hz), 3.60 (2H, q, J = 6.8 Hz), 3.56 (3H, s), 3.13–3.07 (4H, m), 1.24–1.16 (4H, m), 1.11 (3H, t, J = 6.8 Hz); MS (FAB) m/z 494 (M^+); Anal. $C_{26}H_{28}ClN_3O_3S_2{\cdot}0.8H_2O$; C, 57.35; H, 5.48; N, 7.72. Found: C, 57.43; H, 5.72; N, 7.61.

4.4. Typical procedure for the synthesis of [0,0,0]-rhodacyanines (class III) outlined in Scheme 3 (for 3c, 3h, and 3i)

4.4.1. 2-(3-Ethyl-4-oxothiazolidin-2-ylideneamino)-3-methyl-3-benzothiazolium bromide (18). To a mixture of 2-amino-3-methyl-3-benzothiazolium *p*-toluenesulfonate (**16**; 1.74 g, 4.70 mmol) and ethyl isothiocyanate (0.42 mL, 4.70 mmol) in pyridine (2.5 mL) was added dropwise triethylamine (0.66 mL, 4.70 mmol). The reaction mixture was stirred at 110 °C for 45 min and then poured into a mixture of crushed ice and water. The precipitate formed was collected and washed with water and then dried up. To the residue obtained were added bromoacetic acid (1.29 g, 9.26 mmol) and acetic acid (4 mL), and then the mixture was stirred at 90 °C for 30 min. After being cooled to ambient temperature, to the mixture was added diethyl ether (10 mL). The resulting mixture was stirred for 30 min at the same temperature. The precipitate formed was collected and washed with diethyl ether. The crude product was recrystallized from methanol to give **18** (1.72 g, 90%) as yellow solids. Mp 233–235 °C (decomp.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51 (1H, d, J = 2.0 Hz), 8.14 (1H, d, J = 9.0 Hz), 7.88 (1H, dd, J = 9.0, 2.0 Hz), 4.57 (2H, s), 4.06 (3H, s), 3.93 (2H, q, J = 7.1 Hz), 1.26 (3H, t, J = 7.1 Hz); MS (FAB) m/z 326 (M^+); Anal. $C_{15}H_{17}BrN_2OS_2{\cdot}0.7H_2O$; C, 45.27; H, 4.66; N, 7.04. Found: C, 45.33; H, 4.69; N, 7.12.

4.4.2. Compound 3h. To a mixture of **18** (106 mg, 0.24 mmol) and compound **19** (91.3 mg, 0.24 mmol) in acetonitrile (2.4 mL) was dropwise added triethylamine (0.71 mmol, 0.10 mL) at ambient temperature. The reaction mixture was stirred at 70 °C for 15 h. After being cooled to ambient temperature, the mixture was stirred for an additional 1 h. The precipitate formed was collected and washed with acetonitrile to give **3h** (108 mg, 73% yield) as green solids. Mp 258–259 °C (decomp.);

IR (KBr) 1657, 1504, 1411, 1367, 1271, 1063 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.29 (1H, d, J = 7.7 Hz), 8.01 (1H, d, J = 8.5 Hz), 7.76 (1H, dd, J = 8.5, 7.2 Hz), 7.58 (1H, dd, J = 7.7, 7.2 Hz), 6.75 (1H, s), 4.75 (2H, q, J = 7.2 Hz), 4.30–4.17 (4H, m), 3.93 (2H, t, J = 7.6 Hz), 3.47 (3H, s), 3.25 (2H, t, J = 7.6 Hz), 1.41–1.32 (6H, m), 1.26 (3H, t, J = 7.2 Hz); MS (FAB) m/z 531 (M^+); Anal. $C_{24}H_{27}BrN_4O_2S_2$; C, 52.65; H, 4.97; N, 10.23. Found: C, 52.73; H, 5.05; N, 10.26.

4.4.3. Compound 3c. Deep purple solids; mp 199–201 °C (decomp.); IR (KBr) 1636, 1545, 1491, 1377, 1283, 1165, 1067 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.60 (1H, d, J = 6.0 Hz), 8.47 (1H, d, J = 8.7 Hz), 8.23 (1H, dd, J = 8.7, 7.3 Hz), 8.13 (1H, d, J = 6.0 Hz), 7.70 (1H, dd, J = 7.3, 6.0 Hz), 7.61 (1H, d, J = 8.7 Hz), 7.44–7.33 (3H, m), 7.31–7.20 (3H, m), 6.94 (1H, dd, J = 7.8, 6.8 Hz), 5.82 (1H, s), 5.42 (2H, s), 4.14 (3H, s), 4.06 (3H, s), 3.99 (2H, q, J = 7.2 Hz), 1.18 (3H, t, J = 7.2 Hz); MS (FAB) m/z 515 (M^+); Anal. $C_{28}H_{27}ClN_4O_2S_2 \cdot 1.1H_2O$; C, 58.90; H, 5.15; N, 9.81. Found: C, 58.97; H, 5.11; N, 9.89.

4.4.4. Compound 3i. Green solids; mp >300 °C; IR (KBr) 1664, 1641, 1539, 1478, 1318, 1275, 1065 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.29 (1H, d, J = 8.0 Hz), 7.93 (1H, d, J = 8.4 Hz), 7.87 (1H, d, J = 7.7 Hz), 7.72 (1H, dd, J = 8.4, 7.6 Hz), 7.57 (1H, dd, J = 8.0, 7.6 Hz), 7.49 (1H, d, J = 8.2 Hz), 7.35 (1H, dd, J = 8.2, 7.4 Hz), 7.26 (1H, dd, J = 7.7, 7.4 Hz), 6.66 (1H, s), 4.65 (2H, m), 4.27 (4H, m), 4.10 (3H, s), 1.42 (3H, t, J = 6.9 Hz), 1.34 (3H, t, J = 6.9 Hz), 1.28 (3H, t, J = 7.1 Hz); MS (FAB) m/z 579 (M^+); Anal. $C_{28}H_{27}BrN_4O_2S_4 \cdot 0.5H_2O$; C, 50.29; H, 4.22; N, 8.38. Found: C, 50.27; H, 4.22; N, 8.41.

4.5. Typical procedure for the synthesis of [0,0,0]-rhodacyanines (class III) outlined in Scheme 5 (for 3a–3d, 3f, 3g, 3i, and 3j)

4.5.1. *N*-Ethyl-2-pyridylthioacetamide (23). To anhydrous ethylamine, which was collected from the heating of 70% aq $EtNH_2$ (14 mL) on KOH (0.50 g) at 70 °C for 1 h, was added ethyl pyridylacetate (**20**, 1.52 mL, 10 mmol) at −78 °C. After being stirred at the same temperature for 5 h, the mixture was allowed to warm to ambient temperature and was stirred for an additional 4 days. The mixture was evaporated to give *N*-ethyl-2-pyridylacetamide as a pure form (1.7 g, quant.). ^1H NMR (300 MHz, $CDCl_3$) δ 8.56 (1H, d, J = 6.3 Hz), 7.67 (1H, dd, J = 7.8, 7.5 Hz), 7.28 (1H, d, J = 7.8 Hz), 7.21 (1H, dd, J = 7.5, 6.3 Hz), 3.72 (2H, s), 3.29 (2H, q, J = 7.2 Hz), 1.12 (3H, t, J = 7.2 Hz); MS (EI) m/z 164 (M^+).

A solution of the amide (1.55 g, 9.3 mmol) and Lawesson's reagent (1.88 g, 4.7 mmol) in toluene (10 mL) was refluxed for 1 h. After the solvent was removed under reduced pressure, 20% aq K_2CO_3 was added to the residue. Then, the mixture was extracted with $CHCl_3$ three times. The combined organic layer was washed with 1 N HCl. The resulting aqueous phase separated was brought to pH 10 with K_2CO_3 and then extracted again with $CHCl_3$. The combined organic layers were dried over Na_2SO_4 and concentrated to yield **23** as yellow oil (1.45 g, 86% yield): ^1H NMR (300 MHz, $CDCl_3$) δ 9.82 (1H, s), 8.55 (1H, d, J = 5.8 Hz), 7.69 (1H, dd, J = 7.7, 7.6 Hz), 7.30 (1H, d, J = 7.7 Hz), 7.22 (1H, dd, J = 7.6, 5.8 Hz), 4.21 (2H, s), 3.67 (2H, q, J = 7.2 Hz), 1.26 (3H, t, J = 7.2 Hz); MS (EI) m/z 180 (M^+); Anal. $C_9H_{12}N_2S$; C, 59.96; H, 6.71; N, 15.54. Found: C, 60.12; H, 6.91; N, 15.63.

4.5.2. 3-Ethyl-2-pyridin-2-ylmethylenethiazolidin-4-one (24). To a solution of **23** (2.70 g, 15 mmol) in dichloromethane (20 mL) was added bromoacetyl chloride (3.75 mL, 45 mmol) at 0 °C. The reaction mixture was stirred at ambient temperature for 2 days. After addition of saturated $NaHCO_3$, the mixture was extracted with EtOAc and washed with brine. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give **24** as colorless oil: IR (KBr) 1701, 1603, 1545, 1391 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.57 (1H, d, J = 5.7 Hz), 7.55 (1H, dd, J = 7.8, 7.5 Hz), 7.11 (1H, d, J = 7.8 Hz), 6.97 (1H, dd, J = 7.5, 5.7 Hz), 6.08 (1H, s), 3.83 (2H, q, J = 7.2 Hz), 3.68 (2H, s), 1.28 (3H, t, J = 7.2 Hz); MS (EI) m/z 220 (M^+); Anal. $C_{11}H_{12}N_2OS$; C, 59.97; H, 5.49; N, 12.72. Found: C, 59.78; H, 5.46; N, 12.62.

4.5.3. 2-(3-Ethyl-4-oxothiazolidin-2-ylidenemethyl)-1-methylpyridinium iodide (25). A mixture of **24** (363 mg, 1.65 mmol) and methyl iodide (0.64 mL, 9.89 mmol) in acetone (4 mL) was stirred at 60 °C for 3 h. The mixture was concentrated under reduced pressure, and then EtOAc (5 mL) was added to the residue. The precipitate was collected and washed with EtOAc to give **25** (0.56 g, 93% yield) as light brown amorphous: IR (KBr) 1711, 1545, 1383, 1119 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.87 (1H, d, J = 6.3 Hz), 8.43 (1H, dd, J = 8.0, 7.4 Hz), 8.13 (1H, d, J = 8.0 Hz), 7.73 (1H, dd, J = 7.4, 6.3 Hz), 6.23 (1H, s), 4.20 (3H, s), 4.16 (2H, s), 3.87 (2H, q, J = 7.2 Hz), 1.18 (3H, t, J = 7.2 Hz); MS (FAB) m/z 235 (M^+); Anal. $C_{12}H_{15}IN_2OS \cdot 0.5H_2O$; C, 38.82; H, 4.27; N, 7.43. Found: C, 39.01; H, 4.46; N, 7.51.

4.5.4. Compound 3a. To a mixture of **25** (109 mg, 0.30 mmol) and compound **26** (132 mg, 0.30 mmol) in acetonitrile (3 mL) was dropwise added triethylamine (0.13 mL, 0.90 mmol). The mixture was stirred at 70 °C for 15 h. After being cooled to ambient temperature, EtOAc (3 mL) was added to the mixture, and the resulting was stirred for 1 h. The precipitate formed was collected and washed with EtOAc. The crude product was recrystallized from MeOH/EtOAc to give **3a** (118 mg, 68% yield) as green solids. Mp 251–252 °C (decomp.); IR (KBr) 1634, 1543, 1489, 1375, 1167, 1003 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.62 (1H, d, J = 6.3 Hz), 8.43 (1H, d, J = 8.8 Hz), 8.23 (1H, dd, J = 8.8, 7.5 Hz), 8.05 (1H, d, J = 6.6 Hz), 7.95 (1H, d, J = 8.8 Hz), 7.64 (1H, dd, J = 8.8, 7.8 Hz), 7.37 (1H, dd, J = 7.5, 6.6 Hz), 6.87 (1H, dd, J = 7.8, 6.3 Hz), 5.91 (1H, s), 4.20–3.95 (10H, m), 1.34–1.18 (6H, m);

MS (FAB) *m/z* 453 (M^+); Anal. $C_{23}H_{25}IN_4O_2S_2 \cdot 1H_2O$; C, 46.15; H, 4.55; N, 9.36. Found: C, 46.09; H, 4.60; N, 9.40.

4.5.5. Compound 3b. Deep purple solids; mp 246–247 °C (decomp.); IR (KBr) 1649, 1545, 1491, 1375, 1283, 1167, 1076 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.57 (1H, d, J = 6.3 Hz), 8.38 (1H, d, J = 8.5 Hz), 8.12–8.03 (2H, m), 7.72–7.58 (4H, m), 7.52–7.46 (2H, m), 7.32 (1H, dd, J = 6.3, 6.3 Hz), 7.21 (1H, d, J = 8.5 Hz), 6.89 (1H, dd, J = 8.0, 6.8 Hz), 5.75 (1H, s), 4.11 (3H, s), 4.01 (3H, s), 3.98 (2H, q, J = 7.0 Hz), 1.16 (3H, t, J = 7.0 Hz); MS (FAB) *m/z* 501 (M^+); Anal. $C_{27}H_{25}ClN_4O_2S_2 \cdot 2.5H_2O$; C, 55.71; H, 5.19; N, 9.62. Found: C, 55.67; H, 5.28; N, 9.74.

4.5.6. Compound 3d. Green solids; mp 211–212 °C (decomp.); IR (KBr) 1639, 1541, 1489, 1373, 1165 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.61 (1H, d, J = 6.1 Hz), 8.44 (1H, d, J = 8.5 Hz), 8.23 (1H, dd, J = 8.5, 7.6 Hz), 8.08 (1H, d, J = 6.1 Hz), 7.90 (1H, d, J = 8.5 Hz), 7.65 (1H, dd, J = 8.5, 8.5 Hz), 7.37 (1H, dd, J = 6.1, 7.6 Hz), 6.89 (1H, dd, J = 8.5, 8.5 Hz), 6.10–5.99 (1H, m), 5.89 (1 H, s), 5.18 (1H, d, J = 11.2 Hz), 4.93 (1H, d, J = 17.1 Hz), 4.77 (2H, m), 4.10 (3H, s), 4.09 (3H, s), 4.04 (2H, q, J = 7.1 Hz), 1.23 (3H, t, J = 7.1 Hz); MS (FAB) *m/z* 465 (M^+); Anal. $C_{24}H_{25}IN_4O_2S_2$; C, 48.65; H, 4.25; N, 9.46. Found: C, 48.67; H, 4.18; N, 9.55.

4.5.7. Compound 3e. Green solids; mp 284–285 °C (decomp.); IR (KBr) 1639, 1541, 1489, 1435, 1367, 1271 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.46 (1H, d, J = 8.5 Hz), 8.25 (1H, d, J = 8.0 Hz), 8.16 (1H, d, J = 6.5 Hz), 7.93 (1H, d, J = 8.5 Hz), 7.75 (1H, dd, J = 8.5, 7.3 Hz), 7.69 (1H, dd, J = 8.5, 7.3 Hz), 7.51 (1H, dd, J = 8.0, 7.3 Hz), 6.98 (1H, dd, J = 7.3, 6.5 Hz), 6.66 (1H, s), 4.69 (2H, q, J = 7.2 Hz), 4.33–4.20 (4H, m), 4.41 (3H, s), 1.43–1.32 (m, 6H), 1.27 (3H, t, J = 7.2 Hz); MS (FAB) *m/z* 523 (M^+); Anal. $C_{26}H_{27}BrN_4O_2S_3$; C, 51.73; H, 4.51; N, 9.28. Found: C, 52.04; H, 4.49; N, 9.48.

4.5.8. Compound 3f. Green solids; mp 259–261 °C (decomp.); IR (KBr) 1647, 1504, 1375, 1168 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.68 (1H, d, J = 6.5 Hz), 8.29 (1H, dd, J = 8.5, 7.2 Hz), 8.00 (1H, d, J = 8.5 Hz), 7.77–7.66 (3H, m), 7.50–7.43 (2H, m), 7.40 (1H, dd, J = 7.2, 6.5 Hz), 7.10 (1H, d, J = 8.0 Hz), 6.01 (1H, s), 4.15 (3H, s), 4.13–4.02 (4H, m), 1.32–1.21 (6H, m); MS (FAB) *m/z*503 (M^+); Anal. $C_{27}H_{27}IN_4O_2S_2 \cdot 1.1H_2O$; C, 49.86; H, 4.53; N, 8.61. Found: C, 49.79; H, 4.62; N, 8.91.

4.5.9. Compound 3g. Red solids; mp >300 °C; IR (KBr) 1655, 1497, 1379, 1285, 1173, 1065 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.67 (1H, d, J = 6.5 Hz), 8.30 (1H, dd, J = 8.2, 7.7 Hz), 7.99 (1H, d, J = 8.2 Hz), 7.83 (1H, d, J = 7.7 Hz), 7.57–7.40 (4H, m), 7.25 (1H, dd, J = 7.5, 7.5 Hz), 5.97 (1H, s), 4.20–4.09 (5H, m), 4.08–3.98 (5H, m), 1.29 (3H, t, J = 7.0 Hz), 1.24 (3H, t, J = 7.0 Hz); MS (FAB) *m/z* 509 (M^+); Anal. $C_{25}H_{25}IN_4O_2S_3 \cdot 1H_2O$; C, 45.87; H, 4.16; N, 8.56. Found: C, 45.68; H, 4.32; N, 8.61.

4.5.10. Compound 3j. Deep purple solids; mp 242–243 °C (decomp.); IR (KBr) 1626, 1541, 1485, 1369, 1285, 1155, 1003 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.61 (1H, d, J = 6.0 Hz), 8.24 (1H, dd, J = 8.2, 7.5 Hz), 7.95 (1H, d, J = 8.2 Hz), 7.78–7.69 (2H, m), 7.50–7.41 (2H, m), 7.36 (1H, dd, J = 6.0, 7.5 Hz), 5.91 (1H, s), 4.24–4.05 (7H, m), 3.84 (6H, s), 1.31 (3H, t, J = 7.0 Hz), 1.25 (3H, t, J = 7.0 Hz); MS (FAB) *m/z* 506 (M^+); Anal. $C_{26}H_{28}IN_5O_2S_2 \cdot 0.5H_2O$; C, 48.60; H, 4.55; N, 10.90. Found: C, 48.49; H, 4.57; N, 10.81.

4.6. Biological experiment (malaria parasites)

4.6.1. In vitro antimalarial assay. *Plasmodium falciparum* K1 strain (a clone originating from Thailand) was used in this study. The strain was maintained in RPMI 1640 medium with 0.36 mM hypoxanthine, supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 25 mM $NaHCO_3$, neomycin (100 U/mL), and 5 g/L of Albumax® II (lipid-rich bovine serum albumin, GIBCO, Grand Island, NY, USA), together with 5% washed human A^+ erythrocytes. All cultures and assays were conducted at 37 °C under an atmosphere of 4% CO_2, 3% O_2, and 93% N_2. Cultures were kept in incubation chambers filled with the gas mixture. Subcultures were diluted to a parasitemia of between 0.1% and 0.5% and the medium was changed daily. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL and heated or sonicated if necessary to dissolve the sample. For the assay, the compound was further diluted in serum-free culture medium and finally to the appropriate concentration in complete medium without hypoxanthine. The DMSO concentration in the wells with the highest drug concentration did not exceed 1%.

Assays were performed in sterile 96-well microtiter plates, each well containing 200 μL of parasite culture (0.15% parasitemia, 2.5% hematocrit) with or without serial drug solutions. Seven 2-fold dilutions were used, covering a range from 5 to 0.078 μg/mL. Each drug was tested in duplicate and the assay was repeated for active compounds showing an EC_{50} below 1.0 μg/mL. After 48 h of incubation at 37 °C, 0.5 mCi 3H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before being harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results are recorded as counts per minute per well at each drug concentration and expressed as percentage of the untreated controls. EC_{50} values are calculated from the sigmoidal inhibition curves.

4.6.2. In vitro cytotoxicity assay. The rat skeletal myoblast cell line (L-6 cells) was used to assess cytotoxicity in host cells. The cells were grown in RPMI 1640 medium supplemented with 1% L-glutamine (200 nM) and 10% fetal bovine serum in T-25 tissue culture flasks at

37 °C in 5% CO_2 in air. The cultures were subpassaged three times a week using trypsin to detach the cells and split in a 1:2 or 1:3 ratio depending on the density of the parent culture. Stock drug solutions were prepared in 100% DMSO at 10 mg/mL. For the assays, the compound is further diluted to the appropriate concentration using complete medium. The DMSO concentration in the wells with the highest drug concentration did not exceed 1%.

Assays were performed in 96-well microtiter plates, each well receiving 100 μL of culture medium with 4×10^4 cells. After 24 h, the medium was removed from all wells and replaced by 100 μL of fresh medium in all wells except for those in row H of the plate. Fresh medium (150 μL) containing the highest drug concentration was added to wells of row H. Serial drug dilutions were prepared by transferring 50 μL from wells of row H to wells of row G. After gentle mixing 50 μL from row G was transferred to row F, and so on. The highest concentration for the test compounds was 200 μg/mL. Seven 3-fold dilutions were used, covering a range from 200 to 0.274 μg/mL. Each drug was tested in duplicate. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Then, 10 μL of Alamar blue (12.5 mg resazurin dissolved in 100 mL distilled water) was added to each well and the plates were incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. EC_{50} values are determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

4.6.3. In vivo antimalarial assay. In vivo antimalarial activities of β-carbolinium salts were determined in mice infected with *P. berghei* (NK 65 strain). Five-week-old ICR male mice obtained in sterile containers from Japan SLC, Inc. (Hamamatsu, Japan) weighing 22–25 g were used. They were housed under a natural day–night cycle at 25 °C. The mice were randomly assigned to treated groups and housed in cages each containing five individuals. The heparinized blood collected by cardiac puncture from a donor mouse with approximately 20% parasitemia was taken and diluted in saline to 5×10^6 parasitized erythrocytes per mL. An aliquot (0.2 mL) of this suspension was injected intravenously (iv) into experimental groups of five mice and a control group of five mice. Test compounds were prepared at each dose in saline or SSV and administered once a day from day 0 to 3. The first administration of the test compound started intraperioneally or intravenously 2 h post-infection. Test compounds were formulated in saline (0.9% NaCl) for intravenous dosing and in SSV (0.5% sodium carboxymethylcellulose, 0.5% benzyl alcohol, and 0.4% Tween 80 in 0.9% NaCl) for intraperioneal dosing. Parasitemia levels were determined on day 4. To evaluate the antimalarial activity of the compounds, the tail blood smears were prepared and stained with Diff-Quik. Total 1000 erythrocytes per thin blood film were examined under microscopy (Leica, CTR 6000). The difference between the mean of the control group and those of the experimental groups was calculated and expressed as a percent relative to the control group (= %suppression). The care and treatment of mice were in accordance with the guidelines (No. 141, 1987) issued by the Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Science and Technology.

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isomers of rhodacyanines, the structures shown in the Schemes and Charts are depicted as a single geometrical isomer. It is well known that the conjugated double bonds can be easily isomerized in the solution, and the trans geometrical isomers would be thermodynamic products.

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