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Antimalarial Activity of 9a-*N* Substituted 15-Membered Azalides with Improved in Vitro and in Vivo Activity over Azithromycin

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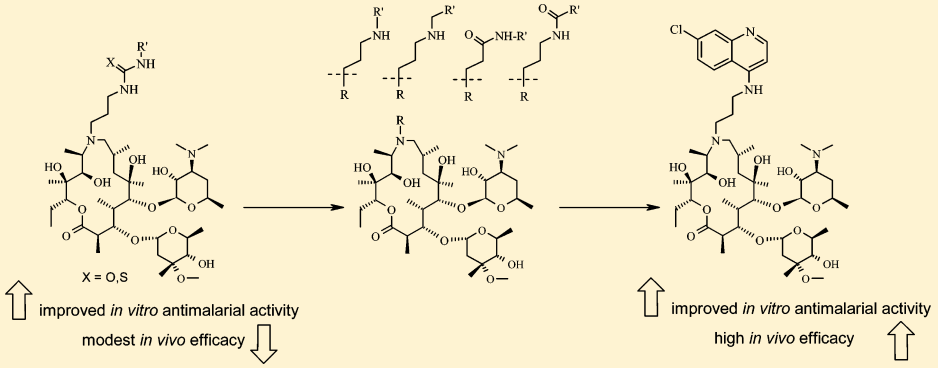
Supporting Information



ABSTRACT: Novel classes of antimalarial drugs are needed due to emerging drug resistance. Azithromycin, the first macrolide investigated for malaria treatment and prophylaxis, failed as a single agent and thus novel analogues were envisaged as the next generation with improved activity. We synthesized 42 new 9a-*N* substituted 15-membered azalides with amide and amine functionalities via simple and inexpensive chemical procedures using easily available building blocks. These compounds exhibited marked advances over azithromycin in vitro in terms of potency against *Plasmodium falciparum* (over 100-fold) and high selectivity for the parasite and were characterized by moderate oral bioavailability in vivo. Two amines and one amide derivative showed improved in vivo potency in comparison to azithromycin when tested in a mouse efficacy model. Results obtained for compound **6u**, including improved in vitro potency, good pharmacokinetic parameters, and in vivo efficacy higher than azithromycin and comparable to chloroquine, warrant its further development for malaria treatment and prophylaxis.

INTRODUCTION

Malaria continues to be one of the most widespread infectious diseases of our time. This disease is caused by infection with one or more protozoan parasites of the genus *Plasmodium* in humans. *Plasmodium falciparum* is responsible for the most serious form of the disease that could be complicated by coma and death if left untreated. The malaria infection is estimated to affect 250 million people worldwide leading to almost 1 million fatal outcomes each year.[1] Sub-Saharan Africa is the most distressed region where the vast majority of deaths occur in children under five years of age and in pregnant women. Even though during the past century a large number of drugs for the treatment of malaria have been developed, their utility in curing malaria is declining due to the selection of single-drug or multidrug resistant parasites. This further fosters the need for the discovery of new antimalarial drugs.[2]

Azithromycin is a semisynthetic macrolide antibiotic that has become a blockbuster drug due to its wide spectrum of activity and superior pharmacokinetic and safety properties. Antimalarial potential of azithromycin was discovered by Walter Reed Army Institute of Research.[3−6] It was demonstrated that azithromycin is a slow acting antimalarial[7,8] that exerts its activity by inhibiting protein synthesis on the prokaryote-like ribosome in *Plasmodium* organelle, the apicoplast.[9] Currently, azithromycin is being extensively evaluated in clinical trials to assess its potential use and combination partner in different

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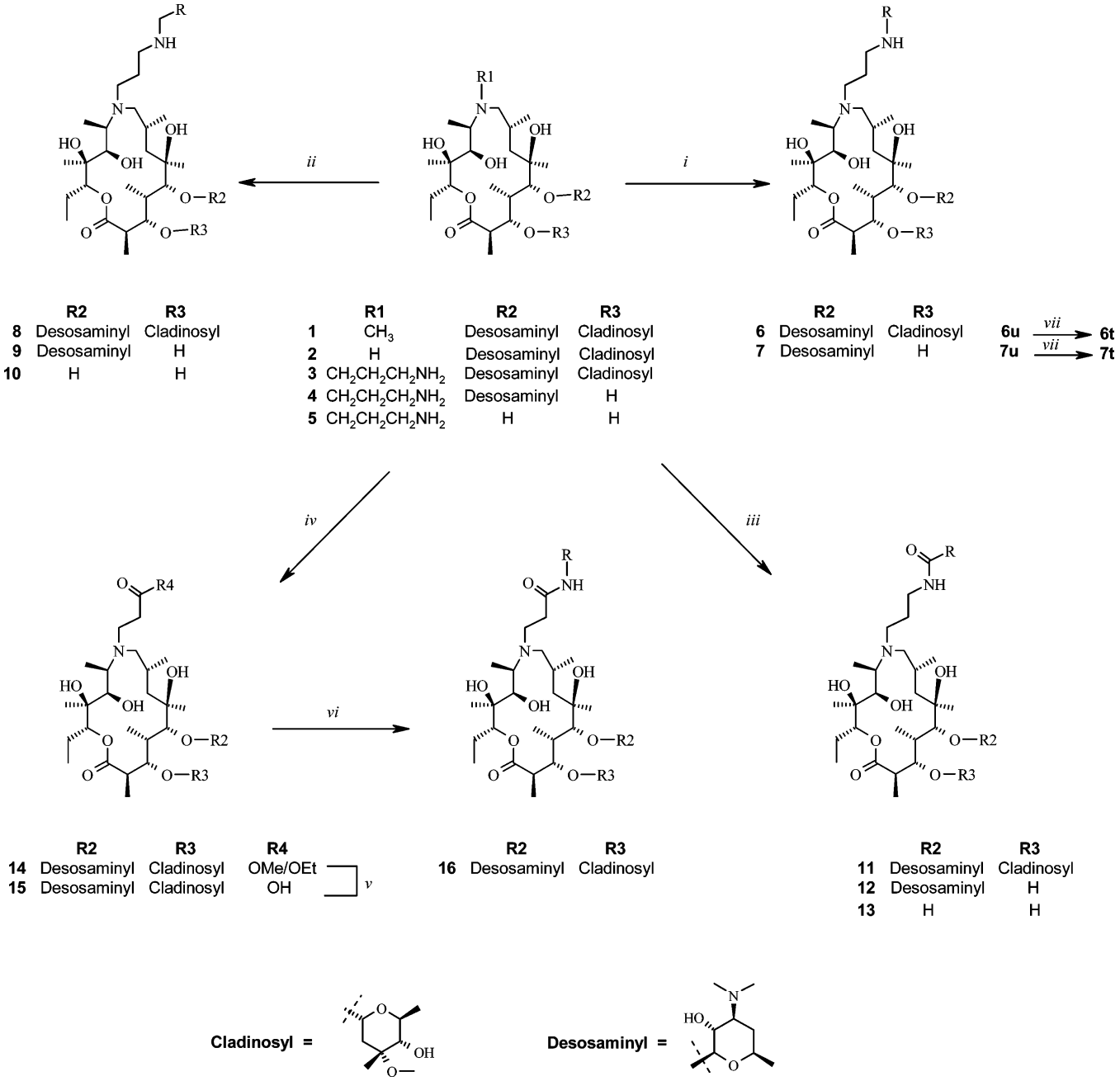
Scheme 1. Synthesis of Novel 15-Membered Azalides[a]



	R2	R3
8	Desosaminyl	Cladinosyl
9	Desosaminyl	H
10	H	H

	R1	R2	R3
1	CH_3	Desosaminyl	Cladinosyl
2	H	Desosaminyl	Cladinosyl
3	$CH_2CH_2CH_2NH_2$	Desosaminyl	Cladinosyl
4	$CH_2CH_2CH_2NH_2$	Desosaminyl	H
5	$CH_2CH_2CH_2NH_2$	H	H

	R2	R3
6	Desosaminyl	Cladinosyl
7	Desosaminyl	H

	R2	R3	R4
14	Desosaminyl	Cladinosyl	OMe/OEt
15	Desosaminyl	Cladinosyl	OH

	R2	R3
16	Desosaminyl	Cladinosyl

	R2	R3
11	Desosaminyl	Cladinosyl
12	Desosaminyl	H
13	H	H

[a]Reagents and conditions: *i*, **3** or **4**, arylhalide, DMSO, 100 °C, 14 h; *ii*, **3**–**5**, aldehyde, TEA, $NaBH_4$, MeOH, rt, 24 h; *iii*, **3**–**5**, carboxylic acid, HOBT, EDCxHCl, TEA, DCM, rt, 24 h; *iv*, **3** (to compound **14**), CH_2=CHCOR4, $CHCl_3$, reflux, 48 h; *v*, LiOH, H_2O, THF, rt, 3 h; *vi*, **15**, (alkyl)aryl amine, HOBT, EDCxHCl, TEA, DCM, rt, 24 h; *vii*, H_2, 10% Pd/C, EtOH, 4 bar, rt.

malaria-related indications.[10−19] As no evidence for the superiority or equivalence to other antimalarials has been confirmed so far, the future of azithromycin for the treatment of malaria is uncertain.[20]

Antimalarial drug discovery today is focusing efforts on creating new molecules that are more potent and safer than currently available drugs and at the same time affordable to the target underprivileged population.[2] These drugs should be dosed orally and infrequently to ensure better patient compliance. A very important feature of any potential new class is the activity against strains resistant to other antimalarial drug classes.[7] Because of azithromycin-specific mode of action,[21] oral bioavailability, long half-life, and good safety record proven in children and pregnant women, we propose more potent analogues from this class could offer properties needed for malaria treatment and prophylaxis.

We recently reported extensive synthesis of new 9a-substituted 15-membered azalide compound libraries comprising of (thio)urea derivatives, investigated their in vitro and in vivo antimalarial activity,[22] and analyzed the structure−activity relationship.[22,23] This research led to the discovery of novel urea and thiourea derivatives of 15-membered azalides with promising in vitro antimalarial activity. Here we report additional work undertaken in this area to avoid initial

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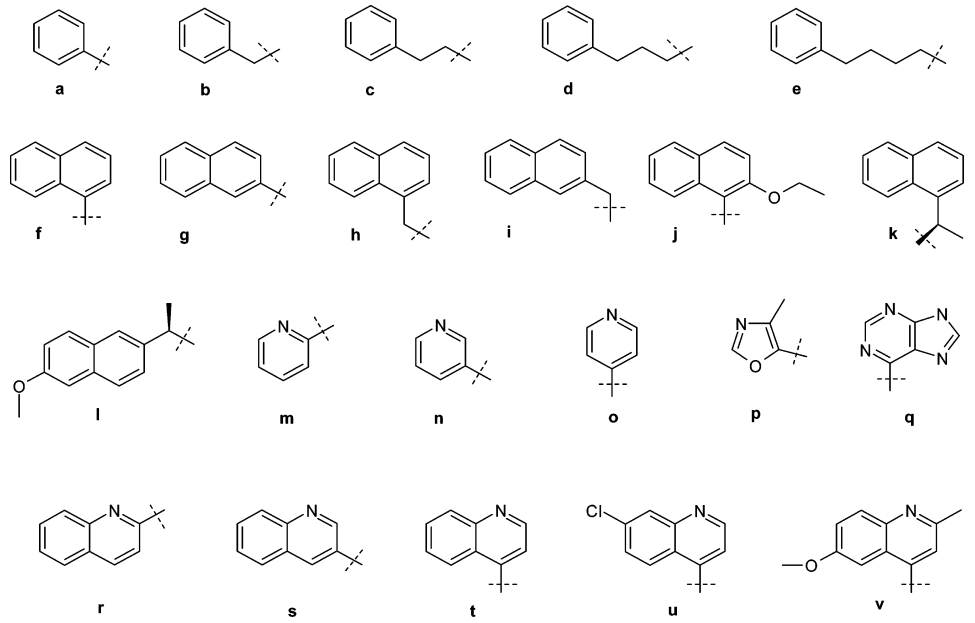
Chart 1. Structures of the Substituents (R) Used in This Study



difficulties with in vivo efficacy. A set of 42 novel 9a-*N* substituted amide and amine azalide compounds were synthesized, their in vitro antimalarial activity was determined, and structure−activity relationship (SAR) was established. Furthermore, five compounds were selected for further profiling by investigating their pharmacokinetic properties and in vivo antimalarial efficacy in an adapted *Plasmodium berghei* mouse model.

CHEMISTRY

Identification of promising lead molecules as starting points for new antimalarial programs represents a persistent challenge for scientists within the field.[24] Because a preliminary library of 9a-*N* substituted urea and thiourea azalide derivatives has set forth compounds with improved in vitro antimalarial activity over azithromycin,[22,23] we expanded research in this field. Here we describe the discovery of novel classes of 9a-*N* substituted azalides that were designed to additionally explore the chemical space around 9a-*N* substituted 15-membered azalide scaffolds. In the view of the stringent requirement for novel antimalarials to enable low cost therapy for uncomplicated malaria and intermittent preventive treatment (less than one US dollar per treatment),[2] we designed novel 9a-*N* substituted derivatives as low cost compounds by using inexpensive starting materials and short synthetic procedures. Structural features that guided the design of novel macrolides included (1) replacement of the ureido/thioureido group with an amido and amino functionality and (2) retaining the aryl or heteroaryl group for improving antimalarial activity.[25]

Here we report the synthesis of 42 novel compounds belonging to four new series of 15-membered azalides (Scheme 1). Three series were completed starting from corresponding primary amine intermediates **3**−**5**, the synthesis and characterization of which was described previously.[26−28] Amine compounds **6** and **7** were prepared by the substitution of primary amines **3** and **4** with aryl and heteroaryl halides at elevated temperature. The chlorine atom from the quinoline moiety was removed by catalytic hydrogenation of compounds **6u** and **7u** yielding compounds **6t** and **7t**. Amine compounds **8**−**10** were obtained by reductive alkylation of amines **3**−**5** with appropriate aldehydes in the presence of triethylamine (TEA) and sodium borohydride. Amides **11**−**13** were prepared by acylation of corresponding amines **3**−**5** with various acids in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) in dichloromethane at room temperature. On the other hand, amides **16** were obtained by esterification of carboxylic acid **15** with various amines using the above-described conditions for the preparation of amides **11**−**13**. Starting carboxylic acid **15** was prepared by the Michael-type addition of amine **2** with methyl or ethyl acrylate followed by ester hydrolysis of **14** under basic conditions.[29]

RESULTS AND DISCUSSION

In Vitro Activity. The in vitro activity of 42 amides and amines of 15-membered azalides was evaluated against *P. falciparum* strain TM91C235, a multidrug resistant clone from Southeast Asia and HepG2 hepatocellular carcinoma cell line. The results presented as IC_{50} values are shown in Table 1 for compounds from two amine classes and in Table 2 for two different amide scaffolds.

Results from antimalarial in vitro screening indicated a high percentage of compounds (>90%) with substantially improved activity over azithromycin (up to >100-fold for the most potent compounds) and a majority of compounds (>60%) with IC_{50} at least 10-fold enhanced. As already observed with (thio)urea derivatives,[25] classes of compounds described here also exhibit higher in vitro potencies against resistant strains than against the sensitive strain (data not shown).

The presented library discloses compounds from an early lead-optimization phase where the influence of major structural features on the antimalarial activity was investigated, primarily the removal of cladinose and desosamine sugars, type of linker at the 9a-*N* position and the nature of the aromatic substituent R (Scheme 1). As sugar substiutents, L-cladinose and D-desosomine, present in azithromycin have been shown to contribute substantially to its antibacterial activity,[30] we investigated the significance of sugar unit(s) on antimalarial

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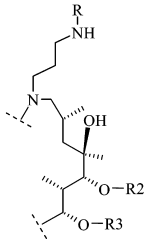
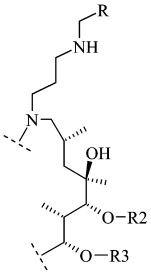
Table 1. In Vitro Activity of 9a-*N* Substituted Amine Derivatives 6−10 Determined against *P. falciparum* TM91C235 Strain and HepG2 Hepatocellular Carcinoma Cell Line

					IC_{50}/nM	IC_{50} /μM
General formula	Cmpd.	R2[a]	R3[b]	R	*P. falciparum* TM91C235	HepG2
azithromycin	**1**	des	clad	-	2654	>50
	**6q**	des	clad		>6043	>50
	6t	des	clad		25	>50
	7t	des	H		437	>50
	6u	des	clad		22	30
	7u	des	H		13	35
	6v	des	clad		854	>50
	8a	des	clad		104	>50
	8c	des	clad		106	40
	8d	des	clad		51	16
	**8f**	des	clad		25	10
	9f	des	H		55	38
	8j	des	clad		26	7
	8m	des	clad		1478	ND
	8n	des	clad		391	>50
	9n	des	H		2506	>50
	8o	des	clad		568	>50
	9o	des	H		3980	>50
	8r	des	clad		62	28
	9r	des	H		164	ND
	10r	H	H		1969	>50
	8s	des	clad		50	45
	9s	des	H		201	ND
	10s	H	H		1886	>50
	8t	des	clad		165	>50
	9t	des	H		264	>50

[a]des = D-desosamine. [b]clad = L-cladinose, ND = not determined.

activity by comparing activities of paired analogues with the same 9a-*N* substituent and different sugar content (**11p**, **13p**; **8o**, **9o**; **8n**, **9n**; **8t**, **9t**; **11h**, **12h**; **8r**, **9r**, **10r**; **10l**, **11l**, **12l**; **8s**, **9s**, **10s**; **8f**, **9f**; **6t**, **7t**; **6u**, **7u**). All aglycones (**10r**, **10s**, and **13p**) were 3−4-fold less active in comparison to their decladinosyl analogues (**9r** and **9s**) and 24−37-fold less active than analogues with both sugars (**8r**, **8s**, and **11p**). Furthermore, the activity of decladinosyl derivatives was lower than the activity of corresponding analogues with both sugars (i.e., IC_{50} of **8o** < **9o**; **11h** < **12h**; **6t** < **7t**) with one exception (IC_{50} of **6u** > **7u**).

The influence of the linker at the 9a-*N* position and the nature of the aromatic moiety on antimalarial activity were analyzed in more detail by examining the amine (**6** and **8**) and amide (**11** and **16**) compounds containing both sugars. Generally, phenyl derivatives (**8a**, **11b**, and **16b**, IC_{50} of 104, 233, and 261, respectively) were significantly more active in comparison to other tested monocyclic aromatic derivatives, that is, five-membered heteroaromatic oxazole derivative **11p** (IC_{50} of 878) and six-membered heteroaromatic pyridine derivatives (**8m**, **8n**, and **8o**, IC_{50} of 1478, 391, and 568, respectively). On the other hand, phenyl derivatives (**8f**, **11i**, **11h**, and **16h**) were less active than their naphthyl analoques

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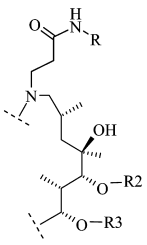
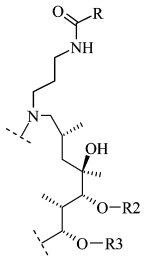
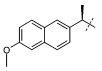
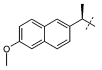
Table 2. In Vitro Activity of 9a-*N* Substituted Amide Derivatives 11−13 and 16 Determined against *P. falciparum* TM91C235 Strain and HepG2 Hepatocellular Carcinoma Cell Line

General formula	Cmpd.	R2[a]	R3[b]	R	IC_{50}/nM *P. falciparum* TM91C235	IC_{50} /μM HepG2
azithromycin	**1**	des	clad	-	2654	>50
	**16b**	des	clad		261	>50
	16c	des	clad		367	>50
	16d	des	clad		89	40
	16e	des	clad		162	40
	16h	des	clad		79	49
	16k	des	clad		95	18
	11b	des	clad		233	>50
	11c	des	clad		70	>50
	11d	des	clad		85	>50
	11e	des	clad		92	49
	**11i**	des	clad		79	40
	11h	des	clad		62	ND
	12h	des	H		1368	ND
	11l	des	clad		58	20
	12l	des	H		520	ND
	11p	des	clad		878	ND
	13p	H	H		>21340	ND

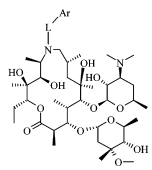
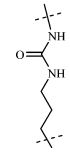
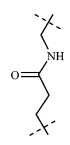
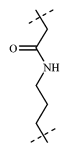
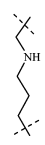
[a]des = D-desosamine. [b]clad = L-cladinose, ND = not determined.

(**8a**, **11b**, and **16b**). Quinolyl derivatives (i.e., **6t**, **6u**, **8r**, and **8s**) showed excellent antimalarial activity, suggesting that steric bulk and heteroatom in bicyclic aromatic system might have a positive impact on the antimalarial potency. Introduction of a chlorine atom on the quinoline moiety retained superior antimalarial activity irrespective of the presence of cladinose (**6u** and **7u**). However, antimalarial activity significantly decreased when the methoxy group was positioned on C-6 of the quinoline ring (compound **6v**, IC_{50} of 854) in contrast to the positive influence of electron donating groups on naphthyl ring derivatives (**8j** and **11l**).

SAR observations regarding linker influence were expanded comparing our earlier published 9a-*N* substituted 15-membered azalides having urea and thiourea moieties in the linker[22,23] with amide and amine analogues reported here. The most populated analogue sets with naphthyl and phenyl aromatic moieties (Table 3) reveal a significant effect of the linker used on antimalarial activity. The activity increased among classes as follows: urea > amide (**16b**, **16h**, **11b**, **11h**) > amine (**8a**, **8f**).

Occasionally, compounds with increased antimalarial potency also showed a slight increase in cytotoxicity (Tables 1 and 2). However, IC_{50} values determined in HepG2 cell line were relatively high (85% of compounds had $IC_{50} \geq 20$ μM) generating favorable selectivity index (ratio between HepG2

Table 3. Structure−Activity Relationship of Amide (16b, 16h, 11b, 11h) and Amine (8a, 8f) Linked Derivatives Compared with Urea Derivatives,[31] All Containing Analogous Aromate Substitutents (IC_{50} Determined against *P. falciparum* TM91C235 Strain)

		IC_{50}/nM			
		L			
					
Ar		911	261 (**16b**)	233 (**11b**)	104 (**8a**)
		131	79 (**16h**)	62 (**11h**)	25 (**8f**)

IC_{50} and *P. falciparum* IC_{50} above 100) in almost all cases where calculations were possible. The overall selectivity index for novel 9a-*N* substituted amides (**11−13** and **16**) and amines (**6−10**) was superior to the selectivity index of previous classes

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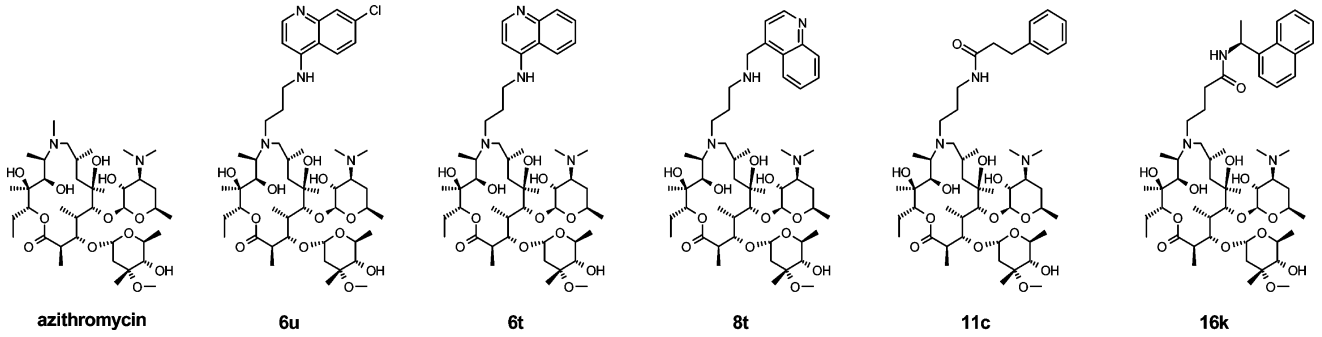
Table 4. Pharmacokinetic Parameters Estimated in Blood after Intravenous (IV) and Oral Gavage (PO) Administration to CD-1 Mice (5 mg/kg IV and 25 mg/kg PO)[a]

	CLs (mL/min/kg)	V_{ss} (L/kg)	$T_{1/2}$ (h)	oral F (%)	fu	CLu (mL/min/kg)
azithromycin	16 ± 5.3	8.8 ± 3.6	8.3 ± 1.1	72	0.69	23.2
6u	4.0 ± 0.2	2.9 ± 1.3	17.8 ± 9.4	20.4	0.42	9.5
6t	7.7 ± 1.5	4.9 ± 1.5	14.4 ± 1.1	1.7	0.39	20
8t	10.2 ± 0.3	4.9 ± 1.1	8.7 ± 1.6	19.5	0.57	18
11c	23.3 ± 3.3	5.0 ± 1.3	6.9 ± 0.7	12.7	0.43	54
16k	10.5 ± 3.3	7.8 ± 2.1	16.5 ± 3.5	23.6	0.13	81

[a]CL = blood clearance, V_{ss} = steady-state volume of distribution, $T_{1/2}$ = half life, F = bioavailability, fu = fraction unbound in mouse plasma, CLu = CLs/fu, assuming Cbl/Cpl = 1, [a] = n = 1, [b] = n = 2.

Chart 2. Structures of the Azalide Compounds Used for in Vivo Study in the Mouse Model



consisting of urea and thiourea derivatives[22,23] due to an overall marked improvement in antimalarial activity accompanied by decrease in cytotoxicity.

Preliminary Pharmacokinetics and in Vivo Antimalarial Activity. The in vivo pharmacokinetic profiles (Table 4) were investigated in the mouse for five compounds (Chart 2). Two amides **16k** and **11c** and three amines **6u**, **6t**, **8t** were selected based on their favorable in vitro profile (IC_{50} against *P. falciparum* < 200 nM and HepG2 IC_{50} > 30 μM) and high solubility when prepared as acetic salts (≥10 mg/kg).

In vitro metabolic stability studies in liver microsomes (mouse, rat, and human) indicated good stability across species, resulting in low intrinsic clearance (CLi < 0.6 mL/min·g liver), with the exception of compound **16k** which showed a low to moderate intrinsic CLi (1.8 mL/min·g liver) in human liver microsomes. Plasma protein binding (PPB) in the mouse suggested these compounds have low PPB ranging from 43 to 61%, with the exception of compound **16k** which was 87% protein bound.

In line with the in vitro mouse microsomal stability results, all compounds had a very low blood CL (<15% LBF), with the exception of compound **11c** which had a slightly higher CL (ca. 26% LBF). When correcting the systemic clearance for protein binding, compound **11c** had a moderate clearance, and compound **16k** had a high clearance. Overall, the tested compounds were characterized by a moderate (compounds **6t**, **8t**, and **6u**) to a large (compounds **11c** and **16k**) volume of distribution and a very long half-life. Compounds with an ureido linker functionality connecting the aromatic moiety with the macrolide 15-membered ring scaffold had a very low oral bioavailability (F_{PO} < 1%) in the mouse, and no improvement was observed with the insertion of a thiourea motif into the linker (data not shown). Replacement of the (thio)urea functionality with an amide in the linker resulted in a significant increase in oral bioavailability (**16k**, **11c**). A substantially improved oral exposure and oral bioavailability were obtained when an aminoquinoline moiety was present as aromatic substituent on the azalide unit (**6u**, **8t**).

In Vivo Efficacy Studies in Mice Models. The in vivo efficacy was evaluated for five selected azalide derivatives using the *P. berghei* mouse model, a model that is routinely used to screen larger numbers of compounds at this stage of discovery. As the efficacy in the *P. berghei* murine model with our previous set of compounds with low to moderate oral bioavailability was modest upon oral administration, the intravenous route was chosen for further screening in order to enable direct delivery of compound to the site of the infection (the blood). In addition, it has been shown that azithromycin can have a lower bioavailability when administered orally after food intake,[32] and as infected animals in this model need to have food ad libidum at all times, the IV route was chosen to avoid potential absorption issues. The dosing regimen included administration of 5 mg/kg of compound twice daily on days 3, 4, and 5 postinfection, while azithromycin, used as a control, was applied at doses 5 mg/kg and 15 mg/kg in the same dosing regimen.

None of the compounds tested, including azithromycin, cured mice (i.e., cleared them of parasitemia until day 31) at the doses administered. In order to evaluate and compare the compound efficacy, survival time of the last animal was used to assess the time needed to delay malaria-related deaths among treated animals (Table 5).

Azithromycin treated mice had a survival time ranging from 17 to 27 days at 5 mg/kg and 19 to 29 days at a dose of 15 mg/kg, depending on the experiment. Animals treated with amine derivatives **6u**, **6t**, and **8t** had longer survival times of +9, +3, and +3 days, respectively, in comparison to the azithromycin-treated group at an equivalent dose. The amide derivatives **11c** and **16k** exhibited shorter survival times than azithromycin group, −12 and −9 days, respectively.

On the basis of this initial efficacy screening result, compound **6u** was selected to be further profiled in a dose ranging study using the same animal model (Table 6) at doses

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Table 5. In Vivo Efficacy of Five Selected Compounds in the *P. berghei* Mice Model (at 5 mg/kg, IV, BID, for 3 days) Expressed As Survival Relative to Azithromycin

	survival relative to azithromycin[a] (± days)
azithromycin	0
6u	+9
6t	+3
8t	+3
11c	−12
16k	−9

[a]Survival of the last animal alive in the compound dosing group compared to the survival of the last animal in the azithromycin group expressed in days (more (+) or less (−) than azithromycin).

Table 6. Efficacy of Compound 6u in *Plasmodium berghei* Infected Mice Following Three Days of Intravenous (IV) and Intraperitoneal (IP) Dosing

		survival of cured animals[a]	
compound	dose (mg/kg)	IV	IP
6u	20	5/5	5/5
	15	5/5	3/5
	10	1/5	0/5
azithromycin	20	2/5	4/5
	15	1/5	3/5
	10	0/5	2/5
chloroquine	10	2/5	2/5
	5	0/5	0/5
	2.5	0/5	0/5
	1.25	0/5	0/5
control		0/5	0/5

[a](no. of mice without parasitemia on day 31)/(total no. of mice at doses).

of 20, 15, and 10 mg/kg BID for 3 days by IV (intravenous) and IP (intraperitoneal) route. Azithromycin and chloroquine were included as comparator drugs and administered in the same dosing. Azithromycin was administered at equivalent doses as **6u**, whereas chloroquine was dosed at lower doses 10, 5, 2.5, and 1.25 mg/kg due to safety issues.

Upon IV administration, compound **6u** cured 5/5 mice at doses of 20 and 15 mg/kg and also IP at a dose of 20 mg/kg. At equivalent doses and administration routes, azithromycin cured only 2/5, 1/5, and 4/5 mice, respectively. Chloroquine only cured mice at the highest dose 10 mg/kg (2 out of 5) both following IV and IP administration. Following IV administration of **6u** and azithromycin at 10 mg/kg, 1/5 mice and 0/5 mice were cured respectively, whereas upon IP administration at this dose 0/5 mice were cured with compound **6u** and 2/5 mice were cured with azithromycin, respectively. These in vivo results demonstrate superior efficacy of compound **6u** to azithromycin upon IV administration. The in vivo efficacy observed in the *P. berghei* murine model confirmed an improvement in potency of this new derivative over azithromycin, as already observed in the in vitro *P. falciparum* test.

DISCUSSION

Novel antimalarial agents are urgently needed, particularly the ones that would enable infrequent dosing, long lasting effect, and parasite clearance efficacy. Macrolides are a class of anti-infective agents that could provide these desired properties, and their chemical modifications seem a valid stream for investigation. The amide and amine series of 15-membered azalides presented in this publication demonstrate a marked improvement in in vitro antimalarial activity in comparison to azithromycin, as well as over our previous set of compounds.[22] Nevertheless, improved in vivo activity in comparison to azithromycin for compounds screened in the *P. berghei* mouse model was only observed with three (**6u**, **6t**, and 8t) of five amine derivatives following intravenous dosing. Although the overall disposition kinetics for all five amine derivatives were similar (low systemic clearance, moderate to large volume of distribution, very long half-lives), unbound clearance values for these compounds differ. The unbound clearance correlates well with the in vivo efficacy results in the *P. berghei* mouse model such that the most efficacious compounds, **6u**, **6t**, and **8t**, have a low clearance, suggesting that the relative efficacy of these compounds may be influenced by the AUC/IC_{50} ratio. Furthermore, it has been reported that macrolides' lipophilicity and cationic nature influence their accumulation and retention in different cell types;[33] thus unequal distribution into different blood cell types could also be the reason for their different efficacy effects in this murine model.

The fact, however, remains that the level of improvement in in vitro activity did not fully translate in vivo. Even though aforementioned differences in unbound clearance in vivo could contribute to this effect, the most likely cause of the lack of in vitro−in vivo correlation is the difference in parasite species used in these two assays: *P. falciparum* vs *P. berghei*. This effect was already presumed with our previous set of compounds and has also been observed with some other compound classes.[22] The *P. berghei* murine model is more convenient for screening of numerous compounds and is thus used frequently in early stages of drug discovery; however it is not necessarily representative of *P. falciparum* human malaria. Thus, future evaluation of the most active compounds should be performed in some of the available *P. falciparum* animal models such as the *Aotus* monkey or its nonprimate replacement the humanized mouse model.[36,37]

Overall, compound **6u** has several interesting properties that justify its progression into further screens as a promising antimalarial drug candidate: it is in vitro superior to azithromycin and has macrolide-like pharmacokinetics and in vivo efficacy in doses lower than azithromycin and comparable to chloroquine (when molar concentrations were considered). Key chemical features of compound **6u** include its two distinctive pharmacologically active elements, the azalide and chloroquinoline moieties, which could both contribute to the overall potency. Some medicinal chemistry approaches advocate hybrid chemical entities, comprising two pharmacophores, that offer a dual mode of action by affecting two different targets. In order to validate if compound **6u** could be considered a hybrid molecule, a set of assays verifying whether it possesses macrolide mode of action (like inhibition of apicoplast protein synthesis and/or delayed death phenotype) as well as aminoquinoline mode of action (inhibition of heme polymerization) will be performed. Further evaluation of compound **6u**, such as extensive evaluation of its ADME properties and safety are also warranted in order to assess its full potential for the clinical use in malaria treatment and prophylaxis.

CONCLUSIONS

We report here the synthesis and antimalarial activity of the novel class of potent macrolides substituted with amide and amine function at the 9a-*N* position of the 15-membered azalide scaffold. The compounds were synthesized via a simple and inexpensive chemical procedure using easily available building blocks to respond to the demand for low-cost novel antimalarial agents. When compared to our previous class of compounds, 15-membered substituted (thio)ureas,[22] these classes exhibited marked improvements with regard to the in vitro antimalarial activity, selectivity, PK properties, and in vivo efficacy. The best compound from this novel set of molecules, compound **6u**, showed improvement over azithromycin >100 fold in the *P. falciparum* in vitro assay that translated in the in vivo efficacy after IV dosing by curing mice more efficiently than azithromycin and comparable to chloroquine. Detailed pharmacology, safety, and mode of action studies are additionally needed to reveal the true potential of this class, and compound **6u** in particular, for its clinical use in the treatment and prophylaxis of malaria.

EXPERIMENTAL SECTION

Chemistry. All commercial reagents (Merck, Sigma-Aldrich) were used as provided unless otherwise indicated, and all solvents are of the highest purity unless otherwise noted. The purity of final compounds was assessed by the analytical LC-MS method and found to be ≥95% unless otherwise stated. The LC-MS analyses were performed using Waters Acquity UPLC instrument equipped with diode-array detector and MS detector, Waters SQD, using the following method: column, Waters Acquity UPLC BEH C18, 2.1 × 50 mm, 1.7 μm particles; mobile phase A, 0.1% HCOOH in water, mobile phase B, 0.1% HCOOH in CH_3CN, isocratic 5% B in 1.5 min, then gradient 5−80% B in 7.25 min followed by 1.25 min at 90% B.

Mass spectra were obtained on a Waters Micromass ZQmass spectrometer for ES^+-MS. Electrospray positive ion mass spectra were acquired using a Micromass Q-Tof2 hybrid quadrupole time-of-flight mass spectrometer, equipped with a Z-spray interface, over a mass range of 100−2000 Da, with a scan time of 1.5 s and an interscan delay of 0.1 s in a continuum mode. Reserpine was used as the external mass calibrant lock mass ($[M + H]^+$ = 609.2812 Da). The elemental composition was calculated using a MassLynx v4.1 for the $[M + H]^+$ and the mass error quoted within ±5 ppm range. NMR spectra were recorded on a Bruker Avance DRX500 or Bruker Avance DPX300 spectrometer in CDCl3 or DMSO and chemical shifts are reported in ppm using TMS as an internal standard. In synthetic procedures, column chromatography was carried out over Merck Kieselgel 60 (230−400 mesh) or on SPE cartrige with average size silica 50 μm. Thin layer chromatography was performed on 0.24 mm silica gel plates Merck TLC 60F254. The eluent used was indicated and solvent ratios refer to volume. In general, organic solutions were dried with anhydrous Na_2SO_4 or K_2CO_3, evaporation and concentration were carried out under reduced pressure below 40 °C, unless otherwise noted.

All final compounds were isolated as amorphous solid.

9a-[3-(7-Chloro-quinolin-4-ylamino)propyl]-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (6u). 4,7-Dichloroquinoline (3.0 g, 15.5 mmol) was added to a solution of intermediate **3** (4.09 g, 5.05 mmol) in DMSO (12 mL). The reaction mixture was stirred at 100 °C for 14 h and then the mixture of H_2O (100 mL) and EtOAc (100 mL) was added. The solvents were separated and the EtOAc layer was washed with brine (50 mL), dried over K_2CO_3, and evaporated in a vacuum. The residue was precipitated from EtOAc-hexane. The precipitated solid was filtered and purified by column chromatography on silica gel using solvent system DCM/MeOH/NH_4OH = 90:9:0.5. Work up of the chromatography fraction and precipitation from EtOAc/hexane yielded compound **6u** (1.2 g, *Y* = 25%); ^1H NMR (500 MHz, DMSO-d_6) δ 8.39 (d, *J* = 5.19 Hz, 1H), 8.27 (d, *J* = 9.16 Hz, 1H), 7.77 (d, *J* = 2.14 Hz, 1H), 7.43 (dd, *J* = 2.14, 9.16 Hz, 1H), 7.28 (t, *J* = 5.34 Hz, 1H), 6.47 (d, *J* = 5.80 Hz, 1H), 4.90 (dd, *J* = 2.80, 9.80 Hz, 1H), 4.79 (d, *J* = 4.88 Hz, 1H), 4.42 (d, *J* = 7.02 Hz, 1H), 4.29 (s, 1H), 4.23 (d, *J* = 7.63 Hz, 1H), 4.07 (dq, *J* = 6.30, 9.60 Hz, 1H), 4.02 (d, *J* = 6.60 Hz, 1H), 3.95−3.97 (m, 1H), 3.61 - 3.70 (m, 1H), 3.53 (d, *J* = 6.90 Hz, 1H), 3.52 (d, *J* = 6.71 Hz, 1H), 3.23−3.27 (m, 2H), 3.22 (s, 3H), 3.03 (dd, *J* = 7.63, 10.07 Hz, 1H), 2.93−2.99 (m, 1H), 2.90 (dd, *J* = 6.71, 9.46 Hz, 1H), 2.76 (q, *J* = 6.71 Hz, 1H), 2.72 (dq, *J* = 6.00, 7.50 Hz, 2H), 2.62−2.65 (m, 1H), 2.57−2.62 (m, 1H), 2.42 (ddd, *J* = 4.12, 10.30, 12.13 Hz, 1H), 2.27 (d, *J* = 14.65 Hz, 1H), 2.21 (s, 6H), 2.09 (dd, *J* = 9.31, 13.28 Hz, 1H), 1.87−1.98 (m, 3H), 1.73−1.83 (m, 2H), 1.55−1.61 (m, 1H), 1.45−1.52 (m, 2H), 1.33−1.43 (m, 2H), 1.17 (s, 3H), 1.15 (d, *J* = 6.41 Hz, 3H), 1.13 (s, 3H), 1.10 (d, *J* = 7.32 Hz, 3H), 1.07 (d, *J* = 6.10 Hz, 3H), 1.04−1.06 (m, 1H), 1.01 (d, *J* = 6.70 Hz, 3H), 1.00 (s, 3H), 0.99 (d, *J* = 7.63 Hz, 3H), 0.84 (d, *J* = 7.02 Hz, 3H), 0.80 (t, *J* = 7.32 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 176.3, 152.0, 150.2, 149.3, 133.4, 127.7, 124.3, 124.1, 117.4, 102.0, 98.8, 95.4, 77.5, 76.6, 75.1, 74.4, 72.6, 70.6, 67.1, 64.9, 64.8, 48.9, 48.7, 41.0, 40.5, 34.9, 30.0, 25.7, 21.4, 21.4, 21.0, 18.6, 18.3, 11.0, 9.3; MS (ESI) *m*/*z* calcd for $C_{49}H_{82}N_4O_{12}Cl$ ($M + H^+$) 953.5618; found 953.5638.

9a-[3-(7-Chloro-quinolin-4-ylamino)propyl]-3-*O*-decladinosyl-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (7u). Compound **7u** (0.32 g, *Y* = 21%) was obtained according to the described procedure for compound **6u**, starting from intermediate **4** (1.5 g, 1.89 mmol) and 4,7-dichloroquinoline (0.75 g, 3.39 mmol); ^1H NMR (500 MHz, DMSO-d_6) δ 8.39 (d, *J* = 5.19 Hz, 1H), 8.29 (d, *J* = 9.16 Hz, 1H), 7.78 (d, *J* = 2.14 Hz, 1H), 7.44 (dd, *J* = 2.14, 9.16 Hz, 1H), 7.20 (t, *J* = 4.88 Hz, 1H), 6.45 (d, *J* = 5.49 Hz, 1H), 5.03 (d, *J* = 9.80 Hz, 1H), 5.00 (d, *J* = 5.80 Hz, 1H), 4.51−4.67 (m, 1H), 4.40 (d, *J* = 7.32 Hz, 1H), 4.29 (s, 1H), 4.22−4.29 (m, 1H), 3.52 (br. s., 1H), 3.46−3.50 (m, 1H), 3.44 (br. s., 1H), 3.34−3.40 (m, 1H), 3.18−3.30 (m, 2H), 3.09 (dd, *J* = 8.00, 10.00 Hz, 1H), 2.83−2.99 (m, 1H), 2.75 (br. s., 1H), 2.63−2.72 (m, 1H), 2.54−2.61 (m, 1H), 2.47−2.50 (m, 1H), 2.45 (ddd, *J* = 3.80, 10.00, 12.50 Hz, 1H), 2.22 (s, 6H), 1.98−2.09 (m, 1H), 1.85−1.97 (m, 3H), 1.74−1.85 (m, 3H), 1.54−1.64 (m, 1H), 1.41 (ddd, *J* = 7.17, 10.68, 14.19 Hz, 1H), 1.14 (d, *J* = 7.02 Hz, 3H), 1.10 - 1.14 (m, 2H), 1.08 (s, 3H), 1.09 (d, *J* = 6.30 Hz, 3H), 1.01 (s, 3H), 0.98 (d, *J* = 6.71 Hz, 3H), 0.84−0.89 (m, 6H), 0.78 (t, *J* = 7.32 Hz, 3H); ^{13}C NMR (500 MHz, DMSO-d_6) δ 7.96, 8.24, 10.60, 15.92, 17.73, 20.94, 21.26, 21.45, 26.50, 26.96, 29.12, 30.12, 36.43, 39.11, 40.30, 40.30, 40.99, 43.72, 50.91, 64.42, 68.29, 70.16, 73.31, 74.35, 75.97, 76.15, 76.15, 90.17, 98.57, 117.39, 123.80, 124.14, 127.35, 133.22, 148.98, 150.08, 151.79, 175.34; MS (ESI) *m*/*z* calcd for $C_{41}H_{66}N_4O_9Cl$ ($M+H^+$) 795.4675; found 795.4683.

9a-[3-(9H-Purin-6-ylamino)propyl]-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (6q). 6-Chloro-9H-purine (0.56 g, 3.61 mmol) and diisopropylethyl amine (1 mL) were added to a solution of intermediate **3** (0.5 g, 0.63 mmol) in *n*-butanole (2 mL). The reaction mixture was stirred at 80 °C for 18 h, than H_2O (100 mL) and CH_2Cl_2 (100 mL) were added, and the pH adjusted to 10, the layers were separated and the CH_2Cl_2 layer was washed with brine (50 mL), dried over K_2CO_3, and evaporated in a vacuum. The residue was precipitated from EtOAc-hexane and purified by column chromatography on silica gel using solvent system DCM/MeOH/NH_4OH = 90:9:0.5 yielding compound **6q** (0.30 g, *Y* = 52%); ^1H NMR (500 MHz, DMSO-d_6) δ 8.17 (br. s., 1H), 8.05 (br. s., 1H), 7.64 (br. s., 1H), 4.90 (dd, *J* = 2.44, 10.07 Hz, 1H), 4.79 (d, *J* = 4.88 Hz, 1H), 4.41 (d, *J* = 7.32 Hz, 1H), 4.28 (s, 1H), 4.21 (d, *J* = 7.63 Hz, 1H), 4.06 (dq, *J* = 6.20, 9.60 Hz, 1H), 4.02 (dd, *J* = 1.68, 5.95 Hz, 1H), 3.94 (d, *J* = 7.00 Hz, 1H), 3.62−3.69 (m, 1H), 3.51 (d, *J* = 7.00 Hz, 1H), 3.51 (d, *J* = 6.41 Hz, 1H), 3.38−3.50 (m, 2H), 3.22 (s, 3H), 3.05 (dd, *J* = 7.63, 9.77 Hz, 1H), 2.91 (dd, *J* = 7.63, 9.46 Hz, 1H), 2.84−2.89 (m, 1H), 2.71 (dq, *J* = 4.00, 7.30 Hz, 1H), 2.66−2.77 (m, 1H), 2.54−2.59 (m, 1H), 2.56 (dd, *J* = 3.00, 11.50 Hz, 1H), 2.42 (ddd, *J* = 4.00, 10.00, 12.00 Hz, 1H), 2.27 (d, *J* = 14.95 Hz, 1H), 2.22 (s, 6H), 2.04 (t, *J* = 11.60 Hz, 1H), 1.92−1.98 (m, 1H), 1.82 - 1.91 (m, 2H), 1.73−1.81 (m, 1H), 1.66−1.74 (m, 1H), 1.54−1.63 (m, 1H), 1.50−1.56 (m, 1H), 1.50 (dd, *J* = 4.88, 14.65 Hz, 1H), 1.34−1.43 (m, 1H), 1.28−1.35 (m, 1H), 1.18 (s, 3H), 1.15 (d, *J* = 6.10 Hz, 3H), 1.13 (s, 3H), 1.10 (d, *J* =

7.32 Hz, 3H), 1.07 (d, J = 5.80 Hz, 3H), 1.05 - 1.07 (m, 1H), 1.00 (s, 3H), 0.97 - 1.00 (m, 6H), 0.83 (d, J = 6.71 Hz, 3H), 0.79 (t, J = 7.32 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 9.31, 9.31, 10.90, 14.92, 18.13, 18.39, 20.89, 21.28, 21.36, 22.09, 26.96, 27.24, 27.72, 29.77, 34.74, 39.43, 40.23, 40.23, 40.23, 40.27, 44.16, 48.74, 49.75, 60.49, 62.86, 64.58, 64.76, 67.03, 70.60, 72.65, 73.38, 74.30, 75.23, 76.32, 77.29, 77.81, 82.53, 94.90, 101.91, 139.10, 152.28, 176.23; MS (ESI) m/z calcd for $C_{45}H_{80}N_7O_{12}$ (M + H$^+$) 910.5865; found 910.5859.

9a-[3-(Quinolin-4-ylamino)propyl]-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (6t). To a solution of compound **6u** (50 mg, 0.05 mmol) in EtOH (15 mL), 10% Pd/C (30 mg) was added and the reaction mixture was hydrogenated in Parr apparatus at 4 bar of hydrogen pressure for 24 h. The catalyst was filtrated off and solvent evaporated under reduced pressure. Product was purified by column chromatography (SPE column 5 g, eluent: DCM/MeOH/NH$_4$OH = 90:9:0.5) and then precipitated from EtOAc:hexane yielding compound **6t** (41 mg, Y = 89%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.38 (d, J = 5.19 Hz, 1H), 8.21 (d, J = 7.63 Hz, 1H), 7.76 (d, J = 8.24 Hz, 1H), 7.59 (td, J = 1.22, 7.63 Hz, 1H), 7.32−7.45 (m, 1H), 7.13 (t, J = 5.19 Hz, 1H), 6.44 (d, J = 5.49 Hz, 1H), 4.91 (dd, J = 2.75, 10.07 Hz, 1H), 4.79 (d, J = 4.88 Hz, 1H), 4.43 (d, J = 7.02 Hz, 1H), 4.29 (s, 1H), 4.23 (d, J = 6.71 Hz, 1H), 4.04−4.10 (m, 1H), 4.04 (dd, J = 2.00, 6.00 Hz, 1H), 4.00 (d, J = 7.30 Hz, 1H), 3.62−3.70 (m, J = 2.00, 6.20, 6.20, 6.20, 11.00 Hz, 1H), 3.54 (d, J = 7.30 Hz, 1H), 3.52 (d, J = 7.50 Hz, 1H), 3.23−3.27 (m, 2H), 3.22 (s, 3H), 3.04 (dd, J = 7.48, 9.92 Hz, 1H), 2.94−3.01 (m, 1H), 2.91 (dd, J = 5.65, 8.70 Hz, 1H), 2.76 (q, J = 7.02 Hz, 1H), 2.71 (dq, J = 1.00, 7.50 Hz, 1H), 2.62−2.66 (m, 1H), 2.60 (dd, J = 3.50, 13.20 Hz, 1H), 2.43 (ddd, J = 3.80, 10.30, 12.00 Hz, 1H), 2.27 (d, J = 14.65 Hz, 1H), 2.22 (s, 6H), 2.10 (dd, J = 9.92, 12.66 Hz, 1H), 1.87 - 2.00 (m, 3H), 1.72 - 1.84 (m, 2H), 1.59 (ddd, J = 1.50, 3.00, 11.00 Hz, 1H), 1.50 (dd, J = 4.88, 14.65 Hz, 1H), 1.45−1.51 (m, 1H), 1.33−1.42 (m, 2H), 1.19 (s, 3H), 1.15 (d, J = 6.10 Hz, 3H), 1.13 (s, 3H), 1.11 (d, J = 7.02 Hz, 3H), 1.08−1.11 (m, 1H), 1.07 (d, J = 5.80 Hz, 3H), 1.01 (d, J = 6.50 Hz, 3H), 1.01 (s, 3H), 0.99 (d, J = 7.63 Hz, 3H), 0.84 (d, J = 6.71 Hz, 3H), 0.80 (t, J = 7.32 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 9.25, 9.33, 10.90, 14.84, 18.09, 18.38, 20.89, 21.25, 21.34, 22.30, 25.65, 27.10, 27.84, 29.86, 34.72, 40.23, 40.23, 40.45, 40.90, 44.16, 48.57, 48.72, 59.58, 62.62, 64.56, 64.79, 66.99, 70.52, 72.66, 73.43, 74.27, 75.03, 76.37, 77.28, 77.82, 82.57, 94.88, 98.04, 101.93, 118.76, 121.65, 123.58, 128.55, 128.79, 148.11, 149.88, 150.47, 176.30; MS (ESI) m/z calcd for $C_{49}H_{83}N_4O_{12}$ (M + H$^+$) 919.6008; found 919.5990.

9a-[3-(Quinolin-4-ylamino)propyl]-3-*O*-decladinosyl-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (7t). According to the procedure described for compound **6t** starting from the **7t** (0.31 g, 0.39 mmol) product **7u** (0.22 g, Y = 75%) was obtained; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.38 (d, J = 5.19 Hz, 1H), 8.23 (d, J = 8.54 Hz, 1H), 7.76 (d, J = 8.24 Hz, 1H), 7.51−7.65 (m, 1H), 7.35−7.44 (m, 1H), 7.03 (t, J = 4.88 Hz, 1H), 6.42 (d, J = 5.49 Hz, 1H), 5.03 (dd, J = 1.00, 12.00 Hz, 1H), 5.00 (d, J = 5.80 Hz, 1H), 4.42 (d, J = 7.32 Hz, 1H), 4.29 (s, 1H), 4.25−4.29 (m, 1H), 3.53 (d, J = 5.80 Hz, 1H), 3.46−3.50 (m, 1H), 3.45 (s, 1H), 3.35−3.43 (m, 1H), 3.17−3.30 (m, 2H), 3.09 (t, J = 8.70 Hz, 1H), 2.85−2.99 (m, 1H), 2.76 (br. s., 1H), 2.65−2.72 (m, 1H), 2.53−2.62 (m, 1H), 2.47−2.50 (m, 1H), 2.45 (ddd, J = 4.00, 10.00, 12.00 Hz, 1H), 2.21 (s, 6H), 2.00−2.08 (m, 1H), 1.93−1.98 (m, 1H), 1.87−1.93 (m, 1H), 1.75−1.86 (m, 3H), 1.67−1.76 (m, 1H), 1.53−1.62 (m, 1H), 1.35−1.47 (m, 1H), 1.14 (d, J = 6.71 Hz, 3H), 1.09 (s, 3H), 1.08−1.13 (m, 2H), 1.09 (d, J = 6.10 Hz, 3H), 1.01 (s, 3H), 0.98 (d, J = 6.71 Hz, 3H), 0.88 (d, J = 7.50 Hz, 3H), 0.87 (d, J = 6.30 Hz, 3H), 0.78 (t, J = 7.48 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 7.96, 8.26, 10.60, 15.91, 17.74, 20.94, 21.25, 21.49, 26.52, 27.02, 29.11, 30.16, 36.37, 39.27, 40.30, 40.30, 40.98, 50.79, 58.19, 60.39, 64.41, 68.27, 70.17, 73.32, 74.34, 75.99, 76.17, 76.42, 89.86, 98.03, 103.37, 118.76, 121.70, 123.52, 128.50, 128.86, 148.18, 149.89, 150.55, 175.31; MS (ESI) m/z calcd for $C_{41}H_{69}N_4O_9$ (M + H$^+$) 761.5065; found 761.5052.

General Procedure for Reductive Alkylation. Appropriate aldehyde (1 equiv), TEA (0.3 equiv), and macrolide intermediate **3**−**5** (1.2 equiv) were added to degasses MeOH solution and the reaction mixture was stirred under N$_2$ at room temperature for 1 h. NaBH$_4$ (2 equiv) was than added and the reaction mixture was stirred overnight at room temperature. The MeOH was evaporated and the residue extracted between DCM and water yielding crude products which were purified on SPE column eluating gradiently starting from 100% DCM and ending with mixture of DCM/MeOH/NH$_4$OH = 90:9:0.5 yielding compounds **8**−**10**.

9a-{3-[(Quinolin-4-ylmethyl)amino]propyl}-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (8t). White powder (Y = 40%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.83 (d, J = 4.27 Hz, 1H), 8.21 (d, J = 8.24 Hz, 1H), 8.02 (d, J = 8.54 Hz, 1H), 7.74 (t, J = 7.48 Hz, 1H), 7.60 (t, J = 7.48 Hz, 1H), 7.55 (d, J = 4.27 Hz, 1H), 4.93 (d, J = 10.38 Hz, 1H), 4.80 (d, J = 4.88 Hz, 1H), 4.43 (d, J = 7.32 Hz, 1H), 4.23 (d, J = 7.32 Hz, 1H), 4.21 (s, 2H), 4.12 (s, 1H), 4.04 (dq, J = 6.30, 9.60 Hz, 1H), 3.96 - 3.99 (m, 1H), 3.99 (d, J = 5.80 Hz, 1H), 3.66 (dq, J = 5.65, 10.53 Hz, 1H), 3.53 (s, 1H), 3.50 (d, J = 6.41 Hz, 1H), 3.21 (s, 3H), 3.03 (ddd, J = 1.00, 7.32, 10.00 Hz, 1H), 2.95−3.02 (m, 1H), 2.82 (t, J = 7.78 Hz, 1H), 2.63−2.76 (m, 3H), 2.53−2.59 (m, 2H), 2.37−2.46 (m, 2H), 2.24 (d, J = 14.00 Hz, 1H), 2.20 (s, 6H), 2.13 (dd, J = 9.16, 12.82 Hz, 1H), 1.86−1.97 (m, 2H), 1.73−1.82 (m, 1H), 1.62−1.72 (m, 1H), 1.54−1.60 (m, 2H), 1.45−1.53 (m, 1H), 1.30−1.44 (m, 3H), 1.17 (s, 3H), 1.08−1.13 (m, 9H), 1.06 (d, J = 6.10 Hz, 3H), 0.96−1.01 (m, 9H), 0.95−0.98 (m, 1H), 0.88 (d, J = 6.71 Hz, 3H), 0.78 (t, J = 7.32 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 176.59, 150.49, 147.93, 146.47, 129.77, 129.25, 127.05, 126.51, 124.25, 120.07, 102.19, 95.32, 82.97, 78.34, 77.62, 76.89, 75.53, 74.53, 73.86, 72.99, 70.96, 67.39, 65.14, 64.87, 63.54, 59.48, 49.23, 49.09, 48.90, 47.46, 44.55, 40.64, 40.64, 40.64, 40.52, 35.05, 30.29, 28.58, 27.68, 26.96, 22.87, 21.73, 21.49, 21.27, 18.76, 18.22, 15.37, 11.23, 9.74, 8.93; MS (ESI) m/z calcd for $C_{50}H_{85}N_4O_{12}$ (M+H$^+$) 933.6164; found 933.6162.

9a-{3-[(3-Phenylpropanoyl)amino]propyl}-9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A (11c). To a solution of 3-phenylpropanoic acid (0.28 g, 1.89 mmol) in dry DCM (100.0 mL), TEA (2.63 mL, 18.9 mmol), HOBT (0.512 g, 3.79 mmol), intermediate **3** (1.5 g, 1.89 mmol) and EDC × HCl (1.45 g, 7.58 mmol) were added. The reaction mixture was stirred at room temperature overnight, and then H$_2$O (50 mL) was added and extracted with DCM (3 × 50 mL). Combined organic layers were washed with brine (50 mL), dried over Na$_2$SO$_4$, and evaporated in vacuum. The residue was purified by column chromatography on silicagel using solvent system DCM/MeOH/NH$_4$OH = 90:7:0.5 to yield compound **11c** (1.29 g, 74%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 0.79 (t, J = 7.3 Hz, 3 H), 0.86 (d, J = 6.7 Hz, 3 H), 0.98 (m, 6 H), 1.00 (s, 3 H), 1.07 (d, J = 5.8 Hz, 3 H), 1.10 (d, J = 7.3 Hz, 4 H), 1.13 (s, 3 H), 1.15 (d, J = 6.1 Hz, 3 H), 1.20 (s, 3 H), 1.36 (m, 2 H), 1.43 (m, 1 H), 1.45 (m, 1 H), 1.50 (dd, J = 14.8, 4.4 Hz, 1H), 1.60 (m, 2 H), 1.77 (m, J = 13.0, 7.3, 7.3, 7.3, 2.0 Hz, 1 H), 1.88 (br. s.,1 H), 1.94 (dq, J = 7.6, 6.0 Hz, 1 H), 2.06 (t, J = 11.4 Hz, 1 H), 2.22 (s, 6 H), 2.27 (d, J = 15.0 Hz, 1 H), 2.34 (t, J = 7.8 Hz, 2 H), 2.44 (m, 2 H), 2.54 (m, 1 H), 2.71 (m, 2 H), 2.80 (t, J = 7.8 Hz, 3 H), 2.91 (t, J = 8.4 Hz, 1 H), 2.98 (m, 2 H), 3.04 (m, 1 H), 3.22 (s, 3 H), 3.50 (m, 2 H), 3.66 (dq, J = 10.0, 5.9 Hz, 1 H), 3.96 (d, J = 6.1 Hz, 1 H), 4.01 (d, J = 4.9 Hz, 1 H), 4.06 (dq, J = 9.6, 6.3 Hz, 1 H), 4.23 (d, J = 7.3 Hz, 1 H), 4.28 (s, 1 H), 4.42 (d, J = 7.0 Hz, 1 H), 4.79 (d, J = 4.3 Hz, 1 H), 4.88 (d, J = 10.1, Hz, 1 H), 7.17 (m, 3 H), 7.25 (m, 2 H), 7.80 (t, J = 5.2 Hz, 1 H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 9.60, 9.63, 11.25, 15.23, 18.42, 18.75, 21.24, 21.59, 21.70, 22.62, 27.35, 27.58, 28.17, 30.33, 31.39, 35.05, 37.32, 40.61, 40.81, 44.49, 48.81, 49.08, 60.13, 63.10, 64.87, 65.11, 67.30, 70.90, 72.99, 73.72, 74.58, 75.32, 76.64, 77.61, 78.13, 82.86, 95.20, 102.27, 126.06, 128.39, 128.47, 141.65, 171.30, 176.61; MS (ESI) m/z calcd for $C_{49}H_{86}N_3O_{13}$ (M + H$^+$) 924.6161; found 924.6139.

9a-{3-[(Naphtalen-1-yl-acetyl)amino]propyl}-3-*O*-decladinosyl-9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A (12h). PS-Carbodiimide resin (PS-CDI, loading: 1.2 mmol/g) (105.2 mg, 0.126 mmol) was added to a dry reaction vessel. The naphtalen-1-yl-acetic acid (186.2 mg, 0.095 mmol) dissolved in dry DCM (1.5 mL), was added to the dry resin. The mixture was stirred at room temperature for 1 h upon which intermediate **4** (50 mg, 0.063 mmol) dissolved in dry DCM (0.8 mL) was added. The reaction mixture was

stirred for 2 h at room temperature, filtered and the resin was washed with DCM (4 × 1.5 mL). The filtrate was evaporated to dryness affording the compound **12h** (57.6 mg, 95%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.76 (t, J = 7.34 Hz, 3 H), 0.83 (d, J = 6.70 Hz, 3 H), 0.87 (d, J = 7.17 Hz, 3 H), 0.93 (d, J = 6.59 Hz, 3 H), 0.99 (s, 3 H), 1.08−1.23 (m, 11 H), 1.30−1.43 (m, 1 H), 1.45 - 1.56 (m, 1 H), 1.56−1.69 (m, 2 H), 1.69−1.84 (m, 3 H), 1.91−2.04 (m, 2 H), 2.28 (s, 6 H), 2.31−2.45 (m, 2 H), 2.52−2.64 (m, 2 H), 2.67−2.76 (m, 2 H), 2.92−3.08 (m, 2 H), 3.08−3.16 (m, 1 H), 3.35−3.60, 3.64 (s, 2 H), (m, 4 H), 4.23 (br. s., 1 H), 4.52 (d, J = 7.17 Hz, 1 H), 5.00 (d, J = 10.98 Hz, 1 H), 7.38−7.50 (m, 3 H), 7.73 (s, 1 H), 7.78−7.88 (m, 3 H), 8.05 (t, J = 5.26 Hz, 1 H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 8.34, 10.67, 15.98, 17.83, 21.07, 21.29, 21.71, 26.61, 28.22, 30.42, 37.24, 40.22, 42.58, 43.65, 64.62, 68.16, 70.11, 73.47, 74.39, 75.89, 76.44, 125.44, 126.01, 127.13, 127.35, 127.45, 127.55, 131.74, 132.98, 134.28, 163.75, 169.78, 175.27. MS (ESI) m/z calcd for $C_{44}H_{71}N_3O_{10}$ (M + H$^+$) 801.5139; found 801.5146.

3-(9-Deoxo-9-dihydro-9a-aza-9a-homoerythromycin A) propionic acid methyl ester (14). Methyl acrylate (24.5 mL, 272.1 mmol) was added to a solution of intermediate 2 (4.0 g, 5.44 mmol) in $CHCl_3$ (80.0 mL). Reaction mixture was stirred under reflux (60 °C) for 2 days. After evaporation of organic solvent, crude product (4.58 g) was obtained. Crude product was purified using a solid phase extraction (SPE) technique on a LC-Si (50 g) cartridge with the FlashMaster II instrument and gradient system for elution: DCM/DCM: MeOH: NH_4OH = 90: 5: 0.5) in which (DCM/MeOH/NH_4OH = 90:5:0.5) was increased from 0 to 100% giving after evaporation of solvent the title product as white powder (2.95 g, 65%); MS (ES+) m/z: [MH]$^+$ = 821.5.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.85 (t, J = 7.40 Hz, 3 H), 0.91 (d, J = 6.90 Hz, 3 H), 1.03 (s, 3 H), 1.04 (d, J = 8.36 Hz, 3 H), 1.09 (d, J = 6.90 Hz, 3 H), 1.17−1.21 (m, 10 H), 1.27 (s, 3 H), 1.28 (d, J = 6.18 Hz, 3 H), 1.31−1.39 (m, 1 H), 1.41−1.49 (m, 1 H), 1.52−1.56 (dd, J = 4.85 Hz, J = 15.20 Hz, 1 H), 1.60−1.70 (m, 2 H), 1.80−1.88 (m, 1 H), 1.90−1.96 (m, 1 H), 1.96−2.00 (m, 1 H), 2.12−2.20 (m, 1 H), 2.25 (s, 6 H), 2.31 (d, J = 15.20 Hz, 1 H), 2.36−2.45 (m, 2 H), 2.58−2.68 (m, 3 H), 2.72−2.75 (m, 1 H), 2.77−2.81 (m, 1 H), 2.99 (t, J = 9.79 Hz, 1 H) 3.20 (dd, J = 7.27 Hz, J = 10.18 Hz, 1 H) 3.29 (s, 3 H), 3.35−3.43 (m, 1 H), 3.44−3.50 (m, 1 H), 3.59 (d, J = 6.91 Hz, 1 H), 3.66 (s, 3H), 3.69 (d, J = 5.09 Hz, 1 H), 4.01−4.06 (m, 1 H), 4.16 (dd, J = 2.42 Hz, J = 6.55 Hz, 1 H), 4.34 (d, J = 5.09 Hz, 1 H), 4.40 (d, J = 7.27 Hz, 1 H), 4.76 (dd, J = 2.55 Hz, J = 10.06 Hz, 1 H), 4.95 (dd, J = 4.61, 1 H),

^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 8.34, 9.61, 11.11, 15.29, 16.36, 18.26, 21.30, 21.31, 21.52, 22.57, 27.11, 26.78, 28.78, 28.94, 32.97, 34.97, 40.32, 40.57, 41.07, 44.85, 46.24, 49.39, 51.90, 60.56, 64.38, 65.51, 65.55, 68.81, 70.88, 72.77, 74.13, 74.26, 74.81, 77.66, 78.02, 79.04, 82.96, 95.57, 103.11, 173.54, 177.39.

3-(9-Deoxo-9-dihydro-9a-aza-9a-homoerythromycin A) propionic acid (15). A solution of LiOH (0.28 g, 6.72 mmol) in water (25.0 mL) was added to a solution of intermediate **14** (2.4 g, 2.92 mmol) in THF (25.0 mL). Reaction mixture was stirred at room temperature for 3 h. Brine was added to the reaction mixture (30 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were dried over anhydrous Na_2SO_4. After evaporation the title product was obtained (2.30 g, 98%); MS (ES+) m/z: [MH]$^+$ = 807.5.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.79 (t, J = 7.45 Hz, 3 H), 0.87 (d, J = 6.75 Hz, 3 H), 0.98 (d, J = 7.45 Hz, 3 H), 1.01 (s, 3 H), 1.02 (d, J = 7.74 Hz, 3 H), 1.07 (d, J = 5.91 Hz, 3 H), 1.10 (d, J = 7.17 Hz, 3 H), 1.13 (s, 3 H), 1.16 (d, J = 6.05 Hz, 3 H), 1.20 (s, 3 H), 1.33−1.42 (m, 2 H), 1.43−1.48 (m, 1 H), 1.51 (dd, J = 4.91 Hz, J = 14.91 Hz, 1 H), 1.58−1.63 (m, 1 H), 1.73−1.81 (m, 1 H), 1.87−1.95 (m, 2 H), 2.13 (dd, J = 10.13 Hz, J = 12.94 Hz, 1 H), 2.24 (s, 6 H), 2.25 − 2.34 (m, 2 H), 2.36−2.45 (m, 2 H), 2.40−2.49 (m, 2 H), 2.57−2.62 (m, 1H), 2.68−2.82 (m, 3 H), 2.91 (d, J = 9.42 Hz, 1 H) 3.05 (dd, J = 7.45 Hz, J = 9.98 Hz, 1 H) 3.08−3.14 (m, 1H), 3.22 (s, 3 H), 3.48 (s, 1 H), 3.51 (d, J = 6.47 Hz, 1 H), 3.64 - 3.70 (m, 1H), 3.99−4.02 (m, 1 H), 4.03−4.09 (m, 1 H), 4.43 (d, J = 7.17 Hz, 1 H), 4.79 (d, J = 4.78 Hz, 1 H), 4.90 (dd, J = 2.39, J = 10.13, 1 H).

13C NMR (101 MHz, DMSO-d_6) δ ppm: 9.41, 10.98, 14.93, 18.19, 18.45, 20.96, 21.35, 21.40, 22.08, 26.53, 27.77, 30.07, 32.37, 34.79, 40.24, 40.38, 44.18, 47.09, 48.79, 59.96, 64.59, 64.88, 67.02, 70.56, 72.72, 73.78, 74.31, 76.42, 77.33, 77.89, 82.60, 94.97, 101.90, 174.20, 176.30.

9a-{1-[(1*S*)-1-(1-Naphthalenyl)ethyl]amino)propanoyl}-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (16k). TEA (62.4 μL, 0.45 mmol), HOBT (12.2 mg, 0.09 mmol), (1*S*)-1-(1-naphthalenyl)ethanamine (8.5 mg, 0.05 mmol), and EDC × HCl (34.5 mg, 0.18 mmol) were added to a solution of intermediate **3** (36.3 mg, 0.045 mmol) in dry DCM (3.0 mL). The reaction mixture was stirred at room temperature overnight. Solvent was evaporated under reduced pressure giving 98.2 mg of yellowish crude product which was purified on preparative LC-MS (XTerra Prep RP_{18} column, 5 μm, 19 × 100 mm) using gradient system for elution: (0.1% HCOOH in H_2O/CH_3CN) in which HCOOH: CH_3CN was changed from 95:5 to 60:40 to give the title compound (14.9 mg, yield 35%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 0.78 (t, J = 7.34 Hz, 3 H), 0.85 (d, J = 6.70 Hz, 3 H), 0.93−1.02 (m, 9 H), 1.04−1.16 (m, 13 H), 1.18 (s, 3 H), 1.30−1.43 (m, 3 H), 1.47 (d, J = 6.82 Hz, 3 H), 1.51 (d, J = 4.39 Hz, 1 H), 1.59 (m, 1 H), 1.69−1.81 (m, 1 H), 1.83−1.98 (m, 2 H), 2.00−2.12 (m, 1 H), 2.23 (s, 6 H), 2.28 (m, 1 H), 2.36−2.46 (m, 2 H), 2.55 (m, 1 H), 2.64−2.78 (m, 3 H), 2.86−2.94 (m, 1 H), 3.01−3.07 (m, 2 H), 3.08−3.17 (m, 1 H), 3.21 (s, 3 H), 3.45−3.53 (m, 2 H), 3.63−3.72 (m, 1 H), 3.95−4.03 (m, 1 H), 4.03−4.10 (m, 2 H), 4.23 (dd, J = 8.09, 3.01 Hz, 1 H), 4.27 (br. s., 1 H), 4.42 (d, J = 7.17 Hz, 1 H), 4.78 (d, J = 4.05 Hz, 1 H), 4.85 - 4.93 (m, 1 H), 5.62−5.72 (m, 1 H), 7.43−7.58 (m, 4 H), 7.81 (d, J = 8.09 Hz, 1 H), 7.92 (d, J = 8.44 Hz, 1 H), 8.08 (d, J = 8.21 Hz, 1 H), 8.44 (d, J = 7.98 Hz, 1 H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 9.44, 9.78, 10.99, 14.87, 18.16, 18.43, 20.96, 21.33, 21.40, 21.49, 22.28, 27.72, 29.99, 33.40, 34.77, 40.29, 43.83, 44.11, 48.79, 64.61, 64.87, 67.04, 70.54, 72.73, 73.45, 74.28, 74.96, 76.44, 77.32, 77.82, 82.60, 94.91, 101.93, 122.27, 123.13, 125.40, 125.53, 126.06, 127.17, 128.58, 130.38, 133.35, 140.23, 170.38, 176.28; MS (ESI) m/z calcd for $C_{52}H_{86}N_3O_{13}$ (M + H$^+$) 960.6161; found 960.6176.

Biology. *Compound Susceptibility Testing.* The in vitro antimalarial activity of novel 15-membered azalide analogues was determined using the tritiated hypoxanthine incorporation assay of Desjardins et al.,[38] as modified by Milhous et al.[39] with the exception of exposing the parasite to the drug for 48 h.[7] The *P. falciparum* clone used was TM91C235,[40] a strain from Southeast Asia that shows high level resistance to mefloquine and a number of other antimalarials. Compounds and control antimalarials (chloroquine, mefloquine, and azithromycin) were diluted 2-fold over 11 different concentration and IC_{50}'s were determined using a nonlinear logistic dose response program. The presented IC_{50}'s represent averaged values in cases where multiple results were generated and relative errors were usually <30% and did not exceed 50%, which is standard for this type of assay.

Cytotoxicity Assay. The assay used to measure the influence of compounds on the proliferation of different human cell lines was described in detail elswhere.[41] In brief, human epithelial cell line HepG2 was purchased from ECACC. Cells were maintained and tested in complete RPMI 1640 medium (Institute of Immunology, Zagreb, Croatia) supplemented with 10% Fetal Bovine Serum (Gibco), penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin B (0.25 mg/mL) at 37 °C in a 5% CO_2 atmosphere. Cytotoxicity assay was performed by using the MTS CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA). Culture wells in 96-well plates contained 50 000 HepG2 cells and were incubated with 10 concentrations of serially diluted compounds (1:2) for 24 h at 37 °C in 5% CO_2. MTS reagent was added directly to the culture wells. Mixture was incubated for the additional 2 h at 37 °C in 5% CO_2, and absorbance recorded at 490 nm using a spectrophotometric plate reader (Ultra, TECAN, USA). The results were expressed as IC_{50} values. In cases where multiple results were generated averaged values are presented.

In Vivo Efficacy Studies in P. berghei Murine Model. A modified Thompson test was used to test the in vivo efficacies of the new compounds.[42] The test measures the survival of the mice and parasite

clearance following administration of the drug on days 3 to 5 postinfection. Male mice that weighed 24−30 g were inoculated with 5 × 10^6 *P. berghei*-infected erythrocytes (KBG-173 strain) into the intraperitoneal cavity. By day 3 postinfection, parasitemia usually ranged from 1.0 to 3.7%. Each drug was administered in an aqueous solution (saline, or saline with addition of 0.2% ethanol and 0.02% acetic acid for less soluble compounds, azithromycin and **8t**) IV twice daily from days 3 to 5 postinfection. In general, the volume of drug suspension is given at 0.01 mL/gram of body weight. Five mice were used in each dosage group and blood films were taken from each mouse on day 6 and biweekly until day 31. Mice that had their blood smears negative on day 31 postinfection were considered cured. Compounds were considered active when the survival time of the treated mice was greater than twice that of the control mice (i.e., 12 to 14 days). Mice losing ≥20% of their body weight were sacrificed.

Pharmacokinetic Studies. *Intrinsic Clearance (CLi) Assay.* Intrinsic clearance (CLi) values were determined in mouse, rat and human liver microsomes. Test compounds (1.0 μM) were incubated at 37 °C for 60 min in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mg microsomal protein/mL. The reaction was started by addition of cofactor (2.0 mM NADP, 20 mM glucose-6-phosphate, 2.0 mM $MgCl_2$ and 4.0 U/mL glucose-6-phosphate dehydrogenase). The final concentration of solvent was 1% of the final volume. Samples were analyzed by LC-MS/MS. The intrinsic clearance (CLi) was determined from the first order elimination constant by nonlinear regression using Excel (Microsoft Exel 2003), corrected for the volume of the incubation and assuming 52.5 mg of microsomal protein/g of liver for all species. Values for CLi were expressed as mL/min/g of liver.

Plasma Protein Binding (PPB). PPB was determined using the ultrafiltration method at concentrations of 1 mg/mL. Test compounds were spiked to mouse plasma (preincubated at 37C for 15 min) to a final concentration of 1 μg/mL. Aliquots (25 ul) were taken at t = 0 and t = 15 min and spiked with 25 μL of PBS. The remaining samples were filtered filtered through a 10 kDa filter (Milipore Multiscreen Ultracel 10 MAU01010 filger, Milipore, USA) while centrifuging for 30 min at 37C at 2850 g. Aliquots of the filtrate were deproteinized and analyzed by LC-MS/MS.

In Vivo Pharmacokinetic Studies. Male CD-1 mice (N = 3 per route) were dosed intravenously (IV) at 5 mg/kg (2 mL/kg dosing volume) and orally (PO), fasted, at a dose of 25 mg/kg (10 mL/kg). Dosing solutions were prepared in 100% saline (2.5 mg/mL) both for IV and PO administration for all compounds with the exception of compounds 9, 12, and 14. Dosing solutions for compounds 9, 12, and 14 were prepared by dissolving Ethanol, acetic acid and saline. After both routes of administration, blood samples were collected from the tail vain up to 24 h and by cardiac puncture at the last time point (30 h), hemolyzed and frozen until analysis. Samples were prepared for analysis by protein precipitation with two volumes of acetonitrile containing internal standard and analyzed by LC-MS/MS in positive ion mode with electrospray. Quantitation was performed using multiple reaction monitoring (MRM) at the specific transitions for each compound. Noncompartmental analysis for all compounds was performed using WinNonlin, version 4.1.1 (Pharsight, Mountain View, CA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional spectroscopic information for selected compounds is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

IV, intravenuously; IP, intraperitoneally; PO, per os (oral gavage); BID, bis in die (twice daily)

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