Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *78* (12) 1847–1864 (2013)
JSCS–4536

UDC **Clostridium botulinum*+547.583.5:
615.285:612.014.46+616.8+615.9+57
Original scientific paper

New 9-aminoacridine derivatives as inhibitors of botulinum neurotoxins and *P. falciparum* malaria•

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(Received 24 September, revised 20 October 2013)

Abstract: Steroidal and adamantane aminoacridine derivatives were prepared and tested as both botulinum neurotoxin (BoNT) inhibitors and antimalarials. Steroid-bound acridines provided good potency against both the BoNT/A and BoNT/B light chains (LCs). The observed inhibition of the BoNT/B LC by *ca*. 50 % is the highest attained inhibitory activity against this serotype by acridine-based compounds to date. With respect to the antimalarial activity, the adamantane acridines were the most potent derivatives (IC_{50} = 6–9 nM, *SI* > 326), indicating that an adamantyl group is a better carrier than a steroidal motif for this indication.

Keywords: antiviral, BoNT/A; BoNT/B; antimalarial; aminoacridine.

INTRODUCTION

Botulinum neurotoxins (BoNTs),** secreted by the anaerobic, spore-forming bacterium *Clostridium botulinum*, are the most potent of known biological tox-

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• This manuscript is dedicated to our dear colleague and friend Prof. Branislav Nikolić on the occasion of his 70th birthday.

doi: 10.2298/JSC130924112T

** Abbreviations: BoNT, botulinum neurotoxin; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; BoNT/A, botulinum neurotoxin serotype A; BoNT/A LC, BoNT/A light chain; SMNPIs, small molecule non-peptidic inhibitors; ACR, 9-amino-2-metoxy-7-chloroacridine; ACR2, *N*-(6-chloro-2-methoxyacridin-9-yl)ethane-1,2-diamine; ACR3, *N*-(6-chloro-2-methoxy-9-anthryl)propane-1,3-diamine; ACR5,

ins.[1,2] The proteolytic activities of these enzymes are responsible for the potentially fatal disease botulism, which is commonly associated with food contamination, wound infection, and colonizing infection in infants. BoNTs can be easily produced and disseminated,[3] and therefore are listed as priority biological threat agents.[4] On the other hand, when dosed locally in minute concentrations, BoNTs are used for the effective treatment of severe diseases.[5]

There are seven known BoNT serotypes identified as A–G. Each cleaves a component of the SNARE complex, which mediates the transport of acetylcholine into neuromuscular junctions.[6,7] BoNTs are secreted as holotoxins composed of a heavy chain (HC) and light chain (LC), which are connected by a disulfide bridge.[8,9] The HC, which transports the LC into the neuronal cytosol *via* an acidic endosome, is comprised of two ≈50 kDa domains. The *C*-terminal domain (H_C), the ganglioside and protein receptor-binding domain, plays a role in binding to the cell membrane and internalization of a toxin into cholinergic neurons; the *N*-terminal domain (H_N) facilitates the release of the LC from endosomes into the cytosol. The LC is a zinc-dependent metalloprotease that cleaves SNARE proteins, thereby inhibiting the formation of the SNARE complex. BoNT serotypes A and E cleave SNAP-25 (synaptosomal-associated protein (25 kDa)),[10] serotypes B, D, F[11] and G cleave VAMP (vesicle-associated membrane protein),[12] and serotype C cleaves both SNAP-25 and syntaxin 1.[13] Serotype A (BoNT/A) is the most potent of the seven serotypes. For example, it is 10^6-fold more potent than cobra toxin and 10^{11}-fold more potent than cyanide,[6] and its lethal dose is estimated to be between 1 and 5 ng kg^{-1} for humans.[14] Moreover, the BoNT/A LC is longest acting of the LC serotypes in the neuronal cytosol.[15]

Currently, there are no therapies available for the treatment of BoNT LC-mediated paralysis post-neuronal intoxication, although several different approaches are currently under development. Two of these approaches include antibody-based therapeutics and small molecule-based therapeutics.[16] However, antibodies cannot reverse the proteolytic effects of the toxins after the LC component has entered the neuronal cytosol.[2] Hence, the development of small molecules that will effectively inhibit proteolytic activity of BoNT LC after post-neuronal intoxication is of special interest, with the BoNT/A LC being the main enzyme of study. At this time, one of the best known inhibitors of the BoNT/A LC is mpp-RATKML, which consists of the oligopeptide RATKML covalently bonded to 2-mercapto-3-phenylpropionyl (mpp) group (K_i = 330 nM).[17] However, peptides are readily hydrolyzed by proteases, thus limiting their application in the therapy.

N-(6-chloro-2-methoxyacridin-9-yl)pentane-1,5-diamine; ACR6, *N*-(6-chloro-2-methoxyacridin-9-yl)hexane-1,6-diamine; CQR, chloroquine resistant strain; CQS, chloroquine susceptible strain; MDR, multi-drug resistant strain; BS, binding site; QA, quinacrine; CQ, chloroquine; MFQ, mefloquine; ART, artemisinin; EA, ethyl acetate; DCM, dichloromethane; PCC, pyridinium chlorochromate; TFA, trifluoroacetic acid.

For this reason, the development of small molecule inhibitors is crucial. To this end, many inhibitors of the BoNT/A LC have been reported,[18] and are composed of compounds that competitively coordinate the catalytic zinc ion,[19] as well as allosteric inhibitors.[20] The most potent possess K_i values ranging from 0.1–10 μM and include zinc (Zn^{2+}) bidentate coordinating hydroxamic acid **1**,[21] 2,5-diphenyl-thiophene derivative **2**,[22] and diazachrysene **3**[23] (Chart 1).

In contrast to BoNT/A LC inhibitors, a paucity of small molecules that inhibit the BoNT/B LC has been reported. These include isocoumarine **4**,[24] aryl--phosphoramidic acid **ICD 2821**[25], bis-benzimidazole-5-carboximidamide **5**[26] (which bidentately coordinates the catalytic Zn ion of enzymes), and L-chicoric acid **6** (which is reported to bond to an exosite of the enzyme).[27] In addition, it has been reported that the reducing agent **TCEP** (tris(2-carboxyethyl)phosphine hydrochloride) reduces the disulfide bond connecting the HC–LC bond of the enzyme both *in vitro* and in neuronal cells, and that it works prophylactically with a maximum activity at 1 mM.[28] In this way, the LC cannot undergo endocytosis, and therefore cannot cleave VAMP.

It is well known that many 4-aminoquinoline antimalarials act as inhibitors of the BoNT/A LC.[29,30] In contrast, little has been reported about the efficacy of acridine-based (ACR) compounds as BoNT inhibitors. However, it was found that quinacrine (QA, Chart 2), effectively prolonged the time of BoNT/A blocking of nerve-elicited muscle twitch in isolated mouse diaphragms.[31] Furthermore, it was found that QA was four times more efficacious than CQ, with a muscle twitch protective index of 2.85 at 10 μM.

Malaria* is a devastating disease and a global health problem. It is estimated to cause 300–500 million clinical cases and *ca*. one million deaths *per annum*.[32] The most successive drugs appeared to kill the parasite either by producing toxic free radicals[33] or by blocking the polymerization of heme, as in the case of the 4-amino-7-chloroquinolines (ACQs).[34] The development of widespread drug-resistance to CQ has resulted in an urgent need for new drug modalities, for example, synthetic peroxide antimalarials,[35] as well as for the development of novel molecules that prevent heme polymerization.[36]

Quinacrine was the first synthetic antimalarial used clinically that acts as a blood schizonticide, but later it was replaced with the more efficient CQ.[37] However, due to the widespread development of CQ resistant (CQR) strains, there is renewed interest for evaluating acridine-based compounds as antimalarials. For example, *N*-sulfonamide derivatives, obtained from various simple 9-aminoacridines, show high antimalarial activity against both CQS and CQR strains.[38] They do not lose activity even when the amino group is stripped of its basic character, which is in contrast to most CQs or other quinoline or acridine derivatives.[39] Qui-

* Malaria is caused by five *Plasmodium* species, *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi*, of which *P. falciparum* causing cerebral malaria is the major death threat.

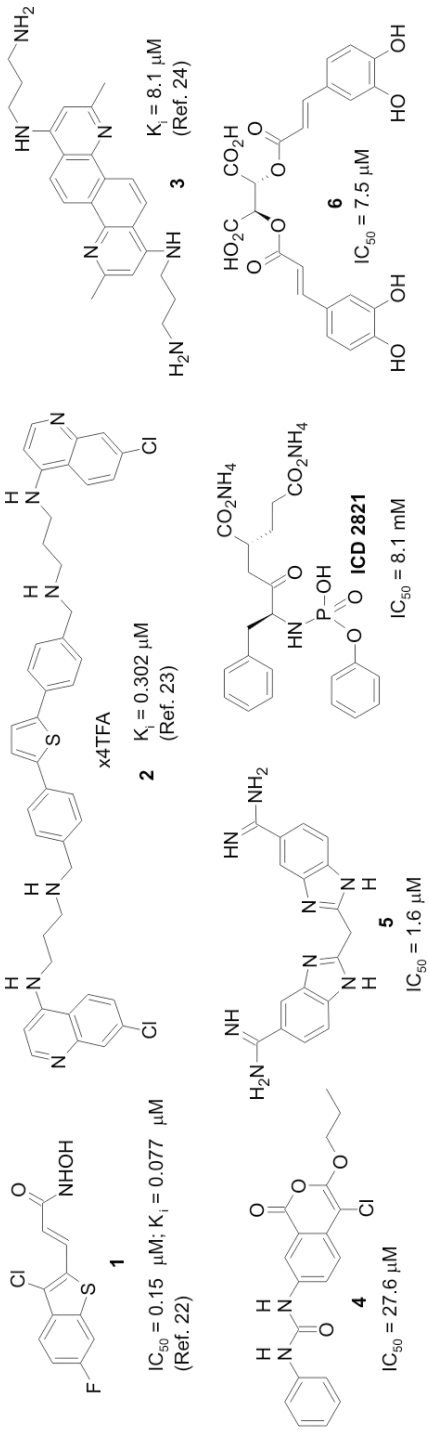


Chart 1. Structures of potent inhibitors of BoNT/A LC and BoNT/B LC.

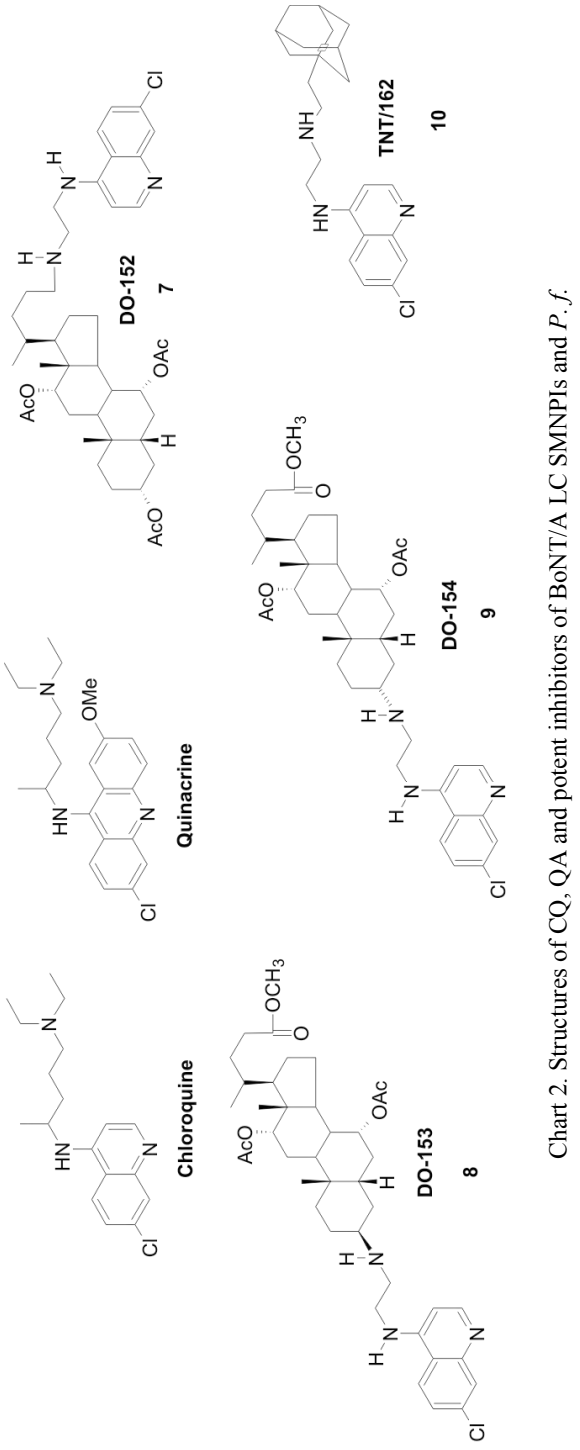


Chart 2. Structures of CQ, QA and potent inhibitors of BoNT/A LC SMNPIs and *P. f.*

nolizidinyl and quinolizidinyl-alkyl derivatives of 9-amino-6-chloro-2-methoxy-acridine were almost 10 times more active than CQ against CQR (W2) strain, and were as active as CQ against the CQS (D10) strain;[40] a series of 9-amino-acridines showed high inhibitory potency.[41] Most active derivatives have diamino-propylidene side chains and exert low nM activity (EC_{50} in range 1–4.1 nM).

Similar to AQ antimalarials, 9-aminoacridines inhibit polymerization of hemozoin. In a β-hematin inhibitory assay (BHIA), it was found that ACR derivatives formed complexes with hematin in a 3:2 molar ratio (hematin:drug).[42] It was shown that the presence of 2-methoxy, 6-chloro substituents and an ionizable terminal amino group are essential for good antimalarial activity. It was also discovered that ACRs inhibit DNA topoisomerase II,[39,43] parasite folate metabolism[44] and plasmepsin II.[45] However, chimeric derivatives with ART[46] or AQ,[47] as well as bis-ACR derivatives,[39,48] showed poor activities.

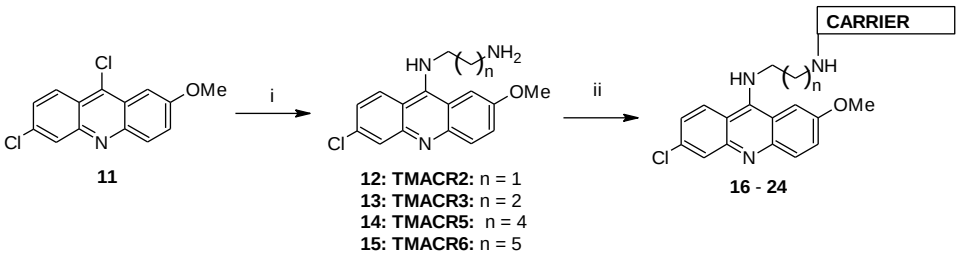
In addition to their significant antimalarial activity, ACR-based compounds exhibit other biological activities, including nuclear localization signal (NLS)--labeling agents for plasmid DNA,[49] photocleavage reagents for DNA,[50] anti-leishmania agents[51] and anti-prion compounds.[52]

Previously, we reported on the inhibitory activities of steroidal 4-amino-quinolines **7**–**9** and adamantyl-aminoquinolines (for example, **10**) against the BoNT/A LC[30] and as antimalarial agents[53] were reported (Chart 2). Specifically, these derivatives provided a high degree of inhibition of BoNT/A metallopro-tease, as well as significant inhibition of CQS and more important, CQR strains of *P. falciparum*. Herein, the synthesis and biological evaluation of new ACR derivatives possessing steroid and adamantane carrier component are presented.

RESULTS AND DISCUSSION

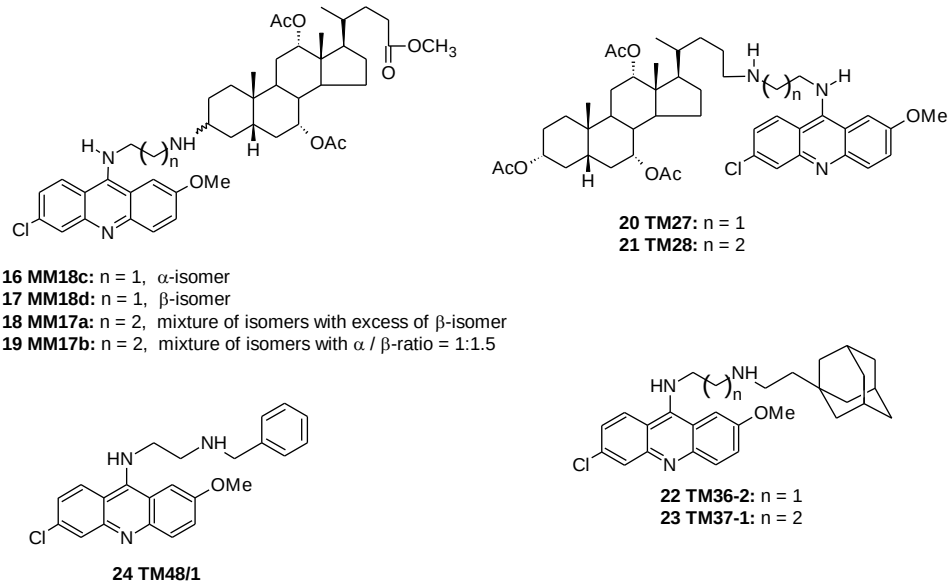
Synthesis

The syntheses of the ACR derivatives examined in this study are provided in Scheme 1, and the corresponding structures are given in Chart 3. First, aminoalk-



i) diamine, Δ;
ii) ketone, $NaBH_3CN$/ MeOH/CH_3CN, or $NaBH_4$ / MeOH or $NaBH_4$ / $Ti(OPr^i)_4$ / EtOH, or $NaB(OAc)_3H$ / DCM

Scheme 1. Synthesis of acridine derivatives **12**–**24**.



16 MM18c: n = 1, α-isomer
17 MM18d: n = 1, β-isomer
18 MM17a: n = 2, mixture of isomers with excess of β-isomer
19 MM17b: n = 2, mixture of isomers with α / β-ratio = 1:1.5

20 TM27: n = 1
21 TM28: n = 2

22 TM36-2: n = 1
23 TM37-1: n = 2

24 TM48/1

Chart 3. Structures of examined derivatives **16**–**24**.

ylamino acridines **12**–**15** were obtained by the heating of 6,9-dichloro-2-methoxyacridine **11** with neat diamines.[50] The spectra of the isolated products were in agreement with literature data, and the compounds were used in the next reaction step without additional purification. Derivatives **16**–**24** were synthesized using procedures described earlier.[30,53] The 3-keto steroidal derivative (7*α*,12*α*-diacetoxy-3-oxo-5*β*-cholan-24-oate)[54] was coupled with aminoalkylamino acridines **12** and **13** to afford the corresponding 3*α*-and 3*β*-diastereomers (**16**–**19**). The crude reaction product obtained from **12** afforded diastereomers **16** and **17** *via* chromatographic separation. However, derivatives obtained from **13** were only partially separated to afford **18** containing *ca*. 20 % of **19**, and the second fraction consisting of both isomers in a 1:1.5 ratio. The configuration at C(3) of the derivatives **16**–**19** was deduced from the respective ^{1}H-NMR spectra. The corresponding H–C(3) atom of derivative **16** had a broad multiplet at 2.50–2.34 ppm, while that of derivative **17** had a narrow signal at 2.93 ppm. Therefore, **16** has an axial H–C(3) (in the *β*-orientation) and acridine substituent in an *α*-orientation (*α*-isomer). Consequently, **17** is the *β*-isomer since it has H_α–C(3) and the acridine substituent in the *β*-orientation. Based on the above correlations, it was possible to establish the approximate composition of mixtures obtained from partially separated isomers **18** and **19**. Steroidal alcohol 3*α*,7*α*,12*α*-triacetoxy-5*β*--cholan-24-ol was oxidized to the corresponding aldehyde,[30] which was further coupled to **12** and **13** to give the corresponding steroidal acridine derivatives **20** and **21**. Following the general approach, 2-(adamantyl)ethanal[53] gave the ada-

mantyl–acridine derivatives **22** and **23**, and the benzaldehyde afforded *N*-benzyl acridine **24**. The structures of all synthesized compounds were confirmed by spectral and analytical techniques, with all details given in the Supplementary material to this paper.

Inhibition of BoNT/A LC

The inhibitory efficacies of synthesized derivatives **16**–**21** and **24** at 20 μM concentration were obtained using a well-established HPLC-based assay for BoNT/A LC inhibition.[55] These data are presented in Table I. The activities of corresponding aminoquinoline derivatives **7**–**9** were established previously,[30] and are provided for comparison. In this study, the inhibitory effects of CQ were also examined. It exhibited only marginal inhibition and thus clearly emphasized the necessity of the cholic acid component for superior inhibitory activity.

TABLE I. *In vitro* inhibitory activities (%, at 20 μM concentration) of the tested compounds against BoNT/A LC and BoNT/B LC; N.T. – not tested

Compound	BoNT/A-LC	BoNT/B-LC
NSC 240898	81.43	77.38
16	58.34	57.03
17	58.25	52.04
18	77.04	47.40
19	59.38	48.10
20	15.07	31.94
21	61.25	33.63
24	5.82	N.T.
12	42	N.T.
13	20	N.T.
14	8	N.T.
15	10	N.T.
7[a]	74	N.T.
8[a]	47.22	N.T.
9[a]	56.26	N.T.
CQ[b]	6.51	N.T.
MQ[c]	28.06	N.T.

[a]Results published earlier;[31] [b]tested as diphosphate salt; [c]tested as HCl salt

The results given in Table I indicate that 9-aminoacridine derivatives coupled to cholic acid derivative as the carrier exhibited moderate to good activities against the BoNT/A LC, with potencies ranging from 58 to 77 % inhibition. The exception is derivative **20,** which provided only 15 % inhibition. The most active derivative was compound **18,** which contains the acridine moiety in the *β*-orientation. However, the results obtained for derivatives **18** and **19** are not so easily interpretable, since both samples were mixtures of diastereomers: **18** contained ≈20 % of the *α*-isomer, and **19** was a mixture of both isomers in 1:1.5

α:β ratio. Nevertheless, from the obtained results, it is plausible to assume that the pure β-isomer would have higher activity *versus* the pure α-isomer (since the former sample showed 20 % higher potency – **18** 77.04 % inhibition *vs*. **19** 59.38 % inhibition).

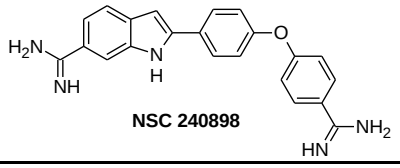
The compounds incorporating acridine **12**, *i.e.*, **16** and **17,** were equally potent, suggesting that the configuration at position C(3), in this instance, is not crucial for BoNT/A LC inhibition. Comparison of the inhibitory activities for steroidal acridines with the corresponding data for steroidal aminoquinolines **7**–**9** indicates that these compounds expressed approximately the same level of inhibitory capacity. This suggests that both acridine and quinoline moieties within the small molecule inhibitors engage in similar types of interactions with the active site of the BoNT LC metalloprotease. However, more reliable correlations will be made on pure isomers after measuring the corresponding K_i values.

The simple 9-aminoacridines **12**–**15** exhibited poor inhibitory activity (8–20 % inhibition) with **12** being an exception (42 % inhibition). In addition, the results showed that elongation of the aminoalkyl chain leads to decreased activity for this class of inhibitor. The low activities of 9-aminoacridines **12**–**15** clearly indicate that for good inhibitory activity, a carrier is necessary in order to enable additional interactions within the active site of a metalloprotease. The poor activity of *N*-benzyl derivative **24** further supports this observation.

Derivatives **16**–**21** were also tested for inhibition of BoNT/B LC, giving rise to some of the most potent inhibitors of this serotype reported to date (Table I). The results indicate that steroidal C(3) derivatives are slightly more active in comparison to C(24) derivatives. Moreover, the results showed no differences between the stereoisomers, *i.e.*, the H_β–C(3) isomers exhibited the same level of activity as the H_α–C(3) isomers. The most active derivative was **16,** which provides 57 % inhibition at 20 μM concentration.

As steroidal 9-aminoacridines inhibited Zn proteases BoNT/A and B LCs, the next step was to examine the activity of synthesized derivatives against a sampling of human metalloprotease – to evaluate selectivity. The four derivatives **16**–**19** were tested at 20 μM concentration against human metalloprotease thermolysin, serine protease trypsin, and cysteine protease papain. The results are given in Table II. The data indicate that the compounds are poor inhibitors of generic human proteases. The exception was derivative **18**, which meaningfully inhibited papain. Nevertheless, in general, it could be concluded that these derivatives selectivity inhibit BoNT LC metalloproteases (as opposed to human proteases). In addition, the low activities of the compounds against the Zn-dependent protease thermolysin indicates the inhibitory potencies against BoNT LCs were not the result of general preferences for interaction with the Zn ion, but that additional specific interactions with the enzymes were involved.

TABLE II. Inhibitory activities (%,at 20 μM concentration) of ACR SMNPIs against human proteases; A: trypsin; B: thermolysin; C: papain



NSC 240898

Compound	A	B	C
NSC 240898	52.07	8.53	–11.78
16	12.36	13.98	5.19
17	17.24	15.42	20.76
18	22.94	22.37	78.54
19	7.48	12.68	–7.85

Antimalarial activity

It was previously shown that aminoquinoline SMNPIs exerted both BoNT inhibiting effects and were simultaneously excellent antimalarials.[30,53] Based on this observation, acridine derivatives were elected for screening against three *P. falciparum* strains: D6 (a CQ and MFQ susceptible strain), W2 (a CQ-resistant, susceptible to MFQ strain), and TM91C235 (Thailand), a multi-drug-resistant strain, using a well-established protocol.[55] The gathered *in vitro* antimalarial activities (Table III) show that all tested derivatives had significantly better activities than CQ against the CQR strain W2 (16–53 times) and the multi-drug resistant TM91C235 strain (2–24 times). Against the CQS strain D6, the steroidal derivatives were less active than CQ; however, the other derivatives were as active as CQ. The steroidal acridines **20** and **21** were less active in comparison to **7** against all the tested strains. However, at the same time, the adamantyl derivatives **22** and **23** were more active when compared to **10** against the CQR W2 and the multi-drug-resistant TM91C235 strains. Importantly, all the new derivatives were significantly more active than *N*-sulfonamides,[38] 9-amino-polyamide acridines,[39] aminoalkylamino acridines,[42] and had activities to those of quinolizidinyl and quinolizidinylalkyl derivatives, which are the second best known acridine antimalarials.[40] The most active of the derivatives was **22,** with IC_{50}(W2) = 8.6 nM, IC_{50}(D6) = 9.3 nM and IC_{50}(TM91C235) = 5.8 nM. Its *in vitro* activity is comparable to that of artemisinin (Table III). In addition, compound **22** had similar activity to those of the most potent diaminopropylidene-aminoacridine derivatives described in the literature.[41]

It should be emphasized that steroidal/adamantane carriers of the 9-aminoalkylamino acridine moiety had significantly improved antimalarial activities. Comparatively, simple acridines such as **12** and **15** showed much lower antimalarial activity against the CQR W2 strain versus new derivatives (**12** (IC_{50} = = 250 nM) and **15** (IC_{50} = 180 nM)).[42] Thus, the attachment of these simple

ACRs to either cholic acid or the adamantyl moiety significantly increased antimalarial activity against CQR strains. The same trend was observed against the CQS strain. The results reported herein indicate that steroidal and adamantyl acridines are highly effective antimalarials.

TABLE III. *In vitro* IC_{50} antimalarial activities (nM) and cytotoxicity of the compounds tested against *P. falciparum* strains; N.D. – not determined

Compd.	W2[a]	D6[b]	TM91C235[c]	*RI*[d]	HEPG2	*SI*[e]
20	23.8	41.5	51.8	1.25 / 0.57	4281	180 / 103 / 83
21	23.3	39.0	53.0	1.36 / 0.60	1030	44 / 26 / 19
22	8.6	9.3	5.8	0.62 / 0.92	>3030	>352 / >326 / >522
23	11.2	15.7	12.2	0.78 / 0.71	3261	291 / 208 / 267
24	28.0	17.9	15.6	0.56 / 1.56	1183	42 / 66 / 76
7[f]	11.4	16.9	27.7	1.64 / 0.67	N.D.	–
10[g]	15.66	8.59	17.76			
12	250[h]	N.D.	N.D.	–	N.D.	–
15	180[h]	N.D.	N.D.	–	N.D.	–
CQ	456.20	12.27	138.82	23.17 / 43.32	N.D.	–
MFQ	4.93	15.70	36.50	3.43 / 0.39	N.D.	–
ART[i]	6.70	9.00	13.40	1.36 / 0.90	N.D.	–

[a]*P. falciparum* Indochina W2 clone; [b]*P. falciparum* African D6 clone; [c]*P. falciparum* multi-drug resistant TM91C235 strain (Thailand); [d]resistance index, defined as the ratio of the IC_{50} for the resistant *versus* sensitive strain, TM91C235/D6 and W2/D6, respectively; [e]selectivity index, defined as the ratio of the IC_{50} for HepG2/W2, HepG2/D6 and HepG2/ TM91C235, respectively; [f]results published earlier;[30] [g]results published earlier;[53] [h]results taken from literature;[42] [i]average of more than eight replicates

The toxicities of the new derivatives were estimated using the human liver carcinoma cell line HEPG2, and the results are given in Table III. The HEPG2 cell line is frequently used for cytotoxicity examination of xenobiotics, since this cell line has low levels of CYP450 enzymatic activities and possible toxic effects are the result of particular molecules and not metabolites.[56] In addition, since all xenobiotics pass through the liver, it is essential to know if they provide heaptotoxic side effects. In this study, it was found that steroidal derivative **20,** containing aminoacridine part **12**, was the least cytotoxic of the series, possessing an IC_{50} = 4281 nM. However, the adamantyl derivatives had a much better selectivity index (*SI*) than the other antimalarials. Thus, **22** has *SI* values 352/326/522 *vs*. 180/103/83 for **20**, and thus have a higher therapeutic potential. It was also observed that elongation of aminoalkylamino chains by only one methylene group in **21** significantly increased cytotoxicity when compared to **20**.

EXPERIMENTAL

Melting points were determined on a Boetius PMHK or a Mel-Temp apparatus and were not corrected. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X. NMR spectra were recorded on Varian Gemini-200, Varian XL-300, and Bruker AM-250 spectrometers in the indicated solvent using TMS as an internal standard. Chemical shifts are

recorded in parts per million (ppm), and coupling constants *J* are expressed in Hz. ESI-MS spectra were recorded on an Agilent Technologies 6210 Time-Of-Flight LC-MS instrument in positive ion mode with CH_3CN/H_2O–0.2 % HCOOH as eluent. The samples were dissolved in CH_3CN or MeOH (HPLC grade purity). The elemental analysis was performed on the Vario EL III – C, H, N, S/O elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Thin-layer chromatography (TLC) was performed on pre-coated Merck silica gel 60 F_{254} and RP-18 F_{254} plates. For column chromatography, Lobar LichroPrep Si 60 (40–63 μm), RP-18 (40–63 μm) columns coupled to a Waters RI 401 detector were used, Biotage SP1 system with UV detector.

*Methyl 7α,12α-diacetoxy-3α-{*N*-{2-[(6-chloro-2-methoxyacridin-9-yl)amino]ethyl}amino}--5β--cholan-24-oate (**16**) and methyl 7α,12α-diacetoxy-3β-(*N*-{2-[(6-chloro-2-methoxyacridin-9--yl)amino] ethyl}amino)-5β-cholan-24-oate (**17**)*

To a solution of methyl 7*α*,12*α*-diacetoxy-3-oxo-5*β*-cholan-24-oate (300 mg, 0.59 mmol) in CH_3CN (6.0 mL), **12** (357.9 mg, 1.19 mmol) in MeOH (3 mL) was added, and after stirring for 30 min at r.t., $NaBH_3CN$ (48.39 mg, 0.77 mmol) was added in one portion. The resulting mixture was further stirred for 2 h at r.t., followed by the addition of glac. AcOH (4 drops) and stirring was continued until TLC indicated the consumption of all starting ketone (*ca*. 1 h). The reaction was quenched with Et_3N, the solvents were evaporated to dryness, and after chromatographic purification (dry-flash, SiO_2, gradient EA → EA/MeOH/NH_3; Lobar RP-18, eluent MeOH/H_2O = 9:1; Biotage flash SP, gradient EA/Hex = 6:4 → EA/Hex = 9:1) **16** (45.0 mg) and **17** (40 mg) were isolated (9.6 and 8.5 % yield, respectively).

*Methyl 7α,12α-diacetoxy-3β-(*N*-{3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl}amino)--5β-cholan-24-oate (**18**) and methyl 7α,12α-diacetoxy-3α-(*N*-{3-[(6-chloro-2-methoxyacridin--9-yl)amino]propyl}amino)-5β-cholan-24-oate (**19**)*

To a solution of methyl 7*α*,12*α*-diacetoxy-3-oxo-5*β*-cholan-24-oate (500 mg, 0.99 mmol) in CH_3CN (9.5 mL), **13** (124.3 mg, 0.39 mmol) in MeOH (5 mL) was added, and after stirring for 30 min at r.t., $NaBH_3CN$ (82.8 mg, 1.32 mmol) was added in one portion. The resulting mixture was stirred for 2 h at r.t. followed by the addition of glac. AcOH (4 drops) and stirring was continued until TLC indicated the consumption of all starting ketone. The reaction was quenched with Et_3N, the solvents were evaporated to dryness and after chromatographic purification (dry-flash, SiO_2, gradient EA → EA/MeOH/NH_3; Lobar RP-18, eluent MeOH/ /H_2O = 9:1; Biotage flash SP, gradient EA/Hex = 6:4 → EA/Hex = 9:1) **18** and **19** were isolated (120.0 mg, 15 % and 90 mg, 11 %, respectively). **18** contained *ca*. 20 % of 3*α*-isomer **19** and **19** was a mixture of a *ca*. 1:1.5 ratio of the *β*- and *α*-isomers.

*3α,7α,12α-Triacetoxy-24-{*N*-{2-[(6-chloro-2-methoxyacridin-9-yl)amino]ethyl}amino}-5β--cholan (**20**)*

The alcohol 3*α*,7*α*,12*α*-triacetoxy-5*β*-cholan-24-ol (540 mg, 1.04 mmol) was dissolved in DCM (50 mL), PCC (185 mg, 1.56 mmol) was added and the mixture was stirred at r.t. for 3.5 h. The reaction mixture was filtered through a short SiO_2 column (eluent CH_2Cl_2/EA = = 95/5). Obtained crude aldehyde (500 mg) was dissolved in MeOH (10.0 mL), followed by **12** (290 mg, 0.96 mmol) and mixture was stirred at r.t. After 12 h, $NaBH_4$ (73 mg, 1.92 mmol) was added in one portion and stirring was continued at r.t. for 2 h. The solvent was evaporated to dryness and after chromatographic purification (dry-flash, SiO_2, eluent EA/MeOH = 8/2), the desired product was isolated as an orange solid. Yield: 325 mg (42 %).

*3α-7α,12α-Triacetoxy-24-{*N*-{3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl}amino}-5β--cholan (**21**)*

The alcohol 3*α*,7*α*,12*α*-triacetoxy-5*β*-cholan-24-ol (540 mg, 1.04 mmol) was dissolved in DCM (50 mL), PCC (185 mg, 1.56 mmol) was added and the mixture was stirred at r.t. for 3.5 h. The reaction mixture was filtered through a short SiO_2 column (eluent CH_2Cl_2/EA = = 95/5). The obtained crude aldehyde (500 mg) was dissolved in MeOH (10.0 mL) followed by **13** (303 mg, 0.96 mmol), and the mixture was stirred at r.t. After 12 h, $NaBH_4$ (73 mg, 1.92 mmol) was added in one portion and stirring was continued at r.t. for 2 h. The solvent was evaporated to dryness and after chromatographic purification (dry-flash, SiO_2, eluent EA/ /MeOH = 8/2), product was isolated as an orange solid. Yield 230 mg (29 %).

N-*[2-(1-Adamantyl)ethyl]*-N′-*(6-chloro-2-methoxyacridin-9-yl)ethane-1,2-diamine (**22**)*

Into stirred mixture of 2-(1-adamantyl)acetaldehyde (140 mg, 0.86 mmol) and **12** (260 mg, 0.86 mmol) in abs. EtOH (10 mL), $Ti(OPr^i)_4$ (381 μL, 1.29 mmol) was added dropwise. After stirring for 1 h at r.t., $NaBH_4$ (65 mg, 1.72 mmol) was added in one portion and when all the aldehyde had been consumed, the reaction was quenched with 0.1 M NaOH (10mL). The reaction mixture was extracted with CH_2Cl_2 (3×30 mL), the combined organic layers were washed with brine and dried over anh. Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by dry-flash chromatography (SiO_2, eluent hexane/EA = = 1/1). Yield 40 mg (10 %).

N-*[2-(1-Adamantyl)ethyl]*-N′-*(6-chloro-2-methoxyacridin-9-yl)propane-1,3-diamine (**23**)*

According to the procedure described for **22**, 2-(1-adamantyl)acetaldehyde (140 mg, 0.86 mmol) was transformed into **23** using **13** (271 mg, 0.86 mmol). The product was isolated after dry-flash chromatography (SiO_2, eluent EA/MeOH = 8/2). Brown–orange amorphous solid. Yield: 190 mg (46 %).

N-*Benzyl*-N′-*(6-chloro-2-methoxyacridin-9-yl)ethane-1,2-diamine (**24**)*

To a stirred mixture of benzaldehyde (100 μL, 0.98 mmol) and **12** (312.0 mg, 1.03 mmol) in CH_2Cl_2 (15 mL), $NaB(OAc)_3H$ (313.0 mg, 1.47 mmol) was added. After stirring for 20 h at r.t., the reaction was quenched with 1 M NaOH (10 mL). The mixture was extracted with CH_2Cl_2 (3×30 mL), the combined organic layers were washed with brine and dried over anh. Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by dry-flash chromatography (SiO_2, eluent EA/MeOH = 95/5). Yield 230.0 mg (60 %).

N-*(6-Chloro-2-methoxyacridin-9-yl)ethane-1,2-diamine (**12**)*[57]

A solution of 6,9-dichloro-2-methoxyacridine **11** (600 mg, 2.15 mmol) in 1,2-diaminoethane (10 mL, 140 mmol) was stirred at 70 °C under an Ar atmosphere. After 1 h, the mixture was poured into ice cold water, the powder was filtered and dried under reduce pressure. The product was obtained as a yellow powder and used in the next reaction step without further purification. Yield 580 mg (89 %).

N-*(6-Chloro-2-methoxy-acridin-9-yl)propane-1,3-diamine (**13**)*[57]

According to procedure described for **12,** 6,9-dichloro-2-methoxyacridine (1 g, 3.59 mmol) was transformed into **13** using 1,3-diaminopropane (20 mL, 232 mmol). The product was obtained as a yellow powder and used in the next reaction step without further purification. Yield 1.08 mg (95 %).

N-*(6-Chloro-2-methoxyacridin-9-yl)pentane-1,5-diamine (**14**)*[49]

According to procedure described for **12** 6,9-dichloro-2-methoxyacridine (500 mg, 1.79 mmol) was transformed into **14** using 1,5-diaminopentane (13 mL, 11.3 g) at 80 °C. The product was obtained as yellow powder and used in next reaction step without further purification.

N-*(6-Chloro-2-methoxyacridin-9-yl)hexane-1,6-diamine (**15**)*[39]

According to procedure described for **12** 6,9-dichloro-2-methoxyacridine (1g, 3.59 mmol) was transformed in **15** using 1,6-diaminohexane (23 g, 197.9 mmol) at 90 °C. Product was obtained as yellow powder and used in next reaction step without further purification.

In vitro *BoNT LC metalloprotease activity*

Determination of BoNT/A LC percent inhibition by SMNPIs was performed as previously described.[55]

For BoNT/B LC percent inhibition, the HTS-assay utilizes a commercially available fluorogenic substrate from List Biological Laboratories (Campbell, CA). Briefly, a multi-channel pipette was used to add fluorescent substrate (final concentration 20 μM) and small molecules (final concentration 20 μM) to a 96 well microplate. The reactions were initiated by adding recombinant BoNT/B LC (final concentration 40 nM) to each well. All reactions were conducted in a buffered solution consisting of 40 mM Hepes pH 7.2, 1 mM DTT and 100 μM $ZnCl_2$. The change in fluorescence intensity over time was continuously monitored in each well using a 96 well plate fluorimeter (Saphire 2, Tecan, Männedorf, Switzerland). The assays were run at 37 °C, quenched by the addition of TFA, and analyzed using reverse-phase HPLC. Potential SMNPIs of the BoNT B LC were identified by comparing the reaction velocities (change in fluorescence intensity over time) of samples assayed in the presence of compounds *versus* control samples.

In vitro *antimalarial activity and toxicity*

The *in vitro* antimalarial drug susceptibility and toxicity screening were realized at the Walter Reed Army Institute of Research.[23]

CONCLUSIONS

Aminoacridine derivatives possessing adamantane and steroid carriers were prepared and tested for BoNT/A LC, BoNT/B LC, and antimalarial activity. Acridines with the cholic acid-derived carrier showed good potency against both the BoNT/A LC and BoNT/B LC. The inhibition of the BoNT/B LC by *ca*. 50 % is the highest inhibitory activity of any acridine reported to date. The antimalarial potency of adamantane acridines (IC_{50} in range 6–9 nM), combined with their low potencies (IC_{50} *ca*. 3000 nM) is a good starting point for further research in this area.

SUPPLEMENTARY MATERIAL

Spectral and analytical data of all the synthesized compounds are available electronically at http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

Acknowledgements. This research was supported by National Institute of Allergy and Infectious Diseases (USA) grant 5-U01AI082051-02, by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 172008) and the

Serbian Academy of Sciences and Arts. Furthermore, for J.C.B., in compliance with SAIC--Frederick, Inc. contractual requirements: this project was funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health (USA), under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services (USA), or the US Army, nor does the mention of trade names, commercial products, or organizations imply endorsement by the US Government, or the US Army. B.Š. is grateful to WRAIR for providing the *in vitro* antimalarial results.

ИЗВОД

НОВИ ДЕРИВАТИ 9-АМИНОАКРИДИНА КАО ИНХИБИТОРИ БОТУЛИНУМ НЕУРОТОКСИНА И *P. falciparum* ПАРАЗИТА МАЛАРИЈЕ

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Синтетисани су деривати стероидних и адамантил-акридина и испитана је њихова инхибиторна активност према ботулинум неуротоксинима (BoNT) и паразиту маларије. Стероидни акридини показују добру инхибицију према кратком низу (LCs) BoNT/A и BoNT/B. Остварена инхибиција BoNT/B LC од око 50 % је највша постигнута вредност акридинских деривата према овом серотипу. Адамантил–акридински деривати су показали највећу антималаријску активност (IC_{50} у опсегу 6–9 nM, $SI > 326$), показујући да је адамантил-група бољи носач фармакофоре у поређењу са стероидним, према овој индикацији.

(Примљено 24. септембра, ревидирано 20. октобра 2013)

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