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Azapropellanes with Anti-Influenza A Virus Activity

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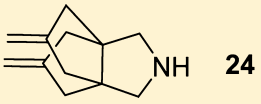
Supporting Information

ABSTRACT: The synthesis of several [4,4,3], [4,3,3], and [3,3,3]azapropellanes is reported. Several of the novel amines displayed low-micromolar activities against an amantadine-resistant H1N1 strain, but they did not show activity against an amantadine-sensitive H3N2 strain. None of the tested compounds inhibit the influenza A/M2 proton channel function. Most of the compounds did not show cytotoxicity for MDCK cells.



KEYWORDS: *Amantadine, influenza, M2 channel, hemagglutinin, propellane*

Human influenza A virus, a member of the *Orthomyxoviridae*, causes significant morbidity and mortality, particularly in infants, elderly people, and those suffering from previous pathology or immunodeficiency.[1] Its capacity to cause widespread epidemics is related to its fast droplet transmission and ability to escape from existing immunity.

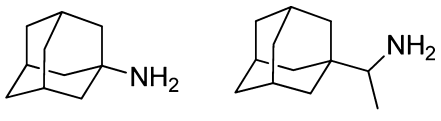
The life cycle of influenza viruses is well documented and most viral proteins are regarded as potential therapeutic targets.[2−6] However, presently available drugs for the treatment of influenza virus infections only comprise the M2 ion channel blockers amantadine (Amt) and rimantadine (Chart 1),[7,8] and the neuraminidase inhibitors oseltamivir, zanamivir, peramivir, and laninamivir.[5] Taking into account that most of the currently circulating influenza strains are resistant to the M2 ion channel blockers and as resistance to the neuraminidase inhibitors (in particular oseltamivir) is also rising,[9,10] novel anti-influenza virus drugs, preferably with a novel mechanism of action, are urgently needed.

The influenza virus enters its target cells by receptor-mediated endocytosis, which is followed by acid-induced fusion of the viral and endosomal membranes. This fusion event is mediated by a conformational change of the influenza hemagglutinin (HA) protein, triggered by the low pH in the endosomal lumen.[11] The endosomal acidic pH also activates the membrane-spanning A/M2 protein, which acts as a proton channel to conduct protons into the virion interior. The decrease in intravirion pH results in uncoating of the viral ribonucleoprotein, which is transferred, through the fusion pore, to the cytoplasm.[12]

Since this endosomal escape of the virus critically depends on the activity of the M2 proton channel and the HA protein, dually acting agents which combine blockade of the M2 channel with an inhibitory effect on HA refolding, appear highly attractive. This dual approach could also, at least in theory, increase the barrier for selecting Amt resistance.[13]

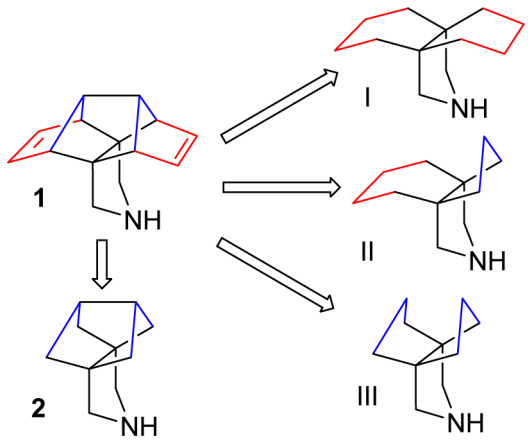
Optimized Amt analogues might be able to exert this dual pharmacological effect, provided that both the M2 and HA inhibition occur at similar and clinically relevant compound

Chart 1. Structures of Amantadine and Rimantadine



amantadine rimantadine

Scheme 1. Simplification of Diene 1 to Tetracyclo 2 and to [4,4,3]-, [4,3,3]-, and [3,3,3]-Azapropellanes of General Structures I, II, and III



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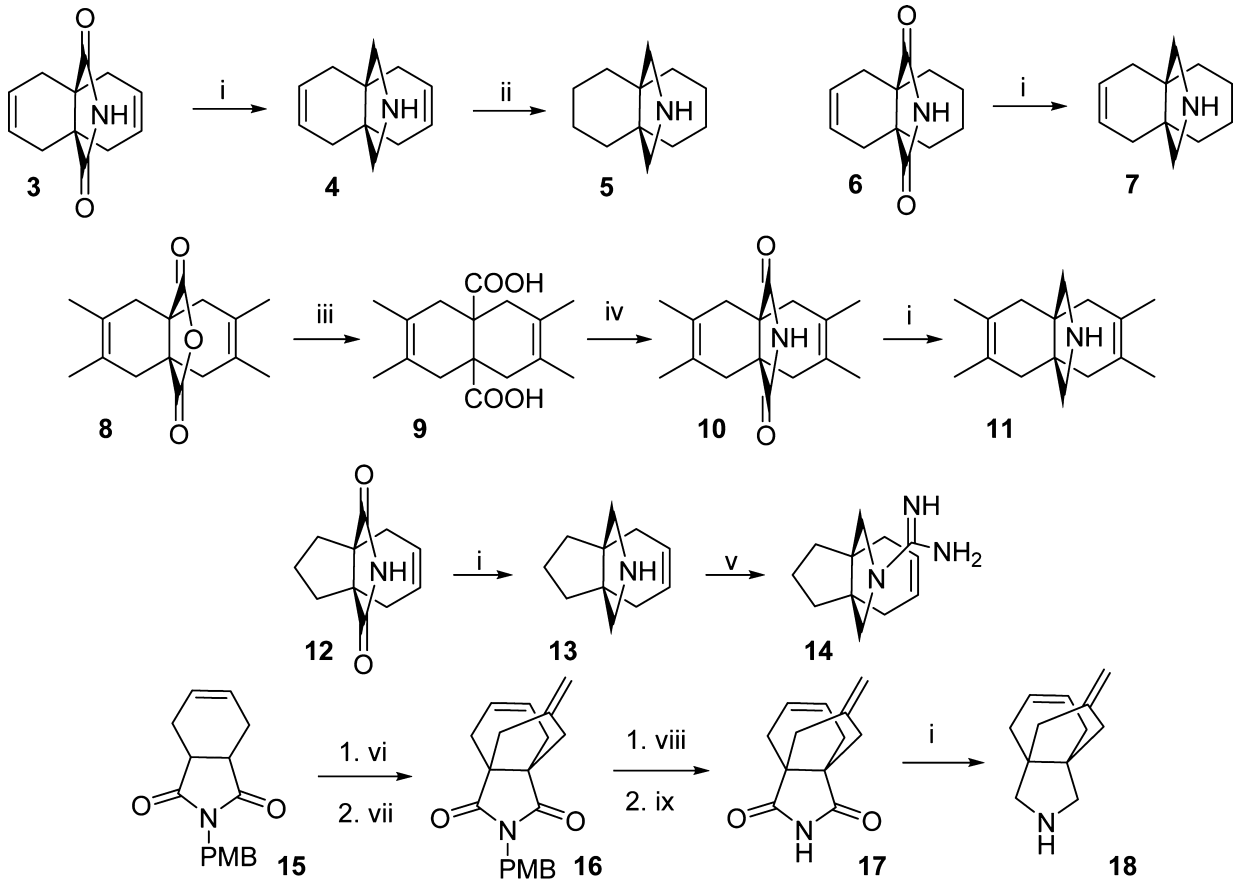
Scheme 2. Synthesis of Novel [4,4,3]- and [4,3,3]-Azapropellanes[a]



[a]Reagents and conditions: (i) $NaAlH_2(OCH_2CH_2OCH_3)_2$, toluene, reflux, overnight, 46% yield for **4**; 71% for **7**; 40% yield for **11**; 82% yield for **13**; 74% yield for **18**. (ii) H_2, Pd/C, methanol, 1 atm, rt, 93% yield. (iii) 5 N NaOH, reflux, overnight, then conc HCl, 81% yield. (iv) urea, 220 °C, 30 min, 80% yield. (v) 1*H*-pyrazole-1-carboxamidine hydrochloride, triethylamine, acetonitrile, reflux, 6 h, 49% yield. (vi) LiHMDS, anhydrous THF, −78 °C. (vii) 3-Chloro-2-(chloromethyl)-1-propene, anhydrous THF, −78 °C to room temperature, overnight, 70% overall yield. (viii) CAN, H_2O, acetonitrile, rt, 2 h. (ix) ethanol, aq NH_4OH, reflux, 2 h, 70% overall yield.

concentrations. For comparison, the M2 blocking effect of Amt is achieved at micromolar concentrations, whereas its effect on HA refolding requires much higher concentrations (i.e., in the range of 100 μM). These high Amt concentrations cannot be achieved *in vivo* with the standard Amt drug regimens, since the reported plasma concentration values for Amt are in the range of 2 μM.[14] Hence, to potentially exploit the effect of Amt analogues on HA refolding, novel derivatives with a more potent activity (i.e., IC_{50} values in the micromolar range) would be required.

It is well-known in medicinal chemistry that for drugs containing cyclic systems, it is generally worth synthesizing analogues where the ring is opened, expanded or contracted by one unit. These analogues show similar activity to the parent compound and sometimes display improved pharmacokinetic profile. Also, it is of interest to simplify a polycyclic active compound by removing some of the rings.[15] Previously we synthesized and evaluated a series of ring-contracted, ring-expanded, and ring-rearranged analogues of Amt and rimantadine. Although we found several compounds with very interesting activities as M2 channel blockers or modifiers of the HA refolding, no dual agents were found.[16−20]

Recently, we have reported that diene **1** displayed similar IC50 values for wild-type (wt) A/M2 to Amt, although it was inactive against the Amt-resistant S31N form of A/M2.[17] As expected, amine **1** was active in a cytopathic effect (CPE) assay (EC_{50} = 14 μM) against the Amt-sensitive A/HK/7/87 strain, an H3N2 virus with a wt-type A/M2 channel. Interestingly, amine **1** was also active (EC_{50} = 16 μM) against the A/PR/8/34 strain, an H1N1 virus carrying two characteristic Amt resistance mutations in its M2 protein (S31N and V27T). A detailed study of the mechanism of action of this and several related analogues suggested that their target was the H1 type HA, through an effect on its refolding.[19] Thus, this compound may be considered a lead for dually acting compounds. Of note, we have also reported that a simplified analogue of diene **1**, 3-azatetracyclo[5.2.1.$1^{5,8}$.$0^{1,5}$]undecane, **2**, displayed a better inhibition of the wt A/M2 channel (IC_{50} = 11.7 μM) than **1** (IC_{50} = 33.5 μM) and was slightly more potent in the CPE assay (EC_{50} = 14.0 μM for 1; EC_{50} = 7.9 μM for **2**) against the Amt-sensitive A/HK/7/87 strain. In sharp contrast with diene **1**, which was also active against the A/PR/8/34 strain, azaundecane **2** was inactive against this Amt-resistant strain (Scheme 1).[20]

In the present work, we report on the synthesis and antiviral activity of further ring-simplified analogues of **1**. We found that several of these novel polycyclic amines, that have considerable higher conformational freedom than **1** and **2**, display low micromolar activity against the Amt-resistant A/PR/8/34 strain (A/H1N1). However, this activity was markedly subtype-

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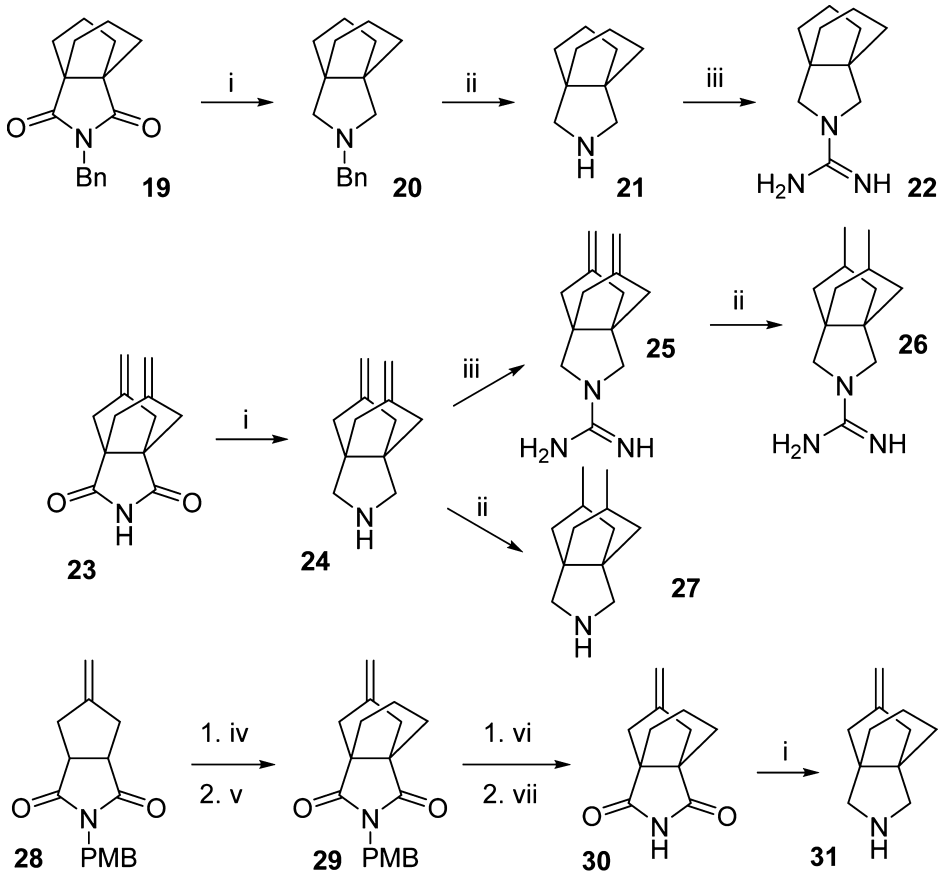
Scheme 3. Synthesis of Novel [3,3,3]Azapropellanes[a]



[a]Reagents and conditions: (i) $NaAlH_2(OCH_2CH_2OCH_3)_2$, toluene, reflux, overnight, 75% yield for **20**; 80% yield for **24**; 93% yield for **31**. (ii) H_2, Pd/C, methanol, 1 atm, rt, 90% yield for **21**; 95% yield for **26**; 87% yield for **27**. (iii) 1*H*-Pyrazole-1-carboxamidine hydrochloride, triethylamine, acetonitrile, reflux, 6 h, 18% yield for **22**; 75% yield for **25**. (iv) LiHMDS, anhydrous THF, −78 °C. (v) 1,3-Dichloropropane, anhydrous THF, −78 °C to room temperature, overnight, 72% overall yield. (vi) CAN, H_2O, acetonitrile, rt, 2 h. (vii) ethanol, aq NH_4OH, reflux, 2 h, 83% overall yield.

dependent, as none of the compounds were active against the Amt-sensitive influenza A/HK/7/87 strain, an H3N2 virus. Seven compounds, including the most potent amine, **24**, were evaluated as M2 channel blockers, but were unable to inhibit the activity of the wt and the V27A mutant, thus suggesting that they interact with a different target in the virus.

We envisaged that the simplification of the hexacyclic amine **1** may lead to three different propellane scaffolds, **I**, **II**, and **III** (Scheme 1). Propellanes are defined as tricyclic compounds in which the three rings have a common carbon−carbon bond.[21] Of note, the use of propellane scaffolds in the field of medicinal chemistry has been rather limited,[22] and, in fact, to the best of our knowledge, the use of [4,3,3] and [3,3,3]azapropellanes is unprecedented, and only two works have been published in connection with the [4,4,3]azapropellane scaffold.[23,24]

The synthesis of azapropellanes related to the general structure **I** was straightforward. Thus, reduction of known imide **3** led to diene **4** in 46% yield.[23,24] The catalytic hydrogenation of **4** led to the [4,4,3]azapropellane **5** in 93% yield. In a similar fashion, starting from the known imide **6**,[23] the azapropellane **7** was isolated in 71% yield.[24] Finally, hydrolysis of the known anhydride **8** led to diacid **9** in 81% yield. Reaction of **9** with melting urea furnished imide **10** that was subsequently reduced to the diene **11** (Scheme 2).

Three compounds related to the general structure **II** were synthesized. Reduction of the known imide **12** furnished **13** in 82% yield. Reaction of **13** with 1*H*-pyrazole-1-carboxamidine led to the guanidine **14** in 49% yield. For the synthesis of the amine **18** featuring a methylidene group we started from the known *N*-(*p*-methoxybenzyl)imide **15**. Alkylation of **15** using lithium hexamethyldisilazane (LiHMDS) and 3-chloro-2-(chloromethyl)-1-propene furnished imide **16** in 70% yield. Removal of the protecting group was accomplished with cerium ammonium nitrate (CAN) in 70% yield. Reduction of the imide gave propellane **18** in 74% yield (Scheme 2).

Finally, seven compounds containing the [3,3,3]-azapropellane scaffold were synthesized (Scheme 3). Reduction of the known imide **19** with sodium bis(2-methoxyethoxy)-aluminum hydride furnished **20** in 75% yield. The hydrogenolysis of the benzyl protecting group furnished the [3,3,3]azapropellane **21** in 90% yield. Finally, reaction of **21** with 1*H*-pyrazole-1-carboxamidine led to the guanidine **22** in very low yield. Similarly, reduction of imide **23**, previously synthesized by our group, gave diene **24** in 80% yield. The guanidine **25** was synthesized following the general procedure in 75% yield. The catalytic hydrogenation of **24** and **25** gave the fully saturated compounds **27** and **26**, respectively, as mixtures of stereoisomers, in very high yields.

For the synthesis of **31** we started from the imide **28**, previously synthesized by our group. Reaction of **27** with LiHMDS and 1,3-dichloropropane gave imide **29** in 72% yield. Deprotection of the imide with CAN led to **30** in 83% yield,

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Table 1. Antiviral Activity in Influenza Virus-Infected MDCK[a] Cells and Inhibitory Effect on A/M2 wt Proton Channel Function[b]

	antiviral EC_{50}[c] (μM)							
	influenza A/H1N1		influenza A/H3N2		inhibitory effect on A/M2 wt proton channel function[d]		cytotoxicity	
compd	CPE	MTS	CPE	MTS	Inh by 100 μM for 2 min (%)	IC_{50} (μM)	MCC[e] (μM)	CC_{50}[f] (μM)
1[17,19]	16		14		82.5 ± 0.8	33.5	>100	>100
2[20]	>100	>100	7.9	7.2	93.9 ± 0.2	11.7	≥59	100
4	38	30	>100	>100	ND	ND	>100	>100
5	5.9	13	>100	>100	ND	ND	>100	>100
7	30	14	>100	>100	19.1 ± 1.8	ND	>100	>100
11	7	5.6	>100	>100	ND	ND	>100	>100
13	70	56	>100	>100	ND	ND	>100	>100
14	>100	>100	>100	>100	ND	ND	>100	>100
18	45	43	>100	>100	14.9 ± 3.9	ND	>100	>100
21	>100	>100	>100	>100	47.1 ± 2.3	ND	>100	>100
22	>100	>100	>100	>100	ND	ND	54	100
24	<0.8	<0.8	>100	>100	29.3 ± 2.3	ND	>100	>100
25	>100	>100	>100	>100	ND	ND	16	20
26	2.0	1.6	>100	>100	8.8 ± 2.2	ND	>100	>20
27	24	20	>100	>100	58.7 ± 2.9	ND	>100	>100
31	>100	>100	>100	>100	29.9 ± 1.1	ND	>100	>100
Amantadine[18,20]	53 ± 11		3.4 ± 1.7		91.0 ± 2.1	16.0	500	>100
Rimantadine[18]	63 ± 18		0.17 ± 0.08				≥100	>100

[a]MDCK: Madin−Darby canine kidney cells. [b]See text and Supporting Information for details. [c]Compounds **7**, **18**, **21**, **24**, **26**, **27**, and **31** were also tested in the V27A mutant and **21**, **24**, and **27** also in the S31N mutant. None of them significantly inhibit these channels. [d]Virus strains: A/PR/8/34 (A/H1N1); A/HK/7/87 (A/H3N2). The EC_{50} represents the 50% effective concentration, or compound concentration producing 50% inhibition of virus replication, as determined by microscopic scoring of the CPE, or by the MTS cell viability test. [e]MCC: minimum cytotoxic concentration, or compound concentration producing minimal alterations in cell morphology. [f]CC_{50}: 50% cytotoxic concentration, as determined by the MTS cell viability test. Values shown are the mean ± SEM of 2−5 determinations. ND: not determined.

whose reduction with sodium bis(2-methoxyethyoxy)aluminum hydride furnished **31** in 93% yield (Scheme 3).

The structure of all new compounds was confirmed by elemental analysis and/or accurate mass measurement, IR, ^{1}H NMR, ^{13}C NMR, and mass spectral data. The amines were fully characterized as their corresponding hydrochloride.

Antiviral cell culture assays were performed to determine the antiviral activity of all the synthesized compounds against a broad panel of DNA and RNA viruses. None of the compounds displayed activity against the enveloped DNA viruses herpes simplex virus or vaccinia virus; the enveloped RNA viruses feline coronavirus, parainfluenza-3 virus, respiratory syncytial virus, vesicular stomatitis virus, sindbis virus or Punta Toro virus; or the nonenveloped RNA viruses Coxsackievirus B4 and Reovirus-1.

In influenza virus-infected MDCK cells, several compounds displayed low micromolar activity against the influenza A/H1N1 subtype, but no compound was active against the influenza A/H3N2 subtype (Table 1). The antiviral data obtained by microscopic inspection of the viral CPE at day 3 post infection were confirmed by a colorimetric cell viability assay.[25] All compounds proved to be inactive against influenza B virus (results not shown). Only two compounds, guanidines **22** and **25** were cytotoxic for the MDCK cells.

Analysis of the data in Table 1 revealed the following trends. First, within the series of [4,4,3]azapropellanes, progressive saturation of the diene **4** to alkene **7** and to the fully saturated **5** increased the antiviral activity against the A/PR/8/34 strain (EC_{50} = 38, 30, and 5.9 μM for **4**, **7** and **5**, respectively). This trend had been previously seen on going from diene **1** (EC_{50} = 16 μM) to its fully saturated derivative (EC_{50} = 2.0 μM).[25] Interestingly, going from diene **4** to its tetramethyl derivative **11** also increased the activity (EC_{50} = 38 μM for **4** vs EC_{50} = 7 μM for **11**). Unfortunately, several attempts to carry out the reduction of **11** either with catalytic hydrogenation or diimide were unsuccessful, likely due to steric hindrance. Second, ring contraction from the [4,4,3]azapropellane to the [4,3,3]-azapropellane did not improve the activity (compare **7** vs **13**), while the introduction of a methylidene group in the five-membered ring slightly increased the activity (compare **13** vs **18**). Third, the smallest member of this series, [3,3,3]-azapropellane **21** was inactive as was its monomethylidene derivative **31**. However, introduction of a second methylidene group, as in **24**, led to an active compound (EC_{50} < 0.8 μM), nearly 2 orders of magnitude more potent than Amt and rimantadine. Finally, with the only exception of compound **26**, that was more potent than amine **27**, the introduction of a guanidine group was clearly deleterious for the activity, leading largely to inactive compounds.

It is well-known that the target of Amt and rimantadine is the influenza A virus M2 channel protein and that a single S31N mutation in M2 is sufficient to render the virus resistant to both drugs.[26−28] As most of the currently circulating strains of influenza A virus of the A/H3N2 or A/H1N1 subtype carry the S31N mutation in M2, there is an urgent need for the development of novel anti-influenza drugs that are effective against the most common amantadine-resistant mutants.

The influenza A/PR/8/34 strain that we used in our CPE assay has an M2 protein carrying two substitutions associated with amantadine resistance (i.e., S31N and V27T). In order to assess if the target of the novel amines was the M2 protein, the inhibitory activity of seven compounds (**7**, **18**, **21**, **24**, **26**, **27**

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and **31**) was tested on A/M2 channels expressed in *Xenopus* oocytes using the two-electrode voltage clamps (TEV) technique. None of them, at 100 μM, was able to significantly inhibit the activity of the wt, the V27A (Amt-resistant) or the S31N (Amt-resistant) mutants, indicating that for the compounds that showed antiviral activity in the CPE assay the viral target is not the M2 protein.

After combining the results that we previously reported for diene **1**,[17,19] those of its simpler analogue **2**,[20] and those of the azapropellane disclosed herein, it seems that keeping a conformationally rigid scaffold is more suitable for targeting the M2 channel.

We previously reported that the strong activity of some polycyclic amine compounds against the A/PR/8/34 strain appears related to the fact that this virus has a lower fusion pH (4.9) compared to other strains.[19] Hence, the A/PR/8/34 strain seems to be more sensitive to slight increases in endosomal pH. The three most active compounds reported here, i.e. **11**, **24**, and **26**, were evaluated in a CPE assay with A/PR/8/34 mutant viruses having an increased fusion pH due to specific changes in HA.[19] More specifically, these mutant viruses have a fusion pH of 5.2 to 5.7 (compared to 4.9 for wt virus) and contain mutations in HA that reduce the stability of this trimeric protein.[19] None of the three compounds was active against these mutant forms of A/PR/8/34 (data not shown).

To sum up, we have synthesized, fully characterized, and evaluated a series of novel azapropellane derivatives. Against the A/PR/8/34 strain, an H1N1 Amt-resistant virus, three compounds (**5**, **11**, and **26**) were endowed with EC_{50} values up to 1 order of magnitude lower than that of Amt, and compound **24** was a submicromolar antiviral agent, while not being cytotoxic. None of the compounds were active against the Amt-sensitive A/HK/7/87 strain. Their mechanism of action is unrelated to the M2 channel and, as in previous compounds developed by our group, seems to be related to the low fusion pH that is characteristic for the HA of the A/PR/8/34 virus strain.

■ ASSOCIATED CONTENT

Supporting Information

Procedures for the synthesis and characterization of novel compounds. Antiviral and cytotoxicity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Amt, amantadine; Bn, benzyl; CAN, cerium ammonium nitrate; CPE, cytopathic effect; HA, hemagglutinin; MDCK, Madin–Darby canine kidney; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PMB, *p*-methoxybenzyl; wt, wild-type

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