

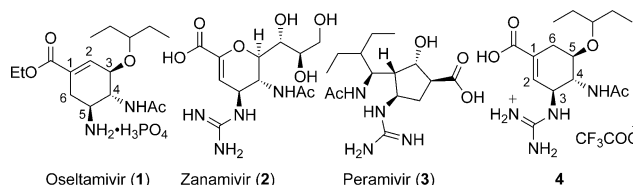
Serendipitous Discovery of a Potent Influenza Virus A Neuraminidase Inhibitor**

Sankar Mohan, Philip S. Kerry, Nicole Bance, Masahiro Niikura, and B. Mario Pinto*

Abstract: We have previously reported a potent neuraminidase inhibitor that comprises a carbocyclic analogue of zanamivir in which the hydrophilic glycerol side chain is replaced by the hydrophobic 3-pentyloxy group of oseltamivir. This hybrid inhibitor showed excellent inhibitory properties in the neuraminidase inhibition assay ($K_i = 0.46$ nM; $K_{i(\text{zanamivir})} = 0.16$ nM) and in the viral replication inhibition assay in cell culture at 10^{-8} M. As part of this lead optimization, we now report a novel spirolactam that shows comparable inhibitory activity in the cell culture assay to that of our lead compound at 10^{-7} M. The compound was discovered serendipitously during the attempted synthesis of the isothioureia derivative of the original candidate. The X-ray crystal structure of the spirolactam in complex with the N8 subtype neuraminidase offers insight into the mode of inhibition.

Despite the continuous research efforts and success in the field of antiviral research, influenza virus continues to pose a major threat to public health. The recent H5N1 and H7N9 avian influenza outbreaks in Asia have caused widespread alarm, with case-fatality rates of 60% and 32%, respectively.^[1] The influenza virus neuraminidase inhibitors oseltamivir (1)^[2] and zanamivir (2)^[3] are the two antiviral drugs stockpiled by most countries in the world in pandemic preparedness planning (Scheme 1). Peramivir (3),^[4] an experimental neuraminidase inhibitor, has also been approved by the U.S. Food and Drug Administration (FDA) for emergency use (Scheme 1). However, appearances of resistant viral strains to these antiviral drugs highlight the urgent need for the next generation neuraminidase inhibitors.

Neuraminidase plays a crucial role in the influenza infection process and is a proven target for antiviral ther-



Scheme 1. Clinically used neuraminidase inhibitors (1–3) and a carbocyclic analogue of zanamivir (4).

apy.^[2–4] There are ten known subtypes of neuraminidase (N1–N10) from influenza A virus which have been characterized. However, the N10 subtype is considered to be a neuraminidase-like protein because of the lack of normal sialidase activity.^[5] Phylogenetically, these subtypes are classified into two groups: N1, N4, N5, and N8 subtypes (group-1) and N2, N3, N6, N7, and N9 subtypes (group-2).^[6] While the residues involved in the catalysis are preserved in subtypes N1–N9, these two groups were also found to be structurally distinct, as revealed by the X-ray crystal structures.^[6] Resistance to known inhibitors arises from mutations in or around the enzyme active site. By far the most common of these mutations, H274Y, restricts the inhibition of oseltamivir by displacing the pentyloxy group out of a hydrophobic pocket close to the active site.^[7] Other mutations, particularly E119 V, E119G, and R292 K can affect binding of both oseltamivir and zanamivir but arise much less frequently.^[8]

Our previous work identified a lead compound 4, which is a carbocyclic analogue of zanamivir in which the hydrophilic glycerol side chain of zanamivir is replaced by the hydrophobic 3-pentyloxy group of oseltamivir (Scheme 1).^[9] This hybrid inhibitor (4) showed excellent inhibition both in neuraminidase activity ($K_i = 0.46$ nM; $K_{i(\text{zanamivir})} = 0.16$ nM)^[9] and in viral replication in cell culture (concentration as low as 80 nM).^[10] More importantly, compared to oseltamivir, this compound (4) retained its inhibitory activity against the oseltamivir-resistant strain [A/Brisbane/59/2007-like oseltamivir-resistant (H274Y) strain] in cell culture assay.^[11] Furthermore, it showed undiminished activities in vitro against the multi-drug resistance mutants N2-E119V and N2-I222L as well as the corresponding double mutant N2-E119V/I222L.^[12] Compound 4 did not show cross-reactivity with human neuraminidase isoforms, NEU3 and NEU4.^[13] Of note, zanamivir inhibits human neuraminidase isoforms in the micromolar range.^[14]

We report herein the serendipitous discovery of a new neuraminidase inhibitor during the course of further optimization of the lead compound 4. Thus, the attempted synthesis of the derivative 5 containing an isothioureia instead of the guanidine group at C-3 resulted in the formation of two new

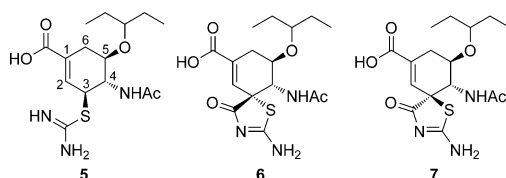
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[**] We thank the Natural Sciences and Engineering Research Council of Canada and Medical Research Council (UK) for financial support. We also thank John Skehel and Patrick Collins of the MRC-National Institute for Medical Research (UK), for the kind provision of N8 protein.

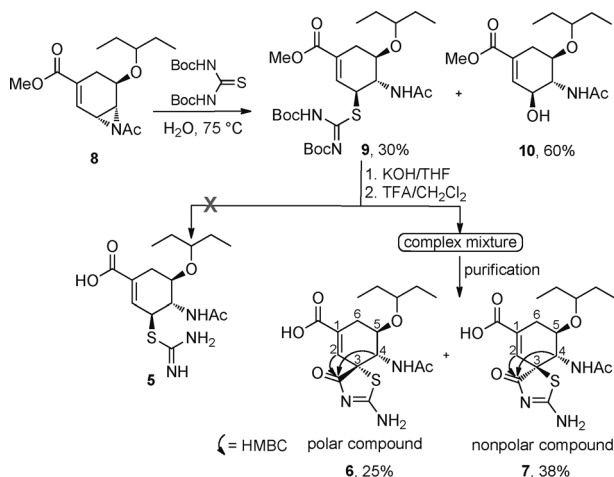
Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201308142>.



Scheme 2. Structures of isothiurea derivative **5** and the spiro compounds **6** and **7**.

compounds **6** and **7**, having an unusual spiro lactam skeleton (Scheme 2). While compound **7** did not show effective inhibition of viral replication, compound **6** showed comparable inhibitory activity in the cell culture assay to that of our lead compound **4**. We also report here the crystal structures of the N8 subtype neuraminidase complexed with these novel spiro compounds **6** and **7**; these crystal structures provide insight into the mode of inhibition.

We envisaged the use of the *N*-acetylaziridine derivative **8** from our previous synthesis,^[9] for the synthesis of the proposed isothiurea derivative **5**, as shown in Scheme 3.

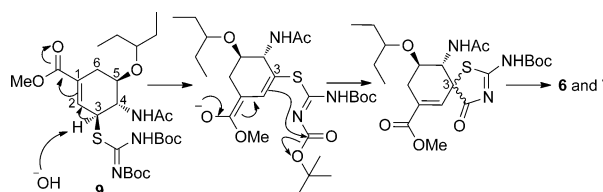


Scheme 3. Attempted synthesis of the isothiurea derivative **5**.

Thus, upon heating compound **8** with *N,N'*-di(Boc)-thiourea in water at 75 °C, the required Boc-protected isothiurea derivative **9** was obtained in 30% yield. The yield of the coupling reaction was low owing to a competing reaction in which water opened the *N*-acetylaziridine ring and led to the formation of the hydroxy compound **10**. We reasoned that this is probably due to the poor solubility of Boc-protected thiourea in water. Attempts to overcome this problem, such as using a mixture of methanol and water in 1:1 ratio as the reaction solvent or using thiourea itself as a nucleophile, were unsuccessful. Compound **9**, upon hydrolysis, followed by treatment with trifluoroacetic acid (TFA), gave a complex mixture. From this mixture, two major compounds were isolated. The first compound (referred to as the nonpolar compound) was isolated by column chromatography using 10% MeOH/CHCl₃ as eluent. The second compound (referred to as the polar compound) was precipitated in pure form by the addition of ethyl acetate to the concentrated

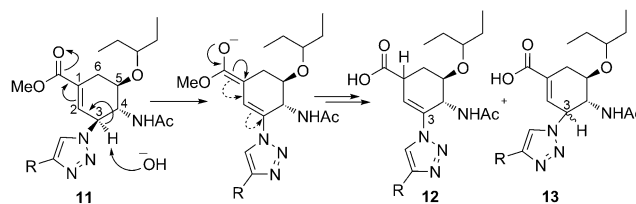
mixture of the polar fractions that were eluted from the column when using 25% MeOH/EtOAc. Characterization of these two compounds by high-resolution mass spectrometry and NMR spectroscopy (see the Supporting Information) led to the assignment of the diastereomeric spiro lactam structures to these compounds (Scheme 3). However, these spectral data were not sufficient to deduce the relative stereochemistry at the spiro center and hence we tentatively assigned the structure of the polar compound as **6** and that of the nonpolar compound as **7** (Scheme 3). This structural assignment was confirmed later by the X-ray crystal structures of these compounds in complex with the N8 subtype enzyme.

The proposed mechanism for the formation of the spiro lactams **6** and **7** is shown in Scheme 4. This mechanism is based on our previous observation with a series of C-3



Scheme 4. Proposed mechanism for the formation of spiro compounds **6** and **7**.

triazole-modified derivatives **11** in which hydrolysis of the methyl ester led to double bond migration (**12**) as well as epimerization at C-3 (**13**; Scheme 5).^[9] However, in the case of intermediate **9**, instead of base-catalyzed epimerization, C-3 acts as a carbon nucleophile and forms a C–C bond with the carbonyl of one of the Boc protecting groups, thereby giving rise to the formation of the diastereomeric spiro lactams (Scheme 4).



Scheme 5. Base-catalyzed double bond migration and epimerization at C-3 in triazole-modified derivatives **11**.

Intrigued by the novel spiro lactam structure, we tested the inhibitory effect of **6** and **7** on influenza A virus replication using two strains, A/Puerto Rico/8/34 (H1N1, PR8) and A/Hong Kong/1/68 (H3N2, HK1). The replication assays were conducted as described in our report.^[10] Briefly, 50 μ L of the virus suspension (50 TCID₅₀) was mixed with test compound in 50 μ L of Dulbecco's minimum essential medium (DMEM) and the virus replication was judged by an immunofluorescent assay (PR8) or by the cytopathic effect (HK1) on day 3 post-infection. The experiments were performed in 96-well plates and each dilution of each compound was tested in quad-

Table 1: Inhibitory effects of the spiro compounds **6** and **7** against HK1 and PR8.

Concentration [M]	Compounds/influenza strain			
	HK1	PR8	HK1	PR8
5×10^{-4}	++++	++++	++++	----
1×10^{-4}	++++	± ± ± ±	++++	----
1×10^{-5}	++++	----	++++	----
1×10^{-6}	++++	----	++++	----
1×10^{-7}	--- ± +	ND	----	ND
1×10^{-8}	----	ND	----	ND

ND = Not determined.

replicate. The inhibitory effects of the spiro compounds **6** and **7** are summarized in Table 1. Compound **6** showed higher efficacy for both HK1 and PR8 strains. The effective concentration was as low as 10^{-6} – 10^{-7} M for HK1. However, for PR8, it required a more than 100 times higher concentration (between 100 to 500 μ M) to be effective. In comparison, the effective concentration for compound **4** was as low as 8×10^{-8} M and 2×10^{-6} M for HK1 and PR8, respectively.^[10] Compound **7** inhibited the replication of HK1 at 10^{-5} M but did not show any inhibitory effect at concentrations as high as 5×10^{-4} M for PR8. A significantly higher concentration of the lead compound **4** was also required for inhibition of the PR8 strain (see above). It is not clear if the difference between strains reflected a true efficacy difference of these compounds on different neuraminidase (NA) subtypes or other factors such as the difference in the binding affinity of the hemagglutinin (HA) of each virus strain to the cellular receptor. It is known that influenza virus strains with lower affinity HA are less dependent on the NA activity.^[15]

To better understand the molecular basis behind the observed differences in the inhibitory activities, crystal structures of the spiro compounds **6** and **7** were obtained in complex with a group-1 NA by soaking crystals of N8 [A/duck/Ukraine/1/63 (Uk1, H3N8)] subtype in 5 mM solutions of inhibitor for 60 min. We note that the critical active-site amino acids of all nine subtypes are highly conserved^[16] (see the Supporting Information, Figure S2 for the sequence alignment of N1, N2, and N8 from PR8, HK1, and Uk1 strains, respectively) and we have chosen N8 from group-1 NAs as a representative subtype for our crystallographic study.

The structures of the bound ligands in each complex were found to be in agreement with our initial assignment of relative stereochemistry at the spiro center. Comparison of the N8–**6** and N8–**7** complexes with the structure of N8–**4**^[11] indicates the binding mode of the shared features is conserved between all three structures (see the Supporting Information, Figure S3), with the majority of the ligand atoms similarly positioned. However, in place of the guanidinium moiety present in N8–**4**, the spirolactam rings of **6** and **7** extend from the cyclohexene ring forming a unique set of contacts with the enzyme, either directly or through ordered water molecules (Figure 1).

Both **6** and **7** may exist in one of several different tautomeric and resonance states (Scheme 6). In the case of

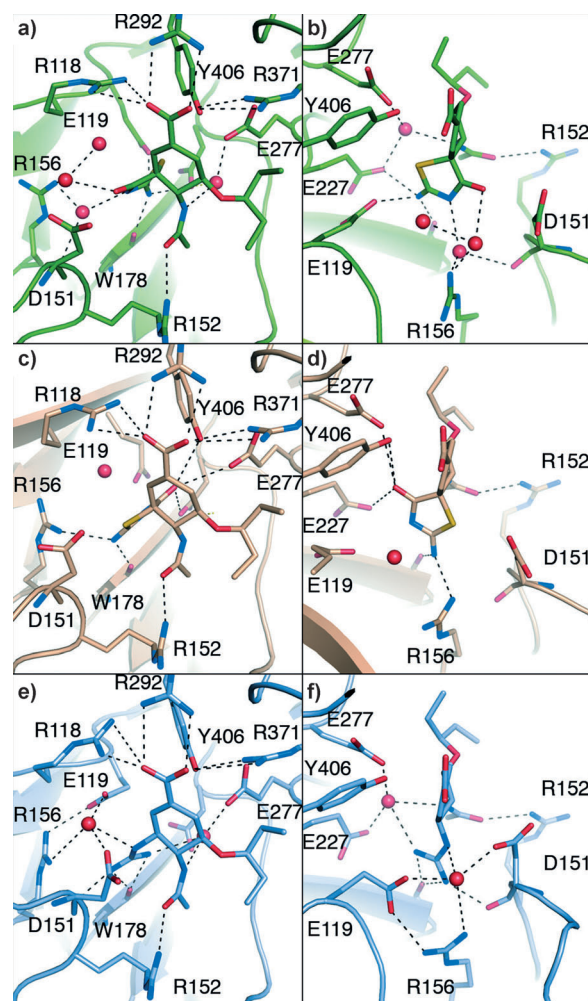
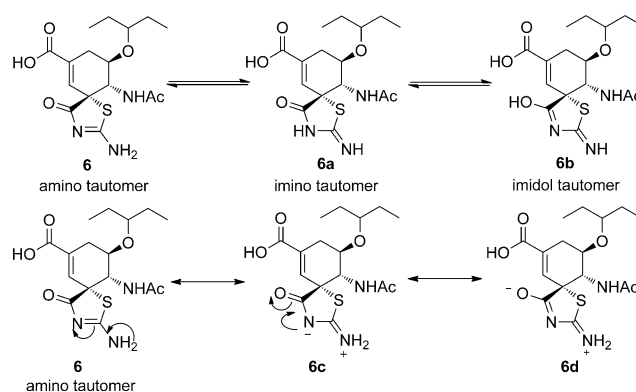


Figure 1. Active-site interactions of **4**, **6**, and **7** in the N8 complex. Representations of N8–**6** (a and b), N8–**7** (c and d), and N8–**4** (e and f), with ligands and interacting residues shown as sticks, interacting water molecules shown as spheres, and H-bonding interactions indicated with dashed lines. All the amino acids numbered are conserved among N8, N1, and N2. Panels (a), (c), and (e) are related to (b), (d), and (f), respectively, by 90° rotations in both x and y axes.



Scheme 6. Different tautomeric (**6**, **6a**, **6b**) and resonance forms (**6c** and **6d**) for the spirolactam ring.

N8–**6**, the interactions between the spirolactam ring and the enzyme appear to be influenced by the resonance state **6c**

adopted by the ligand. Close examination of the N8–6 complex indicates that the sulfur and amine nitrogen of the spirolactam moiety lie out of plane with the other ring atoms, forming a separate plane with the amide nitrogen. Consistent with structures from crystallographic analysis of other thiazolidones,^[17] this conformation would suggest that compound **6** exists predominantly in the form of a zwitterionic resonance contributor **6c** when bound in the enzyme active site, favoring hydrogen-bonding interactions between the iminium nitrogen and an acidic pocket comprised of E119, E227, E277, and the main-chain carbonyl of W178 (Figure 1a). This compound is likely to be held in this zwitterionic resonance state for three reasons: 1) interactions between the iminium nitrogen and the acidic patch stabilizing the positive charge, 2) the proximity of the D151 side chain to the lactam carbonyl which would electrostatically disfavor charge build up at this carbonyl oxygen atom, and 3) a water-mediated hydrogen bond stabilizing the negative charge on the amide nitrogen. Therefore, it seems likely that favorable placement of these charges within the spirolactam structure contribute to the potency of this compound.

In comparison to N8–6, the orientation of the lactam ring within the N8–7 structure is reversed, with the lactam carbonyl oriented towards the acidic patch formed by Y406, E227, and E277 and the amine group positioned close to R156. Both these sets of interactions are likely to be mediated by hydrogen bonds (Figure 1b). While the lower resolution of this structure makes assignment of the exact tautomeric form difficult, it is likely that the hydrogen-bonding network formed between **7** and the enzyme active site is significantly weakened by electrostatic forces working to repel the electronegative carbonyl from the acidic patch. Therefore, while both **6** and **7** form extensive hydrogen bonds with the enzyme, the less favorable orientation of the charges within the spirolactam ring is the likely cause of the greatly reduced potency of compound **7**. In our reports,^[11,12] we have attributed the reduced susceptibility of compound **4** towards known resistance mutations to the change in position of the double bond relative to oseltamivir and its ability to pivot about strong hydrogen-bonding networks at the guanidinium and carboxylate groups. Since these features are largely retained in the N8–6 complex, we speculate that compound **6** will also be less susceptible to drug resistance.

Previous structural analyses of group-1 NA have highlighted the presence of a loop proximal to the enzyme active site (formed by residues 146–152 and termed the 150-loop), which closes upon binding to most ligands.^[6,18] Exploitation of the cavity formed by the open loop conformation (the 150-cavity) has been seen as an avenue to novel inhibitors.^[9,19] In contrast to the structure of N8–4, in which the 150-loop is in a closed conformation, in both N8–6 and N8–7 the 150-loop remains open (Supporting Information, Figure S3). This appears to be due to steric hindrance from the lactam carbonyl group (in N8–6) or the sulfur atom (in N8–7) preventing movement of D151 into a “closed” position. While this characteristic movement is not observed, both **6** and **7** are able to bind to the NA active site and inhibit viral spread efficiently, thereby indicating that the advantages gained from stabilization of a closed state may be offset by other factors

(e.g. additional hydrogen bonds and electrostatic forces). Indeed, as the potency of compound **6** in vitro is comparable to the results of compound **4** (Table 1), closure of the 150-loop may be dispensable for high-affinity interaction. We also note that the 150-loop is dynamic^[20] and may not play a large role in the activity of the spiro compounds. Furthermore, since the 150-loop residues do not seem to engage in stabilizing interactions, we do not expect resistance development in the 150-loop region against the spiro skeleton.

In conclusion, we have discovered a potent neuraminidase inhibitor with a novel spirolactam scaffold. Although this heterocyclic modification in place of the guanidine group alters the strong hydrogen-bond network seen in the case of the N8–4 complex (Figure 1c), it did not affect the inhibitory activity appreciably, thus suggesting that this modification could be useful in mitigating the anticipated poor bioavailability of compound **4**, a drawback also seen in the case of zanamivir with its polar functional groups. Recent structural characterization of various NA subtypes revealed that there are additional cavities near the active site of NA, namely the 150-cavity and 430-cavity,^[6,20] which have not been exploited in the design of currently available drugs. Hence, there is a growing interest in exploring these additional cavities in the design of more potent neuraminidase inhibitors. The highly flexible nature of the 150-loop is responsible for the formation of 150-cavity adjacent to the active site cavity. The influence of the spiro compound **6** in biasing 150-loop dynamics in favor of 150-cavity formation suggests that the spirolactam scaffold might be further exploited in the design of dual-site inhibitors.

Received: September 17, 2013

Revised: November 7, 2013

Published online: December 11, 2013

Keywords: antiviral agents · drug discovery · influenza A · neuraminidase inhibitors · spiro compounds

- [1] World Health Organization. Influenza at the human–animal interface. 29th August 2013 (accessed November 2013).
- [2] C. U. Kim et al., *J. Am. Chem. Soc.* **1997**, *119*, 681–690. For complete citation, see the Supporting Information.
- [3] a) N. R. Taylor, M. von Itzstein, *J. Med. Chem.* **1994**, *37*, 616–624; b) M. von Itzstein, W. Y. Wu, B. Jin, *Carbohydr. Res.* **1994**, *259*, 301–305; c) M. von Itzstein et al., *Nature* **1993**, *363*, 418–423. For complete citation, see the Supporting Information.
- [4] a) Y. S. Babu et al., *J. Med. Chem.* **2000**, *43*, 3482–3486, for complete citation, see the Supporting Information; b) P. Chand, P. L. Kotian, A. Dehghani, Y. El-Kattan, T. H. Lin, T. L. Hutchison, Y. S. Babu, S. Bantia, A. J. Elliott, J. A. Montgomery, *J. Med. Chem.* **2001**, *44*, 4379–4392.
- [5] a) Q. Li, X. Sun, Z. Li, Y. Liu, C. J. Vavricka, J. Qi, G. F. Gao, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 18897–18902; b) X. Zhu, H. Yang, Z. Guo, W. Yu, P. J. Carney, Y. Li, L. M. Chen, J. C. Paulson, R. O. Donis, S. Tong, J. Stevens, I. A. Wilson, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 18903–18908.
- [6] R. J. Russell, L. F. Haire, D. J. Stevens, P. J. Collins, Y. P. Lin, G. M. Blackburn, A. J. Hay, S. J. Gamblin, J. J. Skehel, *Nature* **2006**, *443*, 45–49.
- [7] a) P. J. Collins, L. F. Haire, Y. P. Lin, J. Liu, R. J. Russell, P. A. Walker, J. J. Skehel, S. R. Martin, A. J. Hay, S. J. Gamblin, *Nature* **2008**, *453*, 1258–1261; b) P. J. Collins, L. F. Haire, Y. P.

- Lin, J. Liu, R. J. Russell, P. A. Walker, S. R. Martin, R. S. Daniels, V. Gregory, J. J. Skehel, S. J. Gamblin, A. J. Hay, *Vaccine* **2009**, *27*, 6317–6323; c) L. V. Gubareva, L. Kaiser, M. N. Matrosovich, Y. Soo-Hoo, F. G. Hayden, *J. Infect. Dis.* **2001**, *183*, 523–531; d) A. Lackenby, O. Hungnes, S. G. Dudman, A. Meijer, W. J. Paget, A. J. Hay, M. C. Zambon, *Euro Surveill.* **2008**, *13*(5).
- [8] a) J. L. McKimm-Breschkin, *Influenza Other Respir. Viruses* **2013**, *7*(Suppl 1), 25–36. DOI: 10.1111/irv.12047; b) L. V. Gubareva, *Virus Res.* **2004**, *103*, 199–203.
- [9] S. Mohan, S. McAtamney, T. Haselhorst, M. von Itzstein, B. M. Pinto, *J. Med. Chem.* **2010**, *53*, 7377–7391.
- [10] M. Niikura, N. Bance, S. Mohan, B. M. Pinto, *Antiviral Res.* **2011**, *90*, 160–163.
- [11] P. S. Kerry, S. Mohan, R. J. M. Russell, N. Bance, M. Niikura, B. M. Pinto, *Sci. Rep.* **2013**, *3*, 2871.
- [12] Y. Wu, F. Gao, J. Qi, S. Mohan, B. M. Pinto, C. J. Vavricka, G. F. Gao, unpublished results.
- [13] A. Albohy, S. Mohan, R. B. Zheng, B. M. Pinto, C. W. Cairo, *Bioorg. Med. Chem.* **2011**, *19*, 2817–2822.
- [14] K. Hata, K. Koseki, K. Yamaguchi, S. Moriya, Y. Suzuki, S. Yingsakmongkon, G. Hirai, M. Sodeoka, M. von Itzstein, T. Miyagi, *Antimicrob. Agents Chemother.* **2008**, *52*, 3484.
- [15] R. Wagner, M. Matrosovich, H. D. Klenk, *Rev. Med. Virol.* **2002**, *12*, 159–166.
- [16] E. Garman, G. Laver in *Viral Membrane Proteins: Structure, Function, and Drug Design* (Ed.: W. B. Fischer), Kluwer Academic/Plenum Press, New York, **2005**, pp. 247–267.
- [17] a) P. J. Steel, J. A. M. Guard, *Acta Crystallogr. Sect. C* **1994**, *50*, 1721–1723; b) M. M. Chowdhry, D. M. P. Mingos, A. J. P. White, D. J. Williams, *J. Chem. Soc. Perkin Trans. 2* **2000**, 2265–2268.
- [18] R. E. Amaro, R. V. Swift, L. Votapka, W. W. Li, R. C. Walker, R. M. Bush, *Nat. Commun.* **2011**, *2*, 388.
- [19] S. Rudrawar, J. C. Dyason, M. A. Rameix-Welti, F. J. Rose, P. S. Kerry, R. J. Russell, S. van der Werf, R. J. Thomson, N. Naffakh, M. von Itzstein, *Nat. Commun.* **2010**, *1*, 113.
- [20] a) R. E. Amaro, X. L. Cheng, I. Ivanov, D. Xu, J. A. McCammon, *J. Am. Chem. Soc.* **2009**, *131*, 4702–4709; b) R. E. Amaro, D. D. L. Minh, L. S. Cheng, W. M. Lindstrom, A. J. Olson, J. H. Lin, W. W. Li, J. A. McCammon, *J. Am. Chem. Soc.* **2007**, *129*, 7764–7765; c) U. Grienke, M. Schmidtke, S. von Grafenstein, J. Kirchmair, K. R. Liedl, J. M. Rollinger, *Nat. Prod. Rep.* **2012**, *29*, 11–36.