Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 1419–1424
Bioorganic & Medicinal Chemistry Letters

Novel HCV NS5B polymerase inhibitors derived from 4-(1′,1′-dioxo-1′,4′-dihydro-1′λ^6-benzo[1′,2′,4′]thiadiazin-3′-yl)-5-hydroxy-2*H*-pyridazin-3-ones. Part 2: Variation of the 2- and 6-pyridazinone substituents

Yuefen Zhou,* Lian-Sheng Li, Peter S. Dragovich, Douglas E. Murphy, Chinh V. Tran, Frank Ruebsam, Stephen E. Webber, Amit M. Shah, Mei Tsan,† April Averill,‡ Richard E. Showalter, Rupal Patel, Qing Han,§ Qiang Zhao, Thomas Hermann,¶ Charles R. Kissinger, Laurie LeBrun and Maria V. Sergeeva

Anadys Pharmaceuticals, Inc., 3115 Merryfield Row, San Diego, CA 92121, USA

Received 9 November 2007; revised 23 December 2007; accepted 2 January 2008
Available online 8 January 2008

Abstract—5-Hydroxy-3(2*H*)-pyridazinone derivatives were investigated as inhibitors of genotype 1 HCV NS5B polymerase. The structure–activity relationship (SAR) associated with variation of the pyridazinone 2- and 6-substituents is discussed. The synthesis and metabolic stability of this new class of compounds are also described.

Hepatitis C virus (HCV) infects approximately 170 million individuals worldwide and is a leading cause of chronic liver disease. About 70% of infected people will develop chronic histological changes in the liver (chronic hepatitis) with a high risk of advancing to cirrhosis or hepatocellular carcinoma.[1] The current standard of care for HCV is a combination therapy of pegylated interferon and ribavirin. Unfortunately, this treatment provides benefit for less than 50% of genotype 1 infected patients and is associated with adverse events.[2] Therefore there is an urgent need for the development of more effective HCV therapies especially for genotype 1 patients.

As part of our efforts to develop small molecule non-nucleoside inhibitors of HCV NS5B, a RNA-dependent RNA polymerase (RdRp),[3] we discovered a series of 5-hydroxy-3(2*H*)-pyridazinone derivatives (**1**, Fig. 1) using a structure-based design approach. In a separate communication,[4] we discussed the structure–activity relationships (SAR) associated with variation of the R^3 substituents in this series and identified the $-OCH_2CONH_2$ moiety as a suitable fragment for obtaining potent NS5B inhibition properties. Here we

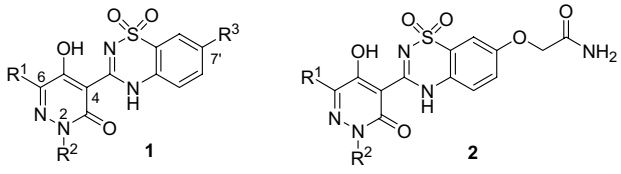


Figure 1. 5-Hydroxy-3(2*H*)-pyridazinone derivatives as HCV NS5B polymerase inhibitors.

Keywords: Pyridazinones; 5-Hydroxy-3(2*H*)-pyridazinone derivatives; Hepatitis C virus (HCV); Structure-based design; RNA-dependent RNA polymerase (RdRp); Small molecule; Non-nucleoside NS5B inhibitors.

* Corresponding author. Tel.: +1 858 530 3693; fax: +1 858 530 3644; e-mail: yzhou@anadyspharma.com
† Present address: Illumina, 9885 Towne Centre Drive, San Diego, CA 92121-1975, USA.
‡ Present address: University of Vermont, Microbiology and Molecular Genetics, 95 Carrigan Drive, Burlington, VT 05401, USA.
§ Present address: Structure Based Design, Inc., 5560 Lusk Blvd. B104, San Diego, CA 92121, USA.
¶ Present address: Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA.

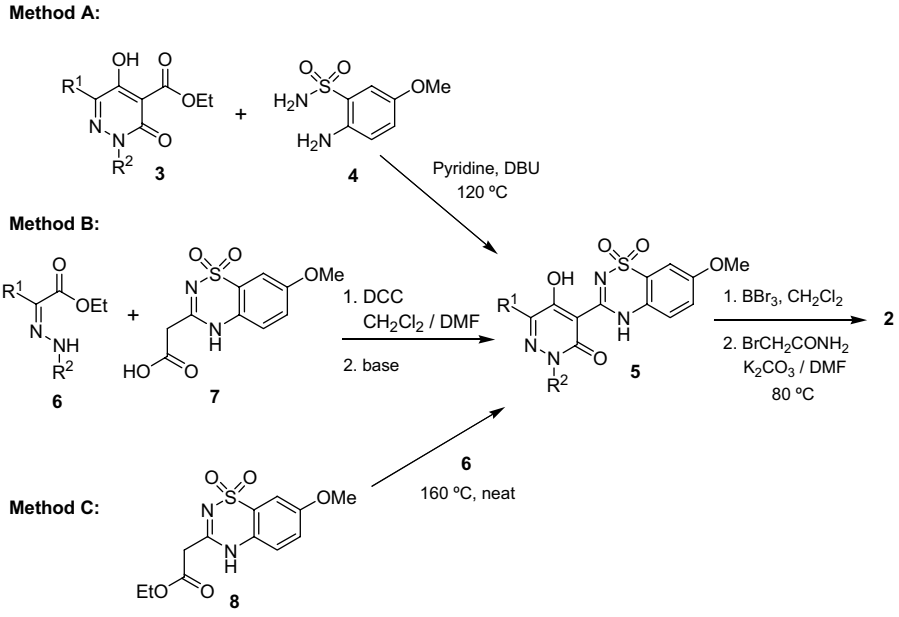
0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.01.005

describe biological properties associated with the systematic alteration of the 2- and 6-pyridazinone substituents in a series of molecules (**2**) which contain this optimal R^3 substituent.

As depicted in Scheme 1, three methods (A, B, and C) were developed to synthesize pyridazinone benzothiadiazinedioxide derivatives (**5**) bearing a methoxy group at the 7′-position. Method A utilized the key 3(2*H*)-pyridazinone ester intermediates (**3**), which were prepared from α-keto-esters via an efficient three-step synthesis.[5] Condensation of **3** with 2-amino-5-methoxy-benzenesulfonamide (**4**)[6] in refluxing pyridine in the presence of DBU afforded the desired products **5**. In method B, hydrazones **6** were prepared by condensation of the corresponding α-keto-esters with alkyl hydrazines (or their oxalic acid salts).[5] Compounds **6** underwent DCC-mediated coupling with (7-methoxy-1,1-dioxo-1,4-dihydro-1λ^6-benzo[1,2,4]-thiadiazin-3-yl)acetic acid (**7**)[6] and the resulting hydrazide intermediates were subsequently cyclized to **5** by treatment with a base such as triethylamine or NaOEt. Alternatively, hydrazones **6** could be converted to **5** directly in one step by heating with (7-methoxy-1,1-dioxo-1,4-dihydro-1λ^6-benzo[1,2,4]thiadiazin-3-yl)acetic acid ethyl ester (**8**)[6] at 160 °C (method C). In general, methods A and B gave higher yields than method C. Compounds **5** were then transformed to the target molecules **2** by treatment with BBr_3 to form the corresponding 7′-phenol intermediates and subsequent O-alkylation with 2-bromoacetamide in the presence of K_2CO_3 at 80 °C.

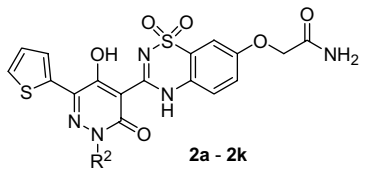
A variety of pyridazinone compounds **2** were synthesized using the methods described above.[7] The inhibitory activities of these compounds in genotype 1a and 1b NS5B enzyme assays as well as in a 1b replicon assay were evaluated. The results of these biological assessments are summarized in Tables 1 and 2.

As previously described in a separate communication,[4] compound **2a** (Table 1) exhibited good inhibitory potency against the genotype 1b enzyme. However, it was 5-fold less potent in the genotype 1a enzymatic assay. Compounds bearing R^2 groups that were comparable in linear length to the isoamyl moiety present in **2a** exhibited slightly improved activity in the biochemical assay (**2b** and **2c**). However, they were significantly less potent in the genotype 1b replicon assay compared to compound **2a**. Adding an F-atom to the terminus of the isoamyl group (**2d**) or introducing a 2,2-dimethylbutyl group (**2e**) led to a significant loss in activity in the biochemical assay when compared with **2a**. Interestingly, compound **2e** exhibited equipotent antiviral properties in the replicon assay as compared to **2a**. In addition, introduction of R^2 alkyl groups that were longer or shorter than the isoamyl moiety led to compounds with NS5B inhibition activities comparable to **2a** against the genotype 1b enzyme (**2f** and **2g**). However, these molecules were less potent than **2a** in both 1b replicon and 1a enzyme assays. Furthermore, compounds bearing a methylene spacer between the pyridazinone core and a cyclic moiety displayed decreasing NS5B inhibition activities with increasing ring size (**2h**–**2j**). When compared to **2j**, compound **2k** having a benzyl R^2 substituent displayed weaker antiviral potency in the 1b replicon assay although the inhibitory activity in the enzyme assay was improved. The different EC_{50}/IC_{50} ratios observed likely resulted from the differences in cell permeability and protein binding.[10] In general, this series of 5-hydroxy-3(2*H*)-pyridazinone derivatives under study was 3- to 14-fold less potent against genotype 1a NS5B relative to the genotype 1b enzyme. We believe that this potency shift was likely due to the amino acid residue difference of Y415F (phenylalanine in genotype 1a vs tyrosine in genotype 1b) located near the R^2 sub-pocket at the palm binding site.[11]

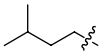
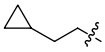
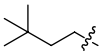
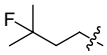
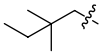
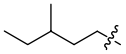
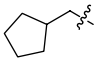
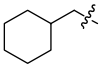
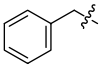


Scheme 1. General methods for the synthesis of pyridazinone derivatives (**2**).[7]

Table 1. R^2 optimization with 2-thiophene as R^1 substituent



2a - 2k

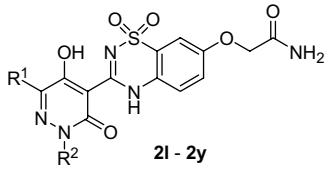
Compound[a]	R^2	IC_{50} (1b)[b] (μM)	IC_{50} (1a)[c] (μM)	EC_{50} (1b)[b] (μM)	CC_{50} (GAPDH)[b] (μM)	HLM $t_{1/2}$[b] (min)
2a		0.07	0.34	0.4	78	23
2b		0.035	0.12	2.1	>33	12
2c		0.046	0.24	1.9	>33	21
2d		0.38	ND[d]	2.3	>33	39
2e		0.18	0.99	0.44	>33	>60
2f[e]		0.07	1.0	1.09	>33	25
2g		0.088	0.84	0.81	>33	27
2h		0.055	0.54	1.6	>33	22
2i		0.28	ND	2.1	>17	26
2j		0.44	ND	1.1	>33	>60
2k		0.15	0.54	5.15	>33	23

[a] See Ref. 8.
[b] See Ref. 4 for assay method and experimental error.
[c] See Ref. 9 for assay method and experimental error.
[d] ND, not determined.
[e] Racemic.

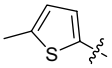
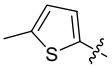
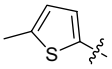
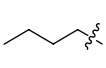
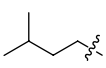
The above efforts led to the identification of the R^2 substituents present in compounds **2a**–**2c** and **2h** as optimal moieties for obtaining potent NS5B inhibitory activity. We then explored combinations of these R^2 fragments with various R^1 substituents in an effort to further improve potency. The impact of R^1 substitution on the inhibitory activity against genotype 1a and 1b enzymes as well as the 1b replicon is summarized in Table 2.

As shown in Table 2, the 3-thiophene analog (**2l**) was 9-fold more potent in the 1b replicon assay than the corresponding 2-thiophene-containing compound (**2b**). However, introducing a methyl group at the 5-position of the 2-thiophene ring resulted in a significant loss of activities in both enzyme (30-fold) and replicon assays (4-fold) (compare **2m** with **2b**). This trend was observed with several other inhibitors (**2n** and **2o**) bearing different R^2 substituents (compare with **2c** and **2h**, respectively). The co-crystal structure of **2m** bound to NS5B[12] revealed that this methyl group was unable to access the small sub-pocket in the R^1 region as originally intended. Instead, the methyl fragment rested on the NS5B surface and generated unfavorable interactions with nearby polar protein residues. These interactions were the likely cause of the observed decrease in the inhibition activity of **2m**. A similar reduction in potency was observed by introducing a

Table 2. R^1 Variation with optimal R^2 substituents



2l - 2y

Compound[a]	R^1	R^2	IC_{50} (1b)[b] (μM)	IC_{50} (1a)[c] (μM)	EC_{50} (1b)[b] (μM)	CC_{50} (GAPDH)[b] (μM)	HLM $t_{1/2}$[b] (min)
2b			0.035	0.12	2.1	>33	12
2l			0.088	0.14	0.23	>33	16
2m			1.1	ND[d]	7.7	>33	42
2n			1.4	ND	23	>33	45
2o			1.2	ND	17	>33	58
2p			0.43	ND	25	>33	ND
2q			0.039	0.15	1.1	>33	60
2r			0.077	0.14	1.8	>33	>60
2s			2.4	ND	5	>33	>60
2t			1.1	ND	8.7	>33	>60
2u			0.79	ND	6.6	>10	8
2v			2.2	ND	11	>100	ND
2w			0.4	ND	4.5	>33	ND
2x			0.43	ND	3	>33	10
2y			0.74	5.1	9.7	>33	ND

[a] See Ref. 8.
[b] See Ref. 4 for assay method and error.
[c] See Ref. 9 for assay method and error.
[d] ND, not determined.

methyl group at the 3-position of the thiophene ring (compare **2p** with **2a**). The co-crystal structure of **2p** complexed with the NS5B protein[12] showed that the addition of the methyl group caused a change in orientation of the thiophene ring relative to the unsubstituted ring. This change placed the methyl group in

close proximity with polar protein atoms and this may explain the reduction in potency observed for **2p**.

In addition, replacement of the 2-thiophene ring with a 5-thiazole moiety led to very potent compounds (**2q** and **2r**) possessing activities comparable to **2a** and **2b** in both enzyme (1b and 1a) and replicon assays. This result was consistent with the analysis of the co-crystal structure of **2q** bound to the NS5B protein (Fig. 2). The pyridazinone and benzothiadiazine rings of **2q** adopted a nearly co-planar geometry which was highly favorable for binding to the NS5B palm site. The 5-thiazole ring, rotated approximately 16° out of plane relative to the pyridazinone ring, was able to effectively access the sub-pocket in the R^1 region. This rotation is similar to the rotation of the 2-thiophene moiety in **2a**-NS5B X-ray co-crystal structure.[12] These rotations were smaller than the rotation of a phenyl R^1 group (R^2 = isoamyl, R^3 = OMe) as we discussed previously.[4] Due to its larger size, a phenyl ring needs to rotate more to avoid a close interaction with Gly410 and Asn411. This could explain some of the differences between the IC_{50} values of **2q** or **2a** and the phenyl substituted analogs. Additionally, a number of direct or water-mediated H-bonds were formed between the sulfonamide O-atoms and residues Ser556, Ser288, and Gly449 as well as between the acetamide R^3 moiety and residues Asn291 and Asp318. These favorable structure features, which were almost identical to those observed in the X-ray co-crystal structure of **2a**-NS5B,[4] may explain the high potency of compound **2q**. A schematic diagram shown in Figure 3 summarizes the observed interactions in the co-crystal structure of compound **2q** bound to the NS5B protein.

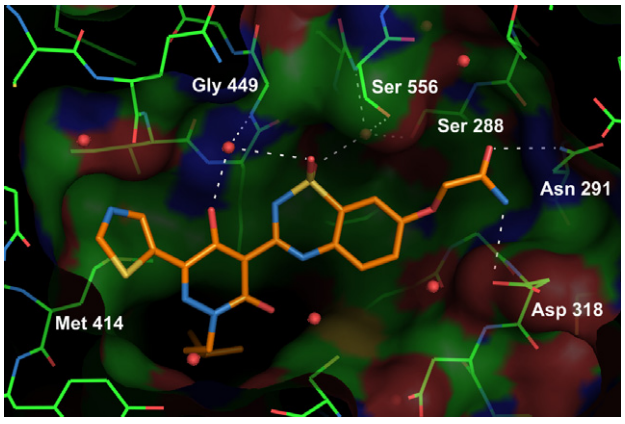


Figure 2. Co-crystal X-ray structure of compound **2q** bound to the NS5B protein (2.3 Å resolution).[13]

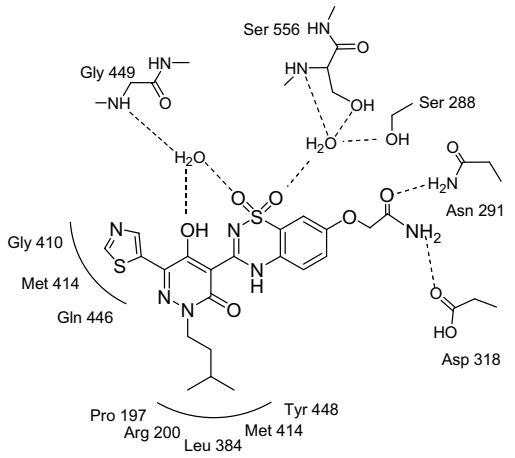


Figure 3. Schematic diagram of compound **2q** bound in the NS5B palm site. Hydrogen bonds are represented as dashed lines, and the residues which make up the enzyme binding subsites are depicted.[14]

Surprisingly, the replacement of the 2-thiophene ring with a 2-thiazole moiety (**2s** and **2t**) caused a significant loss in inhibitory potency in both enzyme and replicon assays when compared with **2a** and **2h**, respectively. The reasons for this loss in biological activity are not yet well understood. Furthermore, introduction of alkyl R^1 substituents into the inhibitor design (**2u**–**2y**) did not afford any improvements in NS5B inhibitory potency as compared with compound **2a**.

In order to better predict the in vivo metabolic stability of this new class of pyridazinone derivatives, the effect of R^1 and R^2 substituents on stability toward human liver microsomes (HLM) was evaluated. As shown in Table 1, the measured HLM half-life for compound **2a** was relatively short (23 min). We reasoned that, in addition to the unsubstituted thiophene ring present in **2a**, the secondary C-atom of the terminal isoamyl group might also be a potential site for cytochrome P450 (CYP) catalyzed metabolism. Accordingly, an F-atom was introduced at the secondary C-atom of the isoamyl fragment and this resulted in a more stable compound (**2d**) ($t_{1/2}$ = 39 min). However, the addition of a methyl group at this position did not improve the stability of the resulting molecule (**2c**) ($t_{1/2}$ = 21 min). Interestingly, inclusion of a more branched R^2 group of the same linear length as the isoamyl moiety or a large cyclohexyl R^2 fragment resulted in very stable compounds (**2e** and **2j**, respectively; $t_{1/2} > 60$ min). In contrast, compound **2k**, bearing a benzyl R^2 moiety, had a short half-life (23 min) possibly due to CYP-mediated modification of the benzene ring (compare **2k** with **2j**). The measured HLM half-lives for the rest of compounds (**2b**, **2f**–**2i**) in Table 1 were relatively short (<30 min).

Alkyl R^1 substituents shown in Table 2 led to the least stable compounds (**2u**, **2x**) with very short half-lives ($\leqslant$10 min). Similar to compound **2b** bearing a 2-thiophene moiety, the 3-thiophene group also led to a compound (**2l**) with a short half-life (16 min). As the 5-position of the 2-thiophene ring is a potential site for oxidation catalyzed by CYPs,[15] we introduced a methyl group at this position to block such metabolism. This change resulted in a significant improvement in the stability of the resulting compounds **2m**, **2n**, and **2o** when compared with **2b**, **2c**, and **2h**, respectively. Both 2-thiazole and 5-thiazole moieties led to very stable compounds (**2q**–**2t**) with $t_{1/2} \geqslant 60$ min. The above results indicated that both R^1 and R^2 substituents significantly affected the metabolic stability of the resulting molecules.

In summary, we synthesized a new class of 4-(1′,1′-dioxo-1′,4′-dihydro-1′λ^6-benzo[1′,2′,4′]thiadiazin-3′-yl)-5-hydroxy-2*H*-pyridazin-3-one derivatives (**2**) as potent inhibitors of genotype 1 HCV RNA-dependent RNA polymerase (NS5B). The optimization of the 2- and 6-substituents of the pyridazinone derivatives resulted in significantly improved potencies in both biochemical (1b and 1a) and replicon (1b) assays. In addition, our SAR studies revealed that both R^1 and R^2 substituents significantly affected the HLM stability of the molecules. Further optimization of this new class of pyridazinone compounds will be discussed in future communications.

Acknowledgments

The authors thank Drs. Devron Averett and Steve Worland for their support and helpful discussions during the course of this work.

References and notes

1. Wong, T.; Lee, S. S. *CMAJ* **2006**, *174*, 649.
2. Hoofnagle, J. H.; Seeff, L. B. *N. Engl. J. Med.* **2007**, *355*, 2444.
3. Koch, U.; Narjes, F. *Curr. Top. Med. Chem.* **2007**, *7*, 1302.
4. Zhou, Y.; Webber, S. E.; Murphy, D. E.; Li, L.-S.; Dragovich, P. S.; Tran, C. V.; Sun, Z.; Ruebsam, F.; Shah, A. M.; Tsan, M.; Showalter, R. E.; Patel, R.; Li, B.; Zhao, Q.; Han, Q.; Hermann, T.; Kissinger, C. R.; LeBrun, L.; Sergeeva, M. V.; Kirkovsky, L. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1413.
5. (a) Li, L.-S.; Zhou, Y.; Zhao, J.; Dragovich, P. S.; Stankovic, N.; Bertolini, T. M.; Murphy, D. E.; Sun, Z.; Tran, C. V.; Ayida, B. K.; Ruebsam, F.; Webber, S. E. *Synthesis* **2007**, 3301; (b) Murphy, D. E.; Dragovich, P. S.; Ayida, B. K.; Bertolini, T. M.; Li, L.-S.; Ruebsam, F.; Stankovic, N. S.; Sun, Z.; Zhou, Y. *Tetrahedron Lett.* **2008**, *49*, 811.
6. 2-Amino-5-methoxy-benzenesulfonamide **4**, benzothiadiazine acid **7** and its ethyl ester **8** were prepared using modified literature methods as described in (a) Fitch, M. D.; Evans, K. A.; Chai, D.; Duffy, K. J. *Org. Lett.* **2005**, *7*, 5521; (b) Kovalenko, S. N.; Chernykh, V. P.; Shkarlat, A. E.; Ukrainets, I. V.; Gridasov, V. I.; Rudnev, S. A. *Chem. Heterocycl. Comp. (Engl. Transl.)* **1999**, *34*, 791.
7. Detailed procedures for the synthesis of compounds **1** and **2** as well as their analytical data are described in Zhou, Y.; Li, L.-S.; Webber, S. E. WO06066079A2, 2006.
8. All structures of compounds **2** under study were consistent with ^{1}H NMR and LC–MS analysis ($\geqslant$95% HPLC purity). They are arbitrarily drawn in one of the possible tautomer forms.
9. NS5B polymerase inhibition assay (1a IC_{50}, μM): assays were performed in a 96-well streptavidin-coated FlashPlate using 50 nM enzyme (NS5B H77 1a), 0.5 μCi of [α-33P]GTP, 0.6 mM GTP, and 250 nM 5′biotinylated oligo (rG13)/poly rC in 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 g/L bovine serum albumin, and 100 U/mL RNAse inhibitor. The reaction was stopped by aspiration after 75 min at 28 °C and the plate was washed several times with 150 mM NaCl aqueous solution. After washing and drying the plate, incorporated radioactivity was counted using a Microbeta scintillation counter. IC_{50} values were calculated relative to the uninhibited control and inhibition data were fitted to a 4-parameter IC_{50} equation. The estimated average standard deviation for 1a IC_{50} data is 20% from the mean value.
10. The amounts of protein in replicon assay were much higher than that in the NS5B enzyme assay. In the 1b NS5B enzyme assay, there was 0.1 g/L bovine serum albumin, while in the replicon assay there was about 4 g/L of fetal bovine serum (FBS).
11. Pauwels, F.; Mostmans, W.; Quirynen, L. M. M.; Helm, L. V. D.; Boutton, C. W.; Rueff, A.-S.; Cleiren, E.; Raboisson, P.; Surleraux, D.; Nyanguile, O.; Simmen, K. A. *J. Virol.* **2007**, *81*, 6909.
12. The co-crystal structures of **2a**, **2m**, and **2p** bound to the NS5B protein are not shown.
13. Crystals of HCV NS5B polymerase (genotype 1b, strain BK, Δ21) were grown by the hanging drop method at room temperature using a well buffer of 20% PEG 4K, 50 mM ammonium sulfate, 100 mM sodium acetate, pH 4.7, with 5 mM DTT. The crystals formed in space group $P2_12_12_1$ with approximate cell dimensions, $a = 85$ Å, $b = 106$ Å, $c = 127$ Å and two protein molecules in the asymmetric unit. Protein-inhibitor complexes were prepared by soaking these NS5B crystals for 24 h in solutions containing 10% DMSO, 20% glycerol, 20% PEG 4K, 0.1 M Hepes, 10 mM $MgCl_2$ at pH 7.6, and **2q** at a concentration of 3 mM. Diffraction data were collected to a resolution of 2.3 Å on beamline 14IDB at the Advanced Photon Source at Argonne National Laboratory. This crystal structure has been deposited in the Protein Databank (www.rcsb.org) with entry code 3BSA. Full details of structure determination are given in the PDB entry.
14. The O-atom at the 5-position of the pyridazin-3-one in Figure 3 was arbitrarily drawn in the protonated form. The precise extent of the protonation was not determined.
15. O'Donnell, J. P.; Dalvie, D. K.; Kalgutkar, A. S.; Obach, R. S. *Drug Metab. Dispos.* **2003**, *31*, 1369.