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PAPER

Synthesis of Janus type nucleoside analogues and their preliminary bioactivity†

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Novel Janus type nucleoside analogues **1a** and **1b** were synthesized in seven steps from 2-amino-4,6-dihydroxypyrimidine and 4,6-dihydroxypyrimidine. The base moiety of **1a** has one face with a Watson–Crick donor-donor–acceptor (DDA) H-bond array of guanine and the other face with an acceptor-acceptor-donor (AAD) array of cytosine, which might lead to its base pairing with either cytosine or guanine due to the rotating of the glycosyl bond. This property may enable Janus type nucleoside analogues to act as an antiviral compound in a similar way to ribavirin. Both **1a** and **1b** were screened by a *vitro* HBV DNA replication inhibition test and indeed **1a** showed a great potential with IC_{50} = 10 μM and SI = 78.9 for antiviral drug development.

Introduction

Nucleoside analogues have been used as antiviral agents, antineoplastic drugs and immunosuppressive molecules for many years. This diverse family is considered to compete with physiological nucleosides through interaction with a large number of intracellular targets. After being transported into a cell by different membrane carriers like natural nucleosides, these compounds are usually transformed into phosphorylated derivatives[1] and then behave as antimetabolites *via* different mechanisms: inhibiting various enzymes which participate in the synthesis of nucleic acid; incorporating and altering natural DNA/RNA macromolecules and terminating the synthesis of the genome.[2] Among these nucleoside analogues, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximide, also named as virazole) is of particular interest due to its unique antiviral mechanism.[3,4] Nowadays, ribavirin is used as a preferred drug combined with interferon-α to treat HCV infection,[5,6] single therapy for Lassa fever infections[7] and acute RSV infection.[8] However, the exact mechanisms of ribavirin's activity has remained controversial since its discovery 30 years ago. Several distinct indirect or direct mechanisms have been proposed to explain its antiviral properties.[9] Crotty and colleagues have recently demonstrated that ribavirin's antiviral activity can be fully attributed to its lethal mutagenesis.[10] Viruses, particularly RNA viruses, exist as quasispecies, a heterogeneous population with a master sequence.[11] On one hand, the great variability in genomes of these quasispecies will help viruses to adapt quickly to the environments changed by immune response and antiviral drugs.[12,13] On the other hand, the higher frequency of mutations in the viral genome also implies a higher risk of genetic melt-down.[14] The maintenance of genetic information of any organism requires a minimal fidelity level during the process of genome replication. Excess mutations beyond the upper limit (error threshold) of the genome would cause the irreversible loss of genetic information and consequently the loss of specific infectivity of viruses.[15] Ribavirin was first synthesized as an ambiguous purine base analogue.[16] Its pseudo-base moiety pairs with cytidine and uridine by rotating the carboxamide bond (Fig. 1). Crotty and his co-workers have proved that ribavirin can be templated by either uridine or cytidine with equal efficiency and it is actually an RNA virus mutagen.[10] Therefore, the dual base pairing property of ribavirin will cause transition or transversion mutations (A to G and C to U) and finally lead to its antiviral activity *via* lethal mutagenesis by pushing the viral genome beyond the error threshold.[17]

However, the efficiency of ribavirin's incorporating into a viral genome is relatively low.[9] Searching new mutagenic nucleoside analogues which can be specifically and efficiently incorporated into a viral genome is a plausible strategy to develop new drugs for viral disease. Inspired by this consideration, we designed a Janus guanine-cytosine nucleoside (**1a**, J-GC) for this purpose. Janus molecules (from the two-faced Roman god Janus) were first proposed by Cristol to describe a new symmetrical carbocyclic system.[18] The base moiety of J-GC has one face with a Watson–Crick donor-donor-acceptor (DDA) H-bond array of guanine and the other face with an acceptor-acceptor-donor (AAD) array of cytosine. Rotating the glycosyl bond of J-GC will lead to an *anti* or *syn* conformation (Fig. 2). In principle, J-GC could pair with cytidine or guanosine *via* rotating the glycosyl bond in a similar way to ribavirin's pairing with U/C *via* rotating the carboxamide bond (Fig. 1). J-GC is a mimic of natural nucleosides which

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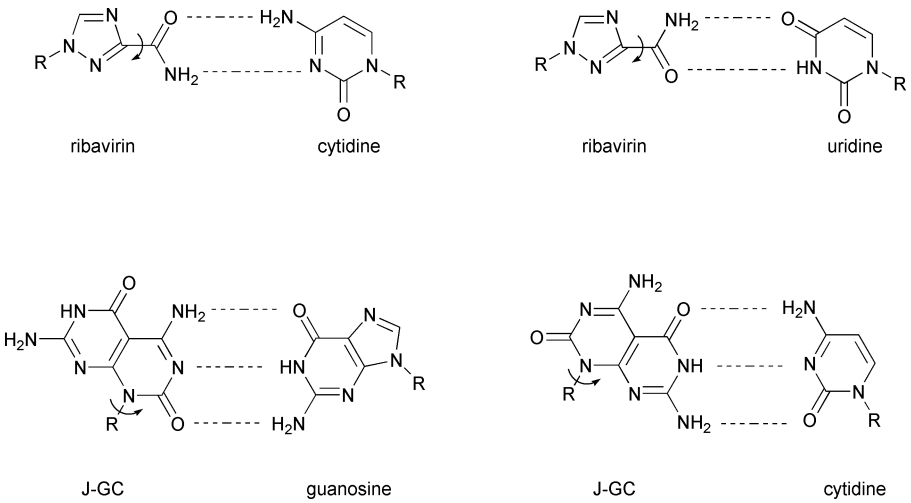


Fig. 1 Ribavirin is an ambiguous purine mimic which pairs with cytidine and uridine. The designed J-GC nucleoside analogue is supposed to pair with either guanosine or cytidine through the specific pattern of hydrogen-bonding patterns (R = ribose).

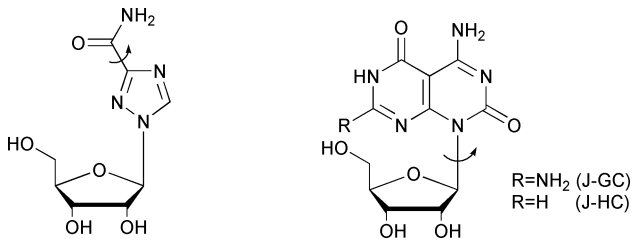


Fig. 2 Ribavirin, J-GC and J-HC nucleoside analogues. The rotation of glycosyl bond will lead to *anti* or *syn* conformation.

preserves the pyrimidine system of the canonical base unit and we hope it could be recognized by viral enzymes more efficiently.

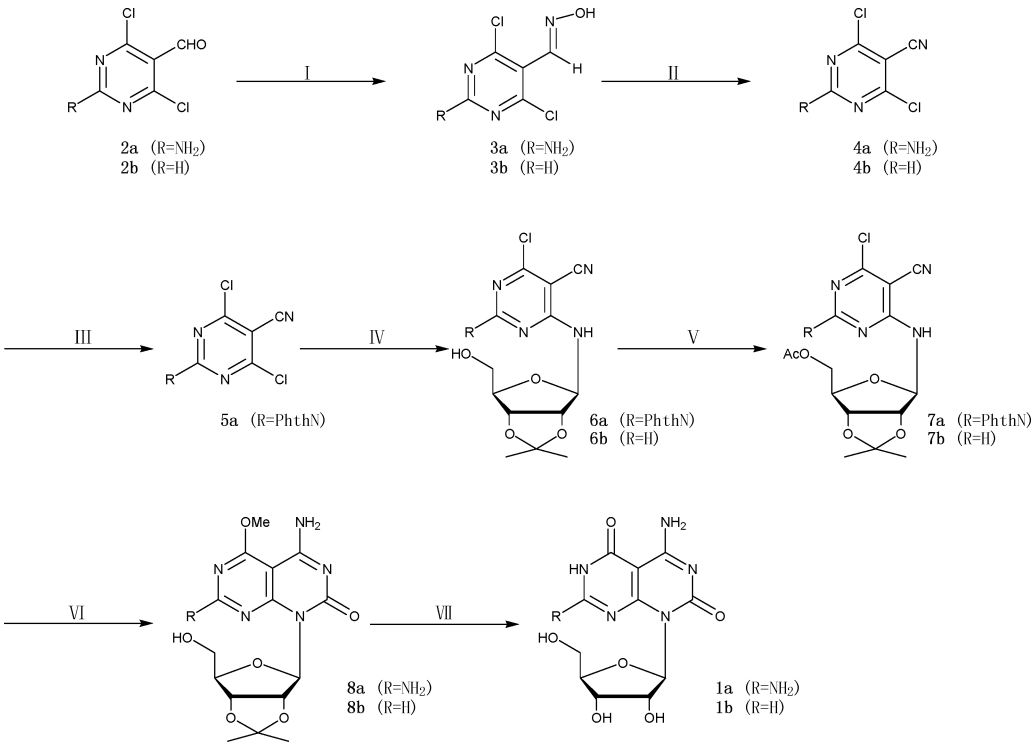
While Janus nucleobase analogues[19–24] have been researched, most studies about these bisfunctionalized molecules were focused on their applications in nanotechnology: to construct architectures such as trimers,[22] rosettes[7,25] and regular noncovalent polymer arrays[19] with different hydrogen bonding codes. Janus guanine-adenine base analogue was first synthesized by Branda and colleagues as a non-self-complementary molecule to avoid self-aggregation.[20] This guanine-adenine system was designed as a base wedge to form cytosine-uracil mismatches, and its interaction with both U and C was observed by ^{1}H NMR titration. Then the same guanine-adenine wedge was proved to also recognize C-T mismatch.[23] After that, Janus guanine-cytosine,[24] Janus adenine-thymine[21] base analogues were reported. These two self-complementary molecules were mostly studied for their self-association and nano-structure formations.

In order to evaluate the base pairing properties of these two-hydrogen-pattern-faced systems in RNA/DNA and to see if they can be used as effective antiviral drugs, their nucleoside derivatives would be needed. Surprisingly, the synthesis of Janus type nucleosides with the aforementioned pyrimidopyrimidine as base moieties has not been reported. Herein, we describe the syntheses and potential application in antiviral treatment of a Janus type nucleoside analogue J-GC (**1a**). Because of its hydrogen self-complementarity, J-GC easily self-associates in solutions leading to bad solubility in different kinds of solvents. Reducing one intermolecular hydrogen bonding interaction may ameliorate this characteristic. Therefore, another Janus type nucleoside analogue (**1b**, J-HC) with a hypoxanthine-cytosine system has also been prepared (Fig. 2).[26–28]

Results and discussion

Chemical synthesis

There are two major approaches to the synthesis of nucleosides. The first approach employs the corresponding base derivatives reacting with an appropriate sugar component (convergent glycosylation reaction). The second one involves the construction of a purine or pyrimidine system from a simple *N*-glycosylated precursor (linear method). Initially, we intended to adopt direct glycosylation reaction through the Vorbrueggen method using corresponding silylated Janus type base moieties. However, it was too difficult to get free bases from some intermediates we synthesized. For instance, the similar pyrimido[4,5-*d*]pyrimidine compounds contain alkyl chains attached to the nitrogen atom,[22] which are hard to remove. In addition, even if we had obtained the free bases, the four ring nitrogen atoms on the pyrimido[4,5-*d*]pyrimidine ring would raise a big problem of regioselectivity during the glycosylation reaction, because the multiple *N*-glycosylation sites will lead to several isomers. For all these reasons, we finally chose to synthesize compounds **1a** and **1b** through the route of Scheme 1. The starting materials **2a** and **2b** are obtained from commercially available 2-amino-4,6-dihydroxypyrimidine and 4,6-dihydroxypyrimidine *via* Vilsmeier–Haack reaction.[29,30] Then, both **2a** and **2b** were treated with hydroxylamine hydrochloride (1.5 equiv.) in acetic acid. After being stirred overnight at room temperature, the oxime derivative **3a** was easily obtained as a precipitate. Then, acetous impurities were washed out by EtOH and the pure product **3a** was dried in vacuum for further use in a yield of 83%. However, when **2b** was reacted under the same conditions, there was no precipitate formed in solution. Alternatively, the solution was evaporated and the residue was treated with ethyl ether. After the insolubles were filtered off, the organic solution was washed with saturated aqueous sodium bicarbonate.



Scheme 1 Sythesis of J-GC and J-HC nucleosides: I. Hydroxylamine hydrochloride, acetic acid, r.t., 16 h. II. SeO_2, pyridine (**3a**), toluene, reflux, 15 min for **3a** and 180 min for **3b**. III. *i*Pr_2EtN, phthaloyl dichloride, dry THF, r.t., 20 min. IV. 2,3-*O*-isopropylidene-D-ribofuranosylamine toluene-*p*-sulfonate, $NaHCO_3$, *i*Pr_2EtN, dry THF, 70 °C, 40–60 min. V. *i*Pr_2EtN, Ac_2O, DMAP, DCM, r.t., 7 min. VI. *i*Pr_2EtN, CCI, dry DCM, r.t., 10–30 min; NaOMe, reflux, 70 min. VII. NaI, TMSi-Cl, acetone, r.t., 20 h, CF_3COOH (10% H_2O), 0 °C, 20 min.

The washing process should be carried out thoroughly otherwise the traces of remaining acid would catalyze the decomposition of the product very fast. It was not practicable for further purification *via* column chromatography due to the lack of a suitable solvent system with reasonable solubility of **3b**. Fortunately, the unpurified compound **3b** was acceptable for next step.

Strangely, the attempt to dehydrate the aldoxime to give nitrile failed under the standard acidic or alkaline conditions. Alternatively, the mild method using selenium dioxide in refluxing toluene was found to be suitable for the transformation leading to compounds **4a** and **4b**. When **3a** was reacted with selenium dioxide (1 equiv.) in boiling toluene, with pyridine (0.5%) added as a base catalyst, product **4a** was formed as pink solid in 73% yield within 15 min. When **3b** was treated with selenium dioxide (1.2 equiv.) in refluxing toluene, no appropriate catalyst was found. So the reaction lasted longer (~3 h) compared to the formation of **4a** and the reaction remains as a homogeneous solution. The solvent was evaporated and product **4b** (yield 82%) was obtained as a white powder after column chromatography.

When **4a** was treated with 2,3-*O*-isopropylidene-D-ribofuranosylamine toluene-*p*-sulfonate[31] directly, no reaction was detected. We postulated that this is caused by the electron pair of the 2-amino group which deactivated the electrophilicity at the 6-position of **4a**. To overcome this problem, the amino group should be protected properly. Finally, compound **4a** was treated with both phthaloyl chloride in dry THF and *i*Pr_2EtN as base catalyst and compound **5a** was obtained with a yield of 60% after flash column chromatography. As expected, protected pyrimidine **5a** can react with 2,3-*O*-isopropylideneribofuranosylamine toluene-*p*-sulfonate successfully, leading the formation of compound **6a** with a yield of 86%. Because compound **4b** has no amino group at the 2-position, it can react with the ribofuranosylamine derivative directly and compound **6b** was obtained in 79% yield *via* similar procedures for the preparation of **6a**.

The anomeric configurations of compounds **6a** and **6b** were firstly determined by the difference in ^{1}H chemical shifts between the two methyl signals of the isopropylidene group, $\Delta\delta$. According to Imbach's rules:[32–34] when $0 < \Delta\delta < 0.10$ ppm, the nucleoside is in the α-configuration, and when $0.17 < \Delta\delta < 0.23$ ppm, the nucleoside is in the β-configuration. The calculated results of both compounds (0.18 ppm. for **6a** and 0.17 ppm. for **6b**) suggest that they are more likely in β-configurations. Then, the single crystals of these two compounds were obtained from methanol. By single crystal X-ray analysis, we confirmed both of the nucleosides adopt β-configurations. The oblique view of crystal structure of **6b** is given here as an example (Fig. 3).

The 5′-OH groups of compounds **6a** and **6b** were protected with acetic anhydride (1.2 equiv.) and the resulting protected nucleosides **7a** and **7b** were then treated with *N*-(chlorocarbonyl) isocyanate (CCI) to give their urea derivatives. Compared to compound **7a**, **7b** reacts with CCI much slower. Due to the neighboring effect of the oxygen of ribose, the urea group of **7a** and **7b** was unstable and decomposed in methanolic ammonia. We have tried several conditions to carry out the ring cyclization reaction of **7a** and **7b** and found NaOMe in methanol was the only way to get the ring closure product (0.5 M for **6a**; 0.15 M for **6b**). During the cyclization reaction both the phthaloyl- and acetyl-group were removed and the 6-chloride on the base moiety

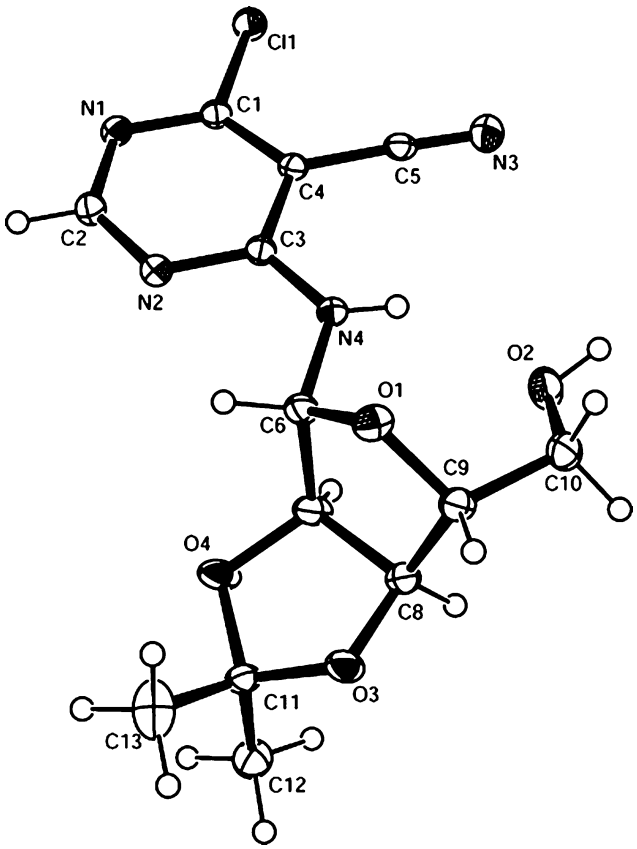


Fig. 3 The oblique view of the crystal structure of **6b**.

was replaced by a methoxy group; compounds **8a** and **8b** were obtained as products.

The simultaneous removal of both the latent carbonyl function group and isopropylidene group proceeded as follows: **8a**/**8b** was dissolved in acetonitrile, NaI (3 equiv.) and TMSi-Cl (3 equiv.) were added and stirred for 20 h at room temperature. The yellowish product (yield 95%) was filtered and washed with acetonitrile and acetone. The product was then added to a solution of CF_3COOH with 10% H_2O at 0 °C, and reacted for 20 min. After the acid was evaporated *in vacuo*, the residue was then co-evaporated with ethanol several times to remove residual acid. The obtained black residue was dissolved in hot water, after a few hours a white precipitate was obtained as our final products **1a** and **1b** (both yield 91%).

Also, to estimate the preferred conformation and the energy differences between *syn* and *anti* conformers, we performed density functional theory (DFT) calculations on **1a** and **1b**. The geometries were optimized at the B3LYP/6-31g (d, p) level. The computational result indicated that the *anti* conformations are more stable than the *syn* conformations with energy differences of 20.49 kJ mol^{-1} for **1a** and 16.90 kJ mol^{-1} for **1b**. These predicted energy differences are rather close to the energy barriers between the different *syn* and *anti* conformational states of natural purine and pyrimidine nucleosides (about 25 kJ mol^{-1}).[35] These results indicate that compounds **1a** and **1b** may rotate their glycosyl bonds freely to adopt both *syn* and *anti* conformations just as natural nucleosides, which will very likely lead to the formation of mismatches in viral genomes as supposed.

Table 1 Inhibition of HBV DNA replication by compounds **1a**, **1b** and 3TC

Compound	IC_{50}/μM	TC_{50}/μM	SI
1a	10	793	79.3
1b	>1000	3200	NA
3TC	0.17	>437	>2500

SI = TC_{50}/IC_{50}; NA: non-applicable

Antiviral activity

The antiviral activity of the compounds was preliminarily screened by a HBV DNA replication inhibition assay.[36] The cell line HepG2.2.15 was previously cultured in the standard condition. Then cells were seeded in a 96-well tissue culture plate for 24 h, treated with test compounds and positive reference compound in gradient concentration (0 days). After 4 days treatment, the culture medium was removed and the test compounds were added to the cultures again in fresh medium. At day 8, the cultures were collected and lysed for the intracellular HBV DNA analysis. The amount of HBV DNA in each condition was measured by real time PCR. Also, the half maximum cytotoxic concentration values (TC_{50}) for each compound were measured by calculating the cell growth rates by a standard procedure.[37] In the test, Lamivudine (3TC) was used as the positive reference compound. The cytotoxicity was monitored with compound concentrations up to 3 mM. The half maximum inhibition concentration values (IC_{50}) and the selected index (SI) were also estimated for all compounds (Table 1).

The results show that compound **1a** really inhibits the viral DNA replication with an IC_{50} of 3.3 μg ml^{-1}. On one hand, compared to 3TC, the inhibition effect of compound **1a** is moderate. The high efficiency of DNA replication inhibition for 3TC is caused by the lack of a 3′-OH. When 3TC is incorporated into viral DNA, the absence of 3′-OH will terminate the 3′–5′ extension of the proviral DNA chain. In our case, compound **1a** possesses an entire ribose structure, therefore its effect of inhibiting viral DNA replication is not due to the chain termination. On the other hand, compound **1a** shows higher efficiency to inhibit viral DNA replication than ribavirin, which showed no inhibition effect for HBV DNA replication at a notable high concentration of 50 μg ml^{-1} under the same assay conditions.[38] The assay shows compound **1b** is devoid of viral DNA replication inhibition effect, which also implies that the canonical cytosine and guanine face in Janus type nucleoside analogues is recognized by enzymes more efficiently. According to these results, the Janus GC compound has strong potential in the application of antiviral drug research. Its base-pairing properties in oligonucleotides and its mechanism for DNA replication inhibition are under further investigation.

Conclusion

We have developed a viable route to two novel Janus type nucleoside analogues of 4,6-diamino-1-(β-D-ribofuranoyl)pyimido[4,5-*d*]pyrimidin-2,5(1*H*,6*H*)-dione (J-GC) and 4-amino-1-(β-D-ribofuranoyl)pyimido[4,5-*d*]pyrimidin-2,5(1*H*,6*H*)-dione (J-HC). These two compounds are designed as genome mutagens and their preliminary antiviral activity test shows that only the J-GC

nucleoside analogue can be used as an HBV DNA inhibitor. The disparity in bioactivity between J-GC and J-HC also provided us with the possibility that in these cases nucleoside analogues with canonical hydrogen face may be recognized more efficiently.

Experimental

General

All chemicals were purchased from Aldrich, Sigma, or Fluka. Solvents were of laboratory grade. Thin-layer chromatography (TLC) was performed on TLC aluminium sheet covered with silica gel 60 F254 (0.2 mm, Merck, Germany). Flash column chromatography (FC): silica gel 60 (Haiyang chemical company, P. R. China) at 0.4 bar. UV-spectra were recorded on a DU-800 spectrophotometer (Beckman, US), $\lambda_{max.}$ in nm, ε in $dm^3\ mol^{-1}\ cm^{-1}$. NMR spectra: *AV II* spectrometers (Bruker, Germany) at 400 MHz for ^1H and 600 MHz for ^{13}C. The δ values in ppm are relative to Me_4Si as internal standard, Elemental analyses were performed by the analysis and testing center, Sichuan University. High resolution mass spectra are measured with mass analyzer (Q-TOF-premier, Waters company, US).

2-Amino-4,6-dichloropyrimidine-5-carbaldehyde oxime (3a). Compound **2a** (10 g, 52 mmol) was firstly dissolved in heated AcOH (800 mL). After cooling to r.t., hydroxylamine hydrochloride (5.4 g, 78.1 mmol) in EtOH (800 mL) was added dropwise over 2 h, then the mixture was stirred at r.t. overnight. Subsequently, the precipitate was filtered and washed with EtOH until neutral. After vacuum drying, compound **3a** (8.8 g, 82.2%) was obtained as a pale yellow solid: UV (MeOH): 267 (28913). δ_H (400 MHz; d_6-DMSO): 7.79 (2H, s, NH_2), 8.03 (1H, s, CH), 11.62 (1H, s, OH). δ_C (600 MHz; d_6-DMSO): 111.30, 142.28, 160.34, 161.39. $C_5H_4Cl_2N_4O$ requires C, 29.01; H, 1.95; N, 27.06%; found C, 29.14; H, 2.13; N, 26.88%.

2-Amino-5-cyano-4,6-dichloroprymidine (4a). Compound **3a** (10 g, 48.4 mmol), selenium dioxide (5.4 g, 48.4 mmol) and pyridine (5 mL) were added in toluene (1200 mL), then the reaction mixture was brought to 110 °C and stirred for 15 min. Whereafter, the supernatant was evaporated to furnish **4a** (6.7 g, 73.2%): UV (MeOH): 260 (24041). δ_H (400 MHz; d_6-DMSO): 8.63 (2H, s, NH_2). δ_C (600 MHz; d_6-DMSO): 94.96, 114.26, 162.12, 162.89. $C_5H_2Cl_2N_4$ requires C, 31.77; H, 1.07; N, 29.64%; found C, 31.45; H, 1.17; N, 29.28%.

2-*N*-Phthaloyl-5-cyano-4,6-dichloroprymidine (5a). Compound **4a** (6.4 g, 33.8 mmol) was dissolved in dry THF (200 mL), iPr$_2$EtN (29.2 mL, 170.0 mmol) was added. A few minutes later, phthaloyl dichloride (9.7 mL, 67.6 mmol) was added dropwise in 20 min. The mixture was stirred at r.t. for 20 min. After evaporation, the residue was dissolved in DCM (250 mL) and washed with H_2O (250 mL × 2). The organic layer was finally dried with anhydrous sodium sulfate and evaporated. Purification was achieved by FC (eluent: DCM) to give a white solid **5a** (6.5 g. 60.3%): UV (MeOH): 210 (42574), 259 (21929), 239 (14642). δ_H (400 MHz; d_6-DMSO): 7.96–8.08 (4H, m, C_6H_4). δ_C (600 MHz; d_6-DMSO): 78.28, 114.39, 125.79, 136.42, 136.64, 163.65, 164.42, 169.00. HRMS (ESI+) m/z: Calc. for $C_{13}H_4Cl_2N_4O_2$: 318.9790 $[M+H]^+$. Found 318.9781 $[M+H]^+$.

2-*N*-Phthaloyl-4-(5′-*O*-acetyl-2′,3′-*O*-isopropylidene-β-D-ribofuransoyl)amino-5-cyano-6-chloroprymidine (7a). Compound **5a** (1.1 g, 3.45 mmol) was dissolved in dry THF (30 mL), 2,3-*O*-isopropylidene-D-ribofuranosylamine toluene-*p*-sulfonate (2.5 g, 6.9 mmol), $NaHCO_3$ (0.6 g, 6.9 mmol) and iPr$_2$EtN (1.2 mL, 6.9 mmol) were added in turn. The mixture was stirred at r.t. for 10 min and then heated to 70 °C, stirred for another 40 min. Subsequently, the solvent was evaporated, the residue was dissolved in DCM (50 mL) and washed with H_2O (50 mL × 2), 0.1 M HCl (50 mL) and finally dried with anhydrous sodium sulfate. The crude product **6a** (2.0 g, 4.2 mmol) was obtained by evaporation and dissolved in DCM (75 mL). Then, iPr$_2$EtN (0.9 mL, 5 mmol), Ac_2O (0.5 mL, 5 mmol) and DMAP (24.6 mg, 0.2 mmol) were added. The mixture was stirred at r.t. for 7 min, whereafter, H_2O (1 mL) was injected to quench the reaction. The mixture was diluted to 150 mL with DCM and washed with 0.1 M HCl (75 mL) and H_2O (75 mL). After drying with anhydrous sodium sulfate, the organic layer was evaporated to afford a crude foam. FC (DCM/Acet. = 97 : 3 as eluent) was applied to obtain the pure product **7a** (1.65 g, 76%): UV (MeOH): 216 (24226), 236 (21872), 300 (4223). δ_H (400 MHz; d_6-DMSO): 1.36 (3H, s, CH_3), 1.54 (3H, s, CH_3), 2.19 (3H, s, CH_3CO), 4.05–4.09 (1H, m, 4′H), 4.45–4.52 (2H, m, 5′H), 4.68–4.71 (1H, q, J = 2.3 Hz, 3′H), 4.87–4.90 (1H, q, J = 2.4 Hz, 2′H), 6.03–6.06 (1H, t, J = 5.0 Hz, 1′H), 6.89–6.91 (1H, d, J = 8.0 Hz, NH), 7.81–7.99 (4H, m, C_6H_4). δ_C (600 MHz; d_6-DMSO): 20.99, 25.41, 27.15, 64.42, 81.70, 83.22, 87.85, 90.32, 113.13, 124.65, 131.37, 136.02, 152.93, 163.19, 163.51, 164.85, 170.54. HRMS (ESI+) m/z: Calc. for $C_{23}H_{20}ClN_5O_7$: 514.1130 $[M+H]^+$. Found 514.1127 $[M+H]^+$.

4,7-Diamino-1-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-5-methyloxy-pyrimido[4, 5-d]pyrimidine-2(1H)-one (8a). Compound **7a** (1.3 g, 2.59 mmol) was dissolved in DCM (46 mL) at 0 °C, iPr$_2$EtN (0.9 mL, 5.18 mmol) was added, 2 min later, CCI (1.4 g, 13 mmol) was added into the solution. Then the ice-box was removed and the reaction was stirred at r.t. for 7 min. Subsequently, 0.1 M HCl (6 mL) was injected carefully to quench the reaction. The resulting mixture was diluted to 150 mL with DCM and washed with H_2O (150 mL). Then the solvent was evaporated and a crude yellowish foam was obtained. The yellowish foam was dissolved in 0.5 M NaOMe (150 mL) and stirred under reflux for 75 min. Whereafter, the precipitate was filtered off when the mixture was still hot and the solvent was removed by evaporation; a white residue was obtained and immediately purified by FC (DCM/Methanol = 95 : 5–9 : 1 as eluent) to afford product **8a** (0.3 g, 30.2%): UV (MeOH): 226 (11181), 287 (9281). δ_H (400 MHz; d_6-DMSO): 1.27 (3H, s, CH_3), 1.47 (3H, s, CH_3), 3.48–3.58 (2H, d, 5′CH_2), 3.92 (1H, s, 4′H), 3.98 (3H, s, OCH_3), 4.67 (1H, s, 3′H), 4.84 (1H, s, 2′H), 5.14 (1H, s, OH), 6.85 (1H, s, 1′H), 7.31–7.58 (3H, m, NH_2+H-NH), 7.89 (1H, s, H-NH). δ_C (600 MHz; d_6-DMSO): 27.73, 54.86, 62.85, 82.53, 84.73, 87.39, 89.00, 112.68, 123.39, 133.08, 134.78, 154.73, 161.31, 162.97, 167.86, 169.68. HRMS (ESI+) m/z: Calc. for $C_{15}H_{20}N_6O_6$: 403.1342 $[M+Na]^+$. Found 403.1336 $[M+Na]^+$.

4,7-Diamino-1-(β-D-ribofuranoyl)pyrimido[4,5-d]pyrimidine-2,5(1*H*,6*H*)-dione (1a). **8a** (3.8 g, 10 mmol) was dissolved in acetonitrile (30 mL); NaI (4.5 g, 30 mmol) and TMSi-Cl (3.3 g, 30 mmol) were then added. The reaction was stirred at r.t. over 20 h, and the precipitate from the resulting mixture was collected,

which was washed with acetonitrile (30 mL × 3) and acetone (30 mL × 3). The treated precipitate (3.6 g, 95%) was then dissolved with CF_3COOH (10% H_2O, 30 mL) and stirred for 20 min at 0 °C. After CF_3COOH was removed by evaporation, the residue was co-evaporated with ethanol (30 mL × 3) to remove the residual acid. The final residue was dissolved in hot water and cooled to r.t.; 8 h later, a white precipitate was found as final product **1a** (3.0 g, 91%): UV (MeOH): 228 (2359), 283 (1216). δ_H (400 MHz; d_6-DMSO): 3.41–3.45 (1H, m, 4′H), 3.59–3.65 (2H, t, 5′CH_2), 4.19 (1H, s, 3′H), 4.54–4.56 (1H, d, J = 8.0 Hz, 3′OH), 4.68 (1H, s, 2′OH), 4.80–4.81 (1H, bd, J = 2.0 Hz, 2′H), 5.02 (1H, s, 5′OH), 6.47 (1H, s, 1′H), 7.57–8.25 (4H, m, NH_2 × 2), 11.72 (1H, br, NH). HRMS (ESI–) m/z: Calc. for $C_{11}H_{14}N_6O_6$: 325.0896 $[M - H]^-$. Found 325.0891 $[M - H]^-$.

4,6-Dichloropyrimidine-5-carbaldehyde oxime (3b). Compound **2b** (1.8 g, 10 mmol) was dissolved in acetic acid (20 mL). Hydroxylamine hydrochloride (1.4 g, 20 mmol) in ethanol (40 mL) was added dropwise, the mixture was stirred at r.t. for 15 h. After the solvent was evaporated, the residue was dissolved in ether (20 mL), insolubles were filtered off and the organic layer was washed with saturated aqueous $NaHCO_3$ (20 mL × 3), then dried with anhydrous sodium sulfate. Finally, a white solid **3b** (1.6 g, 84%) was furnished by evaporation. UV (MeOH): 256 (7662). δ_H (400 MHz; d_6-DMSO): 8.21 (1H, s, NCH), 8.91 (1H, s, CH), 12.28 (1H, s, OH). δ_C (600 MHz; d_6-DMSO): 113.63, 142.03, 149.50, 154.25. $C_5H_3Cl_2N_3O$ requires C, 31.28; H, 1.57; N, 21.89%; found C, 31.24; H, 1.46; N, 22.07%.

4,6-Dichloro-5-cyanopyrimidine (4b). Compound **3b** (1.9 g, 10 mmol), and selenium dioxide (1.3 g, 12 mmol) in toluene (150 mL) were heated under reflux for 3 h. The resulting mixture was then evaporated and applied to FC (DCM as eluent) affording a white powder **4b** (1.4 g, 82%): UV (MeOH): 235 (3418). δ_H (400 MHz; d_6-DMSO): 8.53 (1H, s, CH). δ_C (600 MHz; d_6-DMSO): 101.12, 113.35, 155.25, 163.85. $C_5HCl_2N_3$ requires C, 34.52; H, 0.58; N, 24.15%; found C, 34.61; H, 0.64; N, 24.25%.

4-(5′-*O*-Acetyl-2′, 3′-*O*-isopropylidene-β-D-ribofuransoyl)amino-5-cyano-6-chloropyrimidine (7b). Compound **4b** (1.7 g, 10 mmol) in dry THF (50 mL) was added to 2,3-*O*-isopropylidene-ribofuranosylamine toluene-*p*-sulfonate (7.2 g, 20 mmol). When the solution became homogeneous, $NaHCO_3$ (1.7 g, 20 mmol) and iPr$_2$EtN (3.4 mL, 20 mmol) were added. The mixture was heated to 60 °C and stirred for 1 h. Subsequently, the solvent was removed under reduced pressure, the residue was dissolved with DCM (50 mL), washed with 0.1 M HCl (25 mL × 2) and H_2O (25 mL × 2), and finally dried with anhydrous sodium sulfate. After evaporation, the residue was dissolved in DCM (50 mL); iPr$_2$EtN (2.6 mL, 15 mmol), acetic anhydride (1.4 mL, 15 mmol) and DMAP (12.2 mg, 0.1 mmol) were added. The mixture was stirred at r.t. for 10 min, and washed with 0.1 M HCl (25 mL) and H_2O (25 mL), and finally dried over anhydrous sodium sulfate. After evaporation, the residue was applied to FC (DCM as eluent) to furnish compound **7b** (2.1 g, 58%): UV (MeOH): 216 (24168), 249 (13910), 298 (3333). δ_H (400 MHz; d_6-DMSO): 1.29 (3H, s, CH_3), 1.46 (3H, s, CH_3), 2.02 (3H, s, CH_3CO), 4.13–4.18 (3H, m, 5′CH_2+4′ H), 4.76–4.78 (1H, q, J = 1.4 Hz, 3′H), 5.00–5.02 (1H, q, J = 1.2 Hz, 2′H), 5.87–5.89 (1H, d, J = 8.0 Hz, 1′H), 8.62 (1H, s, CH), 8.99–9.01 (1H, bd, J = 8.0 Hz, NH). δ_C (600 MHz; d_6-DMSO): 21.06, 25.56, 27.21, 64.48, 81.80, 83.13, 83.84, 87.91, 113.20, 159.97, 162.23, 170.57. HRMS (ESI+) m/z: Calc. for $C_{15}H_{17}ClN_4O_5$: 369.0956 $[M+H]^+$. Found: 369.0973 $[M+H]^+$.

4-Amino-1-(2′,3′-*O*-isopropylidene-β-D-ribofuranoyl)-5-methoxylpyrimido[4,5-*d*]pyrimidine-2(1*H*)-one (8b). Compound **7b** (3.7 g, 10 mmol) was dissolved in dry DCM (100 mL), and iPr$_2$EtN (6.5 g, 50 mmol) was added. CCI (5.2 g, 50 mmol) was then added to the mixture carefully at 0 °C. The reaction was brought to r.t. and stirred for 30 min. Subsequently, 0.1 M HCl (5 mL) was injected to quench the reaction. After insolubles were filtered off, the mixture was washed with H_2O (50 mL × 2), dried over anhydrous sodium sulfate and evaporated. The residue was refluxed with 0.15 M NaOMe (50 mL) for 1 h. and a white powder **8b** (1.1 g, 30%) was obtained by purifying the resulting mixture with FC (DCM/methanol = 97 : 3 as eluent): UV (MeOH): 211 (33559), 273 (6213). δ_H (400 MHz; d_6-DMSO): 1.30 (3H, s, CH_3), 1.51 (3H, s, CH_3), 3.53–3.65 (2H, q, 5′CH_2), 3.99–4.00 (1H, d, J = 4.0 Hz, 4′H), 4.12 (3H, s, OCH_3), 4.76 (1H, s, 3′H), 4.89–4.90 (1H, d, J = 4.8 Hz, 2′H), 5.22–5.23 (1H, d, J = 6.4 Hz, 5′OH), 6.87 (1H, s, 1′H), 7.83 (1H, s, H-NH), 8.55 (1H, s, H-NH), 8.73 (1H, s, CH). δ_C (600 MHz; d_6-DMSO): 25.74, 27.68, 55.84, 62.68, 82.67, 84.53, 88.21, 112.96, 154.09, 159.42, 160.12, 161.10, 166.85. HRMS (ESI–) m/z: Calc. for $C_{15}H_{19}N_5O_6$: 364.1256 $[M - H]^-$. Found 364.1282 $[M - H]^-$.

4-Amino-1-(β-D-ribofuranoyl)pyrimido[4,5-*d*]pyrimidine-2,5(1*H*,6*H*)-dione (1b). Compound **8b** (3.7 g, 10 mmol) was dissolved in acetonitrile (30 mL), and NaI (4.5 g, 30 mmol) and TMSi-Cl (3.3 g, 30 mmol) were then added. The mixture was stirred for 20 h, whereafter, the resulting precipitate was collected, and washed with acetonitrile (30 mL × 3) and acetone (30 mL × 3). Finally, a yellowish powder was obtained (3.3 g, 95%). The powder was then dissolved in CF_3COOH (10% H_2O, 30 mL) at 0 °C and stirred at r.t. for 20 min. Subsequently, CF_3COOH was removed by evaporation, and the residue was co-evaporated with ethanol (30 mL × 3). The final mixture was dissolved in hot water and cooled to r.t.. After 8 h, the white precipitate was obtained as final product **1b** (2.7 g, 91%): UV (MeOH): 225 (17184), 277 (2670). δ_H (400 MHz; d_6-DMSO): 3.45 (1H, s, 4′H), 3.61–3.70 (2H, t, J = 7.6 Hz, 5′ H), 4.20 (1H, s, 3′H), 4.56 (1H, s, OH), 4.64 (1H, s, OH), 4.84 (1H, s, 2′H), 5.04 (1H, s, 5′OH), 6.51 (1H, s, 1′H), 8.22–8.42 (3H, m, NH_2), 8.40 (1H, s, CH), 13.15 (1H, s, NH). HRMS (ESI–) m/z: Calc. for $C_{11}H_{13}N_5O_6$: 310.0787 $[M - H]^-$. Found 310.0782 $[M - H]^-$.

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