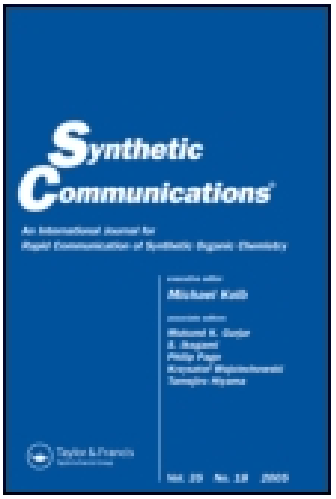

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EXPEDIENT TOTAL SYNTHESIS OF TRICIRIBINE AND ITS PRODRUGS

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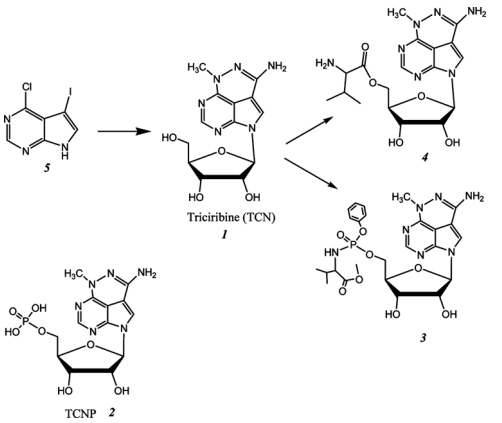
GRAPHICAL ABSTRACT



Abstract *Triciribine (TCN, **1**) and its monophosphate (TCNP, **2**) are tricyclic nucleotide derivatives that have potential antineoplastic activity. Triciribine inhibits the phosphorylation, activation, and signaling of Akt-1, -2, and -3, which may result in the inhibition of Akt-expressing tumor cell proliferation. Both TCN and TCNP have very low bioavailability, and the development of both drugs as intravenous (IV) treatments was halted because of the toxicity induced by the high doses needed for their use as general cytotoxic agents. This publication describes an expedient and straightforward total synthesis of amino acid prodrugs (**3, 4**) of TCN and TCNP. In our study, both the prodrugs significant improved the plasma exposure of the parent drugs and the prodrugs.*

Keywords Amino acid prodrugs; antitumor; bioavailability; phosphoramidate prodrug; triciribine; triciribine monophosphate

INTRODUCTION

Triciribine (TCN, NSC-154020, compound 1, Fig. 1) is a tricyclic nucleotide derivative first synthesized by Schram and Townsend in 1971.[1] The compound showed promise as an anticancer agent but had very poor solubility. In response

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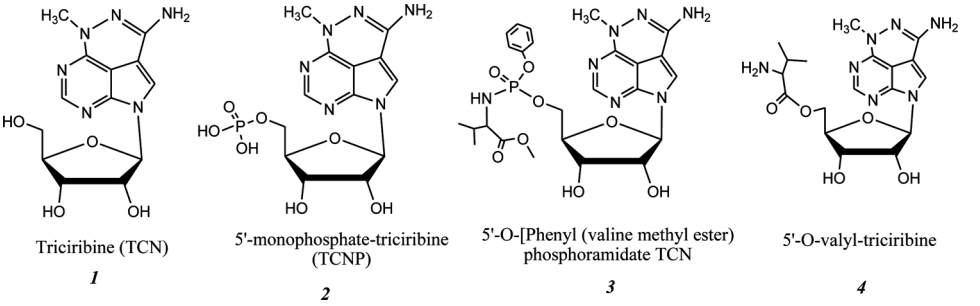


Figure 1. TCN, TCNP, and their prodrugs.

to the poor solubility, they synthesized the 5′-monophosphate of triciribine (TCNP, compound 2, Fig. 1), which was found to be highly soluble. TCN was the most potent AKT inhibitor among 1992 compound library from the National Cancer Institute (NCI) Diversity Set.[2] AKT, also named protein kinase B, a serine/threonine-specific protein kinase, is a member of the PI3K/Akt/mTOR pathway involved in many key cellular processes that are overexpressed in a variety of cancers. Ectopic expression of AKT, especially constitutively active AKT, induces cell survival and malignant transformation, whereas inhibition of AKT activity stimulates apoptosis in a range of mammalian cells.[3–6] Furthermore, activation of AKT has been shown to associate with tumor invasiveness and chemoresistance.[7] These observations establish AKT as an attractive target for cancer therapy. In a screen using the NCI diversity set, TCNP was found to suppress growth and induce apoptosis of cancer cells overexpressing AKT.[2] Furthermore, it was shown to directly inhibit phosphorylation and consequent activation of all three isoforms of AKT.[2] The water-soluble derivative, TCNP, does not inhibit upstream activators of AKT such as PI3K and PDK1, nor does it inhibit other Ser/Thr kinases, making it highly selective and specific for AKT. Consequently, TCNP attenuates the phosphorylation of AKT's downstream target enzymes (substrates). In a xenograft model in nude mice, TCNP treatment significantly inhibited the growth of three cancer cell lines overexpressing AKT, while it had little effect on cancer cell lines expressing low levels of AKT.[2] These results were the first to show that TCNP selectively inhibits the growth of tumors that overexpress AKT.[2]

Neither TCN nor TCNP are orally bioavailable. However, because of the high solubility of TCNP, this compound was suitable for intravenous (IV) formulation and was therefore tested in the late 1980s and early 90s as a general cytotoxic agent for cancer treatment in phase I and phase II clinical trials by the NCI under IND 18,905.[8–11] In a phase I trial of 33 evaluable patients, using a 5-day continuous infusion schedule every 42 days, modest activity was observed by improvement in metastasis in a patient with papillary thyroid cancer, disease stabilization in a patient with mesothelioma, and mixed responses in three patients with sarcoma, colorectal cancer, and tonsillar carcinoma, respectively. The investigators noted cumulative toxicity at the 30 mg/m^2/day cohort with hyperglycemia, myelosuppression, thrombocytopenia, elevated pancreatic enzymes, and hepatotoxicity. Therefore, they recommended a dose of 20 mg/m^2/day for phase II.[10] In a phase II study of 24

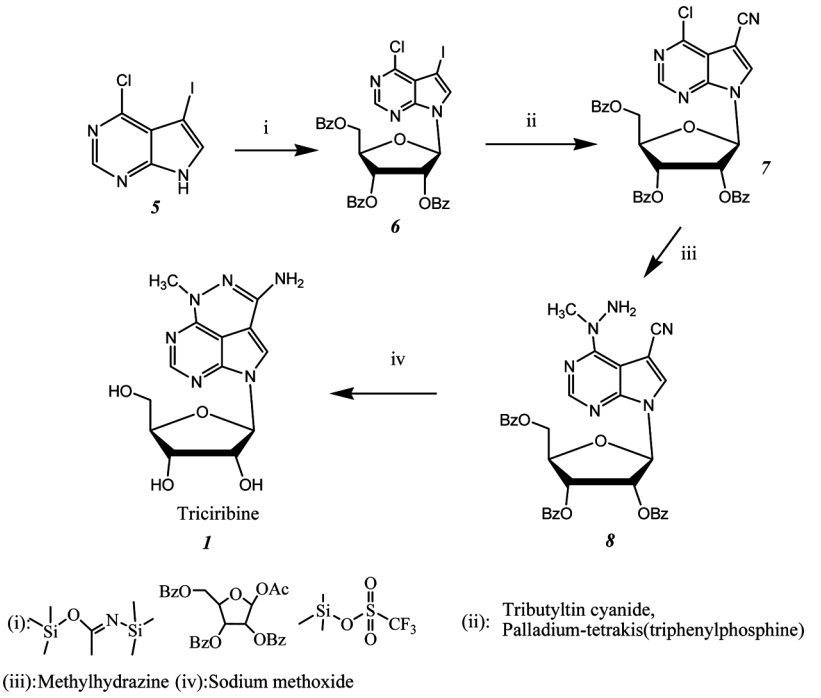
patients with advanced cervical squamous cell carcinoma, the same 5-day continuous infusion schedule was given at a starting dose of 35 mg/m^2/day. Two objective responses were seen: one was a complete response lasting longer than 19 months, and the second was a partial response lasting longer than 5 months. Only one subject developed grade 4 toxicity.[12] Other clinical breast cancer and colorectal cancer studies did not show significant activity following low levels of the drug and were toxic at higher doses.[13–16] The development of TCNP was halted at that time because of the toxicity induced by the high doses needed for its use as a general cytotoxic agent together with its mild efficacy.

The prodrug approach for improvement of oral bioavailability has been highly effective as demonstrated by the success of antiviral prodrugs such as adefovir dipivoxil,[17] famciclovir,[18] tenofovir disoproxil,[19] valacyclovir,[20] and valganciclovir.[21] These prodrugs exhibited 4–10-fold great oral absorption than their parent drug. Interestingly, the amino acid prodrugs valacyclovir and valganciclovir are actively transported by oligopeptide transporters, which are highly expressed in the gastrointestinal tract.[22–25] A prodrug strategy for TCN/TCNP could bolster the intestinal permeability of the drug to maintain the effectiveness of the parent drugs at lower dosage, which can overcome the toxicity induced by the high dose needed during IV treatment. Based on these rationales, prodrugs of TCN and TCNP were synthesized and tested for bioavailability enhancement. In this article, we report the synthesis and bioavailability studies of the prodrugs of TCN.

CHEMISTRY

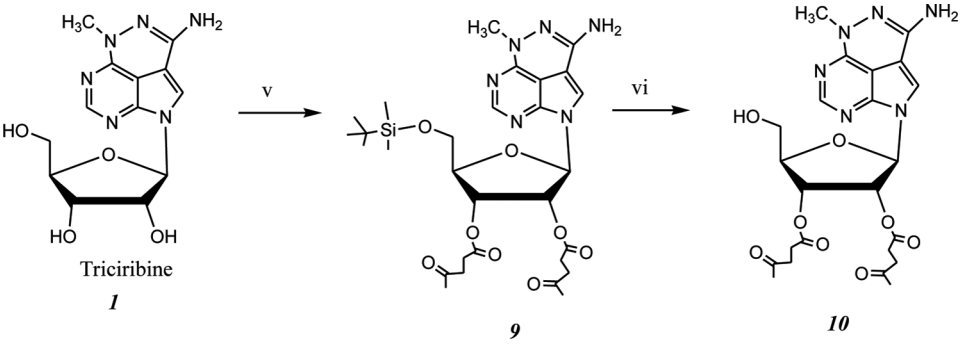
The purpose of developing orally available prodrugs of TCN is to accomplish lower effective dosage, orally available drug delivery, greater flexibility in dose scheduling, and significantly improved patient compliance. The drug should have good permeation across intestinal membranes, relatively good stability in the gastrointestinal tract, and efficiency in the chemical and enzymatic conversion to parent drugs in plasma or intracell. In this case, the parent drugs are TCN and/or TCNP. Two prodrugs were designed based on the mentioned criteria (compounds **3** and **4**, Fig. 1). Compound **1** is a 5′-O-L-valine methyl ester of phosphoramidate of TCN and compound 2 is 5′-O-L-valyl ester of TCN. L-Valine was chosen in our exploration because it is a good substrate of oligopeptide transporters such as hPEPT1 that are highly expressed in the gastrointestinal tract.[26]

During the synthesis of the prodrugs of TCN, a big challenge was lack of efficient synthetic route to TCN itself. Triciribine was originally synthesized from the naturally occurring antibiotic toyocamycin.[1] The toyocamycin is not commercially available anymore. Another method started from tetracyanoethylene via 4-amino-6-bromo-5-cyanopyrrolo[2,3-d]pyrimidine as key intermediate through 10 steps to obtain triciribine at around 10% total yield.[27–29] The reproducibility of the latter method is very difficult in our laboratory. The major barrier was the difficulty in preparation of 4-amino-6-bromo-5-cyanopyrrolo[2,3-d]pyrimidine from tetracyanoethylene. A significant amount of 4-amino-6-bromo-5-cyanopyrrolo[2,3-d] pyrimidine was lost during a tedious workup procedure. To prepare gram or even kilogram amounts of triciribine, we developed a different procedure to avoid using 4-amino-6-bromo-5-cyanopyrrolo[2,3-d]pyrimidine as an intermediate (Scheme 1).



Scheme 1. New method for the synthesis of triciribine.

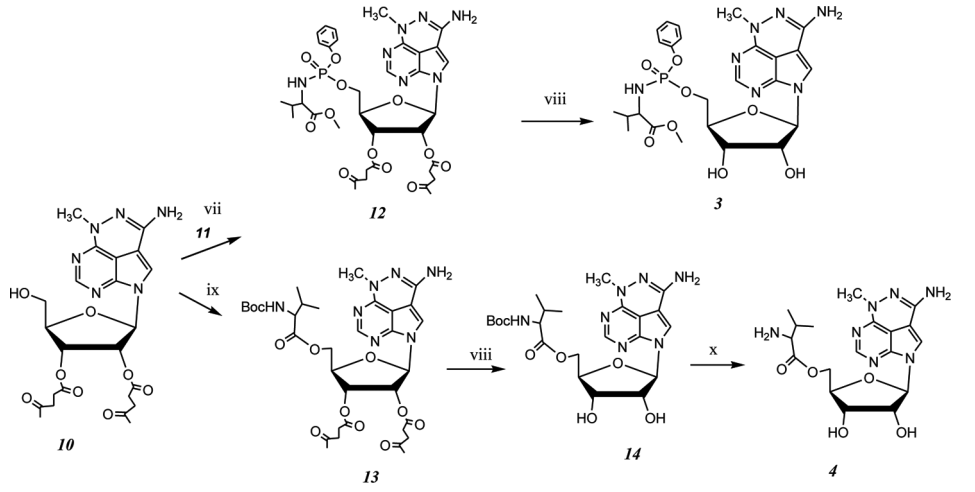
In this novel synthetic route, we chose 6-chloro-7-iodo-7-deazapurine (also called 4-chloro-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidine **5**) as starting material. It is commercially available from several vendors. Glycosylation product (**6**) of 6-chloro-7-iodo-7-deazapurine was obtained with 80% yield by first silylating compound **5** with N,O-bis(trimethylsilyl) acetamide (BSA), then treating it with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, followed by trimethylsilyl trifluoromethanesulfonate (TMSOTf) under argon at 80 °C. The key step of the new method was to selectively introduce the cyano group at the 7-position of the deazapurine ring of compound **6**. Transition-metal-catalyzed cyanation of aryl halides is a common and useful transformation in organic synthesis. The reaction goes smoothly with iodide and bromide at mild condition while more harsh conditions are needed to make reaction on choride occur.[30,31] When palladium complexes were applied as catalyst, the reaction of aryl-chloride cyanation was very slow.[32–34] The difference in reactivity between iodide and chloride is sufficient to permit preferential cyanation of the iodide in the presence of both halogens.[35] In our study, the cyanation was carried out smoothly with 83% yield by treatment with tributyltin cyanide and palladium tetraphenylphosphine in anhydrous tetrahydrofuran (THF) to form 4-chloro-5-cyno-7-[(2,3,5-tri-O-benzoyl)-β-D-ribofuranosyl]-7H-pyrrolo[2,3-d]pyrimidine (**7**). Treatment of compound (**7**) with methylhydrazine in ethanol at room temperature for 1 h afforded 4-(1-methylhydazino)-5-cyano-7-[(2,3,5-tri-O-benzoyl)-b-D-ribofuranosyl]-7H-pyrrolo [2,3-d]pyrimidine (**8**) in 73% yield. Deprotection of compound **8** and subsequent ring



Scheme 2. Selective protection of 2′ and 3′ dihydroxyl groups of triciribine. (v) DMPA, TBDMSCI, levulinic acid, DCC; and (vi) TBAF, glacial acetic acid.

closure were accomplished by following the existing method[8] to obtain triciribine in 78% yield (38% yield from compound **5**).

During the synthesis of the TCN prodrugs, the 2′,3′-dihydroxyl groups of TCN were selectively protected by dilevulinate ester groups with two steps as shown in Scheme 2[36] so that potential side reactions can be avoided and the solubility of the intermediates in organic solvent can be improved. The levulinate group can survive the synthetic conditions for the prodrugs and can be easily removed by treatment with 1 ml of 2 M hydrazine hydrate in pyridine–acetic acid buffer for 10 min.[36] In addition, the levulinate is less prone to migration between adjacent hydroxyl groups on the sugar residue than other ester protection group such as benzoate and acetate.[36] Importantly, the exocyclic amine of the tricyclic base moiety was not levulinated as long as the reaction time was limited to 2 to 3 h.[36] The



Scheme 3. Synthesis of the prodrug of TCN and TCNP. (vii) Methylchlorophenylphosphoryl P-N-L-valine, N-methylimidazolo; (vii) hydrazine hydrate, pyridine–acetic acid buffer; (ix) Box-valine, DMAP, DCC; and (x) TFA, DCM.

protected TCN was coupled with N-Boc-L-valine to form the fully protected 5′-O-valyl ester of TCN. Fully protected 5′-O-[(valine methyl ester) phosphoramidate] of TCN was constructed by reaction of protected TCN with phenyl (valine methyl ester) phosphochloridate **11** that was prepared based on the existing method.[37] The final prodrugs were obtained after removal of the levulinate group by hydrazine and/or removal of Boc group by trifluoroacetic acid (TFA). The entire synthetic route was illustrated in Scheme 3.

RESULTS AND DISCUSSION

Prodrug Stability

The stability of the prodrugs was assessed in 100 mM phosphate buffer, pH 6.5, and rat liver homogenates at 37 °C. For the buffer stability, timed aliquots were taken at 0, 1, 2, and 3 h and mixed with cold 10% trichloroacetic acid (TCA) in preparation for analysis. For the liver homogenates, samples were taken at 0, 5, 10, 20, 30, and 90 min, mixed with cold 10% TCA, and then centrifuged for 10 min to remove precipitated liver homogenate protein. All samples were analyzed by liquid chromatography–tandem mass spectrometry (LC/MS/MS (using an assay developed at TSRL. Briefly, 10μL aliquots of sample were separated on a C18, 2.2 mm × 10 cm column (Higgins Analytical), at a flow rate of 0.2 ml/min over a run time of 3 min. The mobile phase for the separation consisted of an isocratic gradient using the following mobile phases: (A) 70% formic acid in water to (B) 30% acetonitrile. The MS/MS detector was run under MRM positive acquisition mode, with a cone voltage of 30 V and collision energy of 5 V with a collision gas pressure of $1 \times 10-3$ mbar.

According to our study (Table 1), all the compounds were stable in phosphate buffer at pH 6.5. The 5′-O-valyl ester prodrug of TCN broke down rapidly to the parent TCN in liver homogenates, with a $t_{1/2}$ equal to 12 min. In contrast the TCN, TCNP and phosphoramidate prodrugs were stable over a 90 min time course in liver homogenates.

Prodrug Absorption

The oral absorptions of TCN and TCNP were negligible (U.S. patent US2008/058862). This was tested by direct injection of the TCN or TCNP into the duodenum of rats and measuring the resulting systemic plasma levels of the drugs. In this system, male albino Sprague–Dawley rats, 9–10 weeks old and weighing 250–350 g, were fasted for 18 h with free access to water. The rats were anesthetized with 2–5% isofluorane. To withdraw timed plasma samples, a catheter was placed in

Table 1. Stability of TCN, TCNP, and their prodrugs in phosphate buffer and liver homogenates

Compound	Phosphate buffer (pH 6.5)	Liver homogenates
TCN	Stable	Stable
TCNP	Stable	Stable
5′ O-Valyl triciribine	Stable	12 min
5′ Valyl phosphoramidate prodrug	Stable	Stable

the jugular vein. The abdomen was opened by a 4 to 5-cm midline incision and the duodenal segment was located. Three mg (0.5 ml of a 6 mg/ml drug solution of TCNP or suspension of TCN) were injected directly into the duodenal segment, the intestine was placed back into the abdominal cavity, and the incision was covered with gauze. Plasma samples (~0.5 ml) were withdrawn over a 4-h period, and the systemic plasma concentrations of the injected prodrug and/or parent compounds were determined simultaneously using an LC/MS/MS method. In this system, the plasma levels of both TCN and TCNP were minimal; in the case of TCN, measurable levels of drug were below the detection limit of the drug (<1 ng/ml). For TCNP, the resulting Cmax in plasma was only 2.2 ng/ml (Fig. 2A and Table 2).

We then tested the intestinal absorption of the two prodrugs in the same system. In contrast, the prodrug compounds (Fig. 2B and C) showed much greater exposure levels in plasma compared to the two parent compounds, TCNP and TCN. Dosing with the two prodrugs resulted in more complex Cp × time curves, because all of the prodrugs were hydrolyzed in liver, at least in part, to their parent compound. The 5′-O-valyl triciribine had a peak concentration of 53.4 ng/ml and also showed low levels of TCN (5.2 ng/ml). The 5′-O-valyl phosphoramidate triciribine prodrug showed the greatest absorption, and, in this case, the major detectable compound was the parent compound, TCNP, which had a Cmax of 173.6 ng/ml.

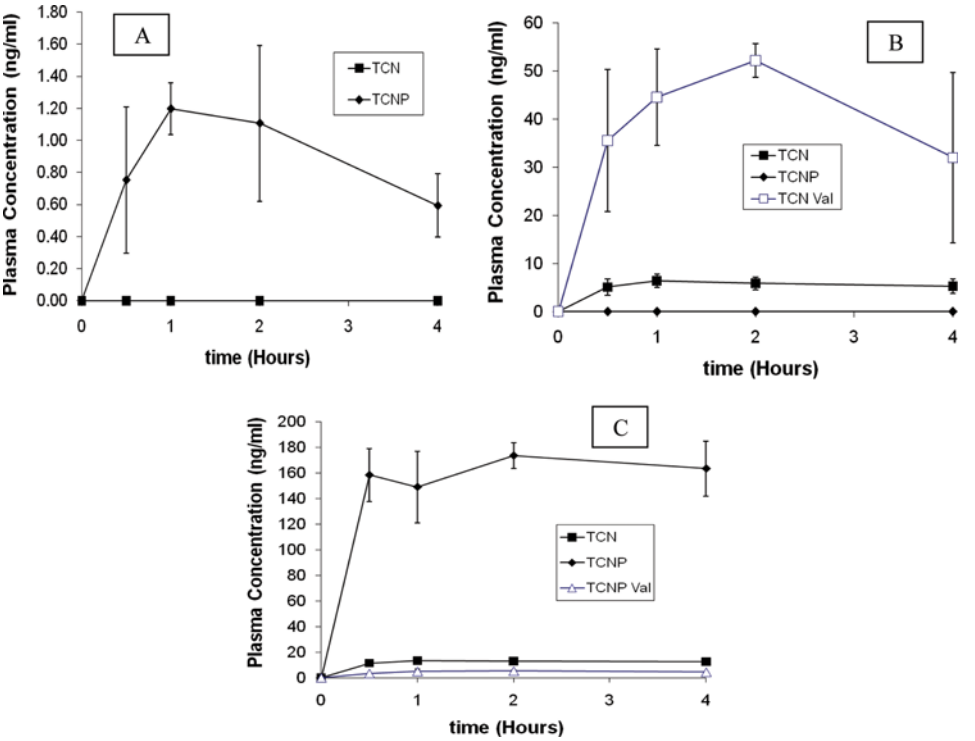


Figure 2. Plasma concentration versus time curves for TCN, TCNP, and their prodrugs. (Figure is provided in color online.)

Table 2. PK parameters of Cmax and AUC after duodenal administration of TCN, TCNP, and their respective prodrugs (Cmax and AUC values were determined from the Cp × time curves shown in Fig. 2)

Compound	TCN	Fold enhancement	TCNP	Fold enhancement	Prodrug
	C_{max} from oral dosing for TCN, TCNP, 5′-O-valyl ester of TCN, and 5′-O-[(valine methyl ester) phosphoramidate] of TCN (ng/ml)				
TCN	BDL	—	—	—	—
TCNP	—	—	2.2	—	—
5′-O-Valyl triciribine	7.2	Significant	BDL	na	52.2
5′-O-Valyl phosphoramidate TCN	13.4	Significant	173.6	78	5.3
	Dose norm. AUC_{0-4} for TCN, TCNP, 5′-O-valyl ester of TCN, and 5′-O-[(valine methyl ester) phosphoramidate] of TCN (ng/ml) · h				
TCN	BDL	—	—	—	—
TCNP	—	—	4.4 ± 0.9	—	—
5′-O-Valyl triciribine	21.5 ± 4.8	Significant	BDL	Na	161 ± 33.7
5′-O-Valyl phosphoramidate TCN	47.7 ± 2.2	Significant	615 ± 11.4	139	17.4 ± 2.5

Total quantification of drug exposure of TCN containing compounds (AUC_{0-4}hr) also showed that the phosphoramidate-linked prodrug had the greatest potential for oral absorption. These results are summarized in Table 2. As can be seen in the data tables, both the TCN prodrugs yielded significant increases in circulating levels of TCN, TCNP, or both as well as high levels of circulating prodrug. With regard to Cmax, L-val ester prodrug yielded the greatest peak concentrations of prodrug, while the valyl phosphoramidate prodrug showed the greatest level of TCNP. More importantly, the total exposure of active TCN and/or TCNP components in plasma was significantly increased for both prodrugs when compared to either TCN or TCNP. This can be seen in the AUC portion of Table 2 and is illustrated graphically in Fig. 3. Thus, the L-valyl ester prodrug yielded significant increase in circulating TCN levels compared with both TCN and TCNP dosing. Similarly, the

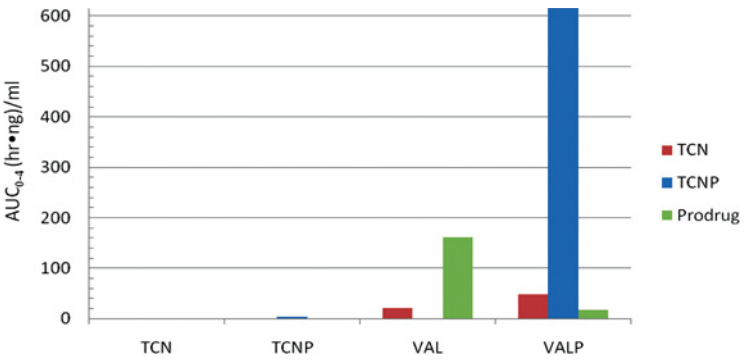


Figure 3. Intestinal dosing of TCN and TCNP prodrugs shows enhanced levels of circulating prodrug and parents compared with TCN and TCNP along. The $AUC_{0-4\,hr}$ of TCN (red bars), TCNP (blue bars), and prodrug (green bars) after intestinal dosing of the prodrugs at a level of 10 mg/kg is compared to AUC levels after TCN and TCNP dosing. The data are taken from Table 1. (Figure is provided in color online.)

(L-valine methyl ester) phosphoramidate prodrugs had very significant increases in TCN levels and 139-fold increase in TCNP levels, which indicated that both TCN and TCNP can be metabolites of the phsophoramidate after passing through the liver. Additionally, both compounds yielded significant levels of circulating prodrugs in the plasma, which may serve as an additional depot of active compounds after entering the caner cell. Taken together, these data strongly support our hypothesis that the amino acid prodrugs of TCN or TCNP can significantly boost the oral availability of active compounds (TCN and TCNP).

EXPERIMENTAL

6-Chloro-7-iodo-7-deazapurine was purchased from Sigma-Aldrich Co. All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. All chemicals were obtained from Sigma-Aldrich Co. For anhydrous reactions, solvents were dried according to Vogel's Textbook of Practical Organic Chemistry.[38] Removal of solvent was performed under reduced pressure using a Büchi rotary evaporator, followed by evacuation (<0.1 mm Hg) to constant sample weight. Deionized water was obtained from a Milli-Q reagent water system (Millipore Co., Milford, MA). All reactions were carried out under an argon atmosphere in dried glassware. All reactions and fractions from column chromatography were monitored by thin-layer chromatography (TLC) using plates with an ultraviolet (UV) fluorescent indicator (normal SiO_2, Merck 60 F254). One or more of the following methods were used for visualization; UV absorption by fluorescence quenching and iodine staining. Flash chromatography was performed according to the method of Still[39] using Merck type 60, 230 to 400-mesh silica gel. Fast atom bombardment mass spectra (FAB-MS) were recorded on a Quattro II mass spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker 400 spectrometer. ^{1}H NMR chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS) using the residual proton resonance of solvents as reference: D_2O δ 4.8 and dimethylsulfoxide (DMSO-d_6) δ 2.50. ^{1}H NMR data are reported in the following order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet, qn, quintet; and m, multiplet), and coupling constant (*J*) in hertz (Hz). When appropriate, the multiplicity is preceded by br, indicating that the signal was broad. Yields refer to isolated yields of compounds estimated to be >95% pure as determined by ^{1}H NMR and analytical high-performance liquid chromatography (HPLC).

4-Chloro-5-iodo-7-[(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (6)

N,O-Bis(trimethylsilyl) acetamide (BSA, 4.8 mL, 20 mmol) was added to a stirred suspension of 6-chloro-7-iodo-7-deazapurine (***5***) (5 g, 18 mmol) in dry acetonitrile (122 mL) under an argon enviroment. After stirring at room temperature for 20 min, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (10 g, 20 mmol) was added, followed by the addition of trimethylsilyl trifluoromethanesulfonate (3.5 mL, 19 mmol). The reaction mixture was stirred at room temperature for 30 min, after which the flask was transferred to a preheated oil bath at 80 °C. After

stirring for 2 h at 80 °C, the reaction mixture was cooled to room temperature and diluted with ethyl acetate (200 mL). The organic phase was sequentially washed with aqueous saturated $NaHCO_3$ and brine, dried with anhydrous Na_2SO_4, and concentrated to provide the crude product, which was purified by flash column chromatography (cyclohexane / ethyl acetate 3/1, v/v) to provide **6** as a pale yellow foam (10 g, 80%). ^{1}H NMR (DMSO-d_6) δ: 8.61 (s, 1H, proton at 2 position of pyrimidine ring), 8.31 (s, 1H, proton at 6 position of pyrimidine), 8.09–7.94 (m, 4H, proton at benzoates), 7.85–7.88 (m, 2H, proton at benzoates), 7.72–7.60 (m, 3H, proton at benzoates), 7.55–7.44 (m, 6H, proton at benzoates), 6.73–6.71 (d, 1H, *J* 4.93 Hz, 1′ proton of ribofuranose); 6.32–6.29 (t, 1H, *J* 6.21 & 5.15 Hz), 6.17–6.14 (t, 1H, *J* 6.00 & 5.79 Hz), 4.90–4.79 (m, 2H), and 4.71–4.67 (m, 1H); 2′, 3′, 4′, and 5′ protons of ribofuranose.

Mass spectrum: calculated for: $C_{32}H_{23}ClIN_3O_7$ (723.90). FAB-MS: m/z 723.86. Analytical calculation for $C_{32}H_{23}ClIN_3O_7$: C, 53.09; H, 3.20; N, 5.80, Found: C, 53.12; H, 3.16; N, 5.74.

4-Chloro-5-cyano-7-[(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (7)

To a deaerated solution of 4-chloro-5-iodo-7-[(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (**6**) (5 g, 6.9 mmol) in 25 ml of anhydrous DMF were added 2.4 g (7.6 mmol) tributyltin cyanide and 0.40 g (0.34 mmol) palladium tetraphenylphosphine. After dearaton for half an hour, the mixture was stirred at 95 °C under protection of argon gas for 6 h. Solvent and volatile component were removed under reduced pressure, and the residue was dissolved in 100 ml ethyl acetate and washed with 100 ml ammonium chloride saturated aqueous solution. After separation of the ethyl acetate layer, the aqueous layer was washed three times with 30 ml ethyl acetate. The combined ethyl acetate solution was washed with 50 ml brine and dried with anhydrous sodium sulfate. After filtration, the ethyl acetated solution was concentrated, and the residue was purified by flash chromatography with hexane/ethyl acetate (3:2) to afford 3.1 g compound **7** as a yellow oil (72% yield). ^{1}H NMR (DMSO-d_6) δ: 8.535 (s, 1H, proton at 2 position of pyrimidine ring), 8.175 (s, 1H, proton at 2 position of pyrimidine), 8.017–8.086 (m, 4H, proton at benzoates), 7.871–7.894 (m, 2H, proton at benzoates), 7.341–7.661 (m, 9H, proton at benzoates), 6.642–6.668 (d, 1H, *J* 5.38 Hz, 1′ proton of ribofuranose), 6.195–6.167 (t, 1H, *J* 5.82 & 5.66 Hz), 6.114–6.088 (t, 1H, *J* 5.30 & 6.02 Hz), and 4.976–4.702 (m, 3H): 2′, 3′, 4′, and 5′ protons of ribofuranose Mass spectrum: calculated for: $C_{33}H_{23}ClN_4O_7$ (623.01). FAB-MS: m/z 623.28. Analytical calculation for $C_{33}H_{23}ClN_4O_7$: C, 63.62; H, 3.72; N, 8.99, Found: C, 63.54; H, 4.00; N, 9.05.

4-(1-Methylhydrazino)-5-cyano-7-[(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (8)

To a solution of 4-chloro-5-cyano-7-[(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine (**7**) (2 g, 3.2 mmol) in 50 ml of 1:1 volume mixture of anhydrous chloroform and anhydrous ethanol was added 0.3 ml methylhydrazine (0.26 g, 5.7 mmol) and stirred under protection of argon gas for 3.5 h. Another 0.3 ml of methylhydrazine was added and the reaction was stirred for another 1.5 h.

Solvent and volatile component were removed under reduced pressure, and the residue was purified by flash chromatography with hexane/ethyl acetate (1:1 and 1:2) to afford 1.9 g compound **8** as an pale yellow oil (93% yield). ^{1}H NMR (DMSO-d_6) δ: 8.100 (s, 1H, proton at 2 position of pyrimidine ring), 7.828 (s, 1H, proton at 6 position of pyrimidine), 8.040–8.017 (m, 2H, proton at benzoates), 7.947–7.923 (m, 2H, proton at benzoates), 7.858–7.828 (m, 2H, proton at benzoates), 7.705–7.607 (m, 3H, proton at benzoates), 7.560–7.411 (m, 6H, proton at benzoates), 6.629–6.615 (d, 1H, *J* 5.46 Hz, 1′ proton of ribofuranose), 6.272–6.243 (t, 1H, *J* 5.82 & 5.66 Hz), 6.112–6.084 (t, 1H, *J* 5.30 & 6.02 Hz), and 4.808–4.650 (m, 3H): 2′, 3′, 4′, and 5′ protons of ribofuranose; 4.938 (s, 2H, 2 proton at hydrazine), 3.264 (s, 3H, methyl group of methylhydrazine). Mass spectrum: calculated for $C_{33}H_{28}IN_5O_7$ (733.51). FAB-MS: *m/z* 734.07. Analytical calculation for $C_{33}H_{28}IN_5O_7$: C, 54.04; H, 3.85; N, 9.55, Found: C, 54.06; H, 4.08; N, 9.50.

4-Methyl-6-amino-8-(β-D-ribofuranosyl)pyrrolo[4,3,2-*d*]pyrimidino [4,5-c]pyridazine (triciribine) (1, TCN or triciribine) (Modified from Existing Method)

4-(1-Methylhydrazino)-5-cyano-7-[(2,3,5-tri-O-benzoyl)-β-D-ribofuranosyl]-7H-pyrrolo[2,3-d]pyrimidine (**8**) (1.0 g, 1.6 mmol) and sodium methoxide (0.38 g, 7.1 mmol) were suspended in methanol anhydrous (100 mL) and stirred at 20 °C with protection of argon. After 1 h, the reaction mixture was heated at reflux temperature with protection of argon for 18 h. The solvent and volatile component were removed under reduced pressure, and the yellow residue was purified by recrstallization from methanol and water to obtain 0.37 g (74%) of pure TCN.

^{1}H NMR (DMSO-d_6) d 8.03 (s, 1H, proton at 2 position of hetero-tricyclic ring), 7.07 (s, 1H, proton at 7 position of hetero-tricyclic ring), 6.25 (s, 2H, NH_2), 5.82 to 5.80 (d, 1H, *J* 6.54 Hz, 1′ proton of ribofuranose), 5.60–5.57 (q, 1H, OH), 5.39–5.37 (d, 1H, *J* 6.63 Hz, OH), 5.18–5.17 (d, 1H, *J* 4.50 Hz, OH), 4.51–4.47 (m, 1H), 4.12–4.08 (m, 1H), 3.98–3.95 (m, 1H), 3.64–3.50 (m, 2H), 2′, 3′, 4′, and 5′ protons of ribofuranose, 3.40 (s, 3H, NCH_3). Mass spectrum: calculated for $C_{13}H_{16}N_6O_4$ (320.12). FAB-MS: *m/z* 321.27.

Analytical calculation for $C_{13}H_{16}N_6O_4 \cdot$ **MeOH**: C, 47.72; H, 5.68; N, 23.86. Found: C, 47.80; H, 5.53; N, 23.75.

4-Methyl-6-amino-8-[5′-O-(*tert*-butyldimethylsilyl)-2′,3′-O-dilevylinyl-β-D-ribofuranosyl]pyrrolo[4,3,2-*d*]pyrimidino[4,5-c]pyridazine (9)

The described procedure[36] was modified. To a stirred suspension of 1.0 g (3.12 mmol) of dry TCN (**1**) in 15 mL of anhydrous N,N-dimethylformamide was added 0.91 g (7.5 mmol) of DMPA, followed by 0.56 g (3.74 mmol) of *tert*-butyldimethylchlorosilane. The mixture was stirred with protection of argon at room temperature for 20 h, at the end of which time TLC (9:1 DCM/MeOH) indicated that the starting material disappeared. A solution of levulinic acid (1.98 g, 17 mmol) in 10 ml of anhydrous ethyl acetate was added followed by solution of N,N′-dicyclohexylcarbodiimide (DCC) (6.7 g, 32.6 mmol) in 10 mL anhydrous ethyl acetate. The

reaction mixture was stirred under protection of argon at room temperature for 1.5 h. TLC (9:1 DCM/MeOH) indicated the reaction was completed. Anhydrous EtOH (4 mL) was added to consume all unreacted levulinic acid. After an additional 30 min, the precipitate (DCU) was filtered off, and the solvent was removed from filtrate in vacuo. The residue was redissolved in 50 ml ethyl acetate and extracted with saturated ammonium chloride aqueous solution (40 mL) once, water (40 mL) twice, and brine (40 ml) once. The organic layer was dried with anhydrous Na_2SO_4 before being evaporated to dryness. The resulted syrup was further purified by silica flash chromatography (4% MeOH in DCM as eluent) to obtain 1.56 g pure product (**9**) (80%).

^{1}H NMR (DMSO-d_6) δ 0.037 (6H, 2 s, $SiMe_2$), 0.846 (9H, s, t-butylSi); levulinate: 2.047 (3H, s CH_3), 2.134 (3H, s, CH_3), 2.457–2.473 (2H, t, CH_2), 2.572–2.752 (4H, m, two CH_2), 2.769–2.784 (2H, m, CH_2); : ribofuranose: 3.781–4.183 (3H, m), 5.495–5.519 (1H, m), 5.808–5.837 (1H, m), 6.156 (1H, d, $^3J = 5.96$ Hz, H_1'); hetero-tricyclic ring: 3.40 (s, 3H, NCH_3), 6.224 (s, 2H, NH_2), 7.014 (s, 1H, proton at 7 position), 8.066 (s, 1H, proton at 2 position).

Mass spectrum: calculated for $C_{29}H_{42}N_6O_8Si$ (630.76). FAB-MS: m/z 631.41

Analytical calculation for $C_{29}H_{42}N_6O_8Si$: C, 55.22; H, 6.71; N, 13.32. Found: C, 55.28; H, 6.68; N, 13.45.

4-Methyl-6-amino-8-[2′,3-O′-dilevylinyl-β-D-ribofuranosyl]pyrrolo[4,3,2-*d*]pyrimidino[4,5-c]pyridazine (10)

One gram (1.59 mmol) of **9**, which had a yellow foamy appearance, was dissolved in a solution of 1 M TBAF in THF (7.2 mL) containing glacial acetic acid (0.67 mL). The reaction mixture was stirred under argon at room temperature for 1.5 h and was then directly filtered through a silica gel plug (4 × 5 cm) with THF as eluant. The collected fractions were evaporated to dryness in vacuo. The residue was redissolved in DCM and purified by silica-gel flash chromatography with 7% methanol in DCM as eluant to obtain 0.61 g (75% yield) of a gumlike product, which was crystallized from ethyl acetate as a pale yellow solid (**10**).

^{1}H NMR (DMSO-d_6): levulinate: 2.010 (3H, s CH_3), 2.143 (3H, s, CH_3), 2.501–2.512 (2H, t, CH_2), 2.601–2.617 (2H, m, CH_2), 2.682–2.698 (2H, m, CH_2), 3.154–3.170 (2H, m, CH_2); ribofuranose: 3.674–3.896 (2H, m), 3.961–4.057 (1H, m), 5.035–5.127 (1H, t, 5′OH, $^3J = 5.48$), 5.409–5.736 (2H, m), 6.137 (1H, d, $^3J = 5.84$ Hz, H1′); hetero-tricyclic ring: 3.38 (s, 3H, NCH_3), 6.215 (s, 2H, NH_2), 7.010 (s, 1H, proton at 7 position), 7.973 (s, 1H, proton at 2 position).

Mass spectrum: calculated for: $C_{23}H_{28}N_6O_8$ (516.5). FAB-MS: m/z 517.96.

Analytical calculation for $C_{23}H_{28}N_6O_8$: C, 53.48; H, 5.46; N, 16.27. Found: C, 53.45; H, 5.61; N, 16.18.

4-Methyl-6-amino-8-[5′-O-(methylphenylphosphoryl P→N-L-valine)-2′,3′-O-dilevylinyl-β-D-ribofuranosyl]pyrrolo[4,3,2-*d*]pyrimidino [4,5-c]pyridazine (12)

Methylchlorophenylphosphoryl P→N-L-valine (**11**)[37] (1.77 g, 5.8 mmol) in 5 ml anhydrous THF was added into a flask containing 0.6 g **10** (1.2 mmol) in 15 ml THF, followed by addition of 1 ml (11 mmol) N-methylimidazole. The reaction

was stirred for 5 h at room temperature. The resultant mixture was concentrated under vacuum. The residue was absorbed in 2 g of silica gel and subjected to silica-gel flash chromatograhph (4% of MeOH in DCM) to obtain 0.62 g **12** (68%).

^{1}H NMR (DMSO-d_6) δ valine methyl ester: 0.733–0.823 (6H, m, two CH_3), 1.871–1.954 (1H, m, CH), 3.462–3.540 (1H, m, CH), 3.541–3.575 (3H, 2s, OCH_3), 5.779–5.935 (1H, m, NH of valine); levulinate: 2.041 (3H, s CH_3), 2.227 (3H, s, CH_3), 2.583–2.742 (4H, m, 2×CH_2), 2.846–2.901 (2H, m, CH_2), 3.301–3.386 (2H, m, CH_2); ribofuranose: 3.953–4.063 (1H, m), 4.102–4.179 (2H, m), 4.836–5.062 (2H, m), 6.312 (1H, d, H1′); C_6H_5: 7.126–7.178 (3H, m), 7.307–7.367 (2H, m); hetero-tricyclic ring: 3.41 (s, 3H, NCH_3), 6.917 (2H, s, NH_2), 7.254 (s, 1H, proton at 7 position), 8.137 (s, 1H, proton at 2 position). Mass spectrum: calculated for: $C_{35}H_{44}N_7O_{12}P$ (785.74). FAB-MS: *m/z* 786.38. Analytical calculation for $C_{35}H_{44}N_7O_{12}P$: C, 53.50; H, 5.64; N, 12.48. Found: C, 53.68; H, 5.81; N, 12.39.

4-Methyl-6-amino-8-[5′-O-(methylphenylphosphoryl P→N-L-valine)-β-D-ribofuranosyl]pyrrolo[4,3,2-*d*]pyrimidino[4,5-c]pyridazine (3)

Compound **12** (0.5 g, 0.63 mmol) was dissolved in 1 ml pyridine. Hydrazine hydrate (1.25 mL, 2 M) in pyridine–acetic acid buffer was added. The reaction mixture was stirred at room temperature for 15 min, after which time the mixture was directly loaded on top of a prepared silica-gel column and eluent with 15% MeOH in DCM to obtain the final product (**10**). Preparative HPLC (gradient, 0 to 35% MeOH in water during 35 min) was used to obtain 0.31 g pure products **3** (83%).

^{1}H NMR (DMSO-d_6) δ valine methyl ester: 0.713–0.821 (6H, m, two CH_3), 1.879–1.895 (1H, m, CH), 3.458–3.519 (1H, m, CH), 3.538–3.570 (3H, 2s, OCH_3), 5.876–5.922 (1H, m, NH of valine); ribofuranose: 4.132–4.188 (3H, m), 4.238–4.276 (2H, m), 5.4–5.6 (2H, broadband 2OH), 5.936 (1H, d, H1′); C_6H_5: 7.144–7.177 (3H, m), 7.305–7.358 (2H, m); hetero-tricyclic ring: 3.455 (s, 3H, NCH_3), 6.4–6.6 (2H, broadband, NH_2), 7.219 (s, 1H, proton at 7 position), 8.135 (s, 1H, proton at 2 position). Mass spectrum: calculated for $C_{25}H_{32}N_7O_8P$ (589.54). FAB-MS: *m/z* 590.75. Analytical calculation for $C_{25}H_{32}N_7O_8P$: C, 50.93; H, 5.47; N, 16.63. Found: C, 51.05; H, 5.64; N, 16.54.

4-Methyl-6-amino-8-[2′,3′-O-dilevylinyl-5′-O-N$^{\alpha}$-Boc-L-valyl-β-D-ribofuranosyl]pyrrolo[4,3,2-*d*]pyrimidino[4,5-c]pyridazine (13)

$^{\alpha}$N-Boc-L-valine, (218 mg, 1 mmol) 15 mg (0.12 mmol) of DMAP, and 260 mg (0.5 mmol) of compound **10** was dried in a 250-ml, single-necked, round-bottom flask under high vacuum overnight. Anhydrous DMF (5 ml) was added. After stirred for 5 min at 0 °C, 208 mg (1.00 mmol) of DCC in 5 ml DMF were added dropwise at 0 °C. After 4 h, TLC indicated completion of the reaction. Anhydrous ethanol (5 ml) was added, and the reaction mixture was stirred overnight. DMF was removed under high vacuum by rotavapor at 40 °C. The residue was dissolved in 50 ml ethyl acetate. The insoluble white precipitate was removed by filtration. The filtrate was consecutively washed with 20 ml saturated ammonium chloride, 20 ml water, and 20 ml brine. After drying with anhydrous sodium sulfate, the solvent was removed in vacuo. The residue was dissolved in a minimum amount of dichloromethane and

absorbed in 2 g of silica gel by removal of dichloromethane with aid of rotavopor. The mixture was loaded on top of a 30 g silica-gel column. Flash chromatography with solvent of 93:7 (v/v) dichloromethane and methanol led to 260 mg compound **13** (72%).

^{1}H NMR (DMSO-d_6): δ valyl: 0.899–0.922 (6H, m, 2×CH_3,), 1.413 (9H, s, Boc), 2.213–2.274 (1H, m, CH), 3.923–3.951 (1H, m, CH), 7.328 (1H, d, $J = 5.0$, NH), levulinate: 2.053 (3H, s CH_3), 2.171 (3H, s, CH_3), 2.457–2.473 (2H, t, CH_2), 2.489–2.494 (2H, m, CH_2), 2.498–2.507 (2H, m, CH_2), 2.691–2.749 (2H, m, CH_2); ribofuranose ring: 3.781–3.883 (1H, m), 4.037–4.153 (2H, m), 4.910–5.127 (2H, m), 6.150 (1H, d, $^2J = 5.96$ Hz, H1′); hetero-tricyclic ring : 3.412 (s, 3H, NCH_3), 6.228 (s, 2H, NH_2), 7.020 (s, 1H, proton at 7 position), 8.104 (s, 1H, proton at 2 position). Mass spectrum: calculated for $C_{33}H_{45}N_7O_{11}$: 715.75. FAB-MS: m/z 716.53 (M + 1). Analyticla calculations for $C_{33}H_{45}N_7O_{11}$: C, 55.38; H, 6.34; N, 13.70. Found: C, 55.42; H, 6.19; N, 13.66.

4-Methyl-6-amino-8-[5′-O-L-valyl-β-D-ribofuranosyl]pyrrolo[4,3,2-*d*]pyrimidino[4,5-c]pyridazine (4)

Compound **13** (200 mg) was dissolved in 2 ml pyridine. (Hydrazine hydrate 1 ml, 2 M) in pyridine–acetic acid buffer was added. After stirring the solution for 10 min at room temperature, the reactant disappeared and a slow migrating fraction was detected by TLC (8:2 dichloromethane/methanol). The excess hydrazine was eliminated through reaction with pentane-2,4-dione for another 10 min. After evaporating the volatile components, the residue was dissolved in EtOAc and washed with 20 ml saturated ammonium chloride, water, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was dissolved in a minimum amount of dichloromethane and absorbed in 2 g of silica gel by removal of dichloromethane with aid of rotavopor. The mixture was loaded on top of a 30 g silica-gel column. Flash chromatography with solvent of 85:15 (v/v) dichloromethane and methanol led to 138 mg compound **4** that still contained a trace of impurity. Without further purification, 6 ml of 1:1 TFA and dichloromethane were added to the residue and stirred for 4 h. Solvent was removed in vacuo. The residue was washed with cold diethyl ether. The resulting white solid was purified by reverse-phase preparative HPLC to obtain 100 mg compound **4** (85% from compound **13**). HPLC condition: gradient, 0 to 85% methanol in water containing 0.02% TFA within 30 min.

^{1}H NMR (D_2O): δ valyl: 0.962–1.012 (6H, m, 2×CH_3,), 2.299–2.482 (1H, m, CH), 4.025–4.088 (1H, d, CH, $^2J = 4.75$ Hz) ribofuranose ring: 3.782–3.802 (2H, m), 4.366–4.378 (1H, m), 4.796–4.827 (1H, m), 5.434–5.453 (1H, m), 6.024 (1H, d, $J = 7.22$ Hz, H1′); hetero-tricyclic ring: 3.482 (s, 3H, NCH_3), 7.376 (s, 1H, proton at 7 position), 8.039 (s, 1H, proton at 2 position). Mass spectrum: calculated for $C_{18}H_{25}N_7O_5$: 419.44. FAB-MS: m/z 420.50(M + 1). Analytical calculations for $C_{18}H_{25}N_7O_5$2TFA: C, 40.81; H, 4.20; N, 15.14. Found: C, 40.65; H, 4.17; N, 15.23.

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