Dipeptidyl-Quinolone Derivatives Inhibit Hypoxia Inducible Factor-1α Prolyl Hydroxylases-1, -2, and -3 with Altered Selectivity

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Intracellular levels of the hypoxia-inducible transcription factor (HIF) are regulated under normoxic conditions by prolyl hydroxylases (PHD1, 2, and 3). Treatment of cells with PHD inhibitors stabilizes HIF-1α, eliciting an artificial hypoxic response that includes the transcription of genes involved in erythropoiesis, angiogenesis, and glycolysis. The different in vivo roles of the three PHD isoforms are not yet known, making a PHD-selective inhibitor useful as a biological tool. Although several chemical series of PHD inhibitors have been described, significant isoform selectivity has not been reported. Here we report the synthesis and activity of dipeptidyl analogues derived from a potent but non-selective quinolone scaffold. The compounds were prepared by Pd-catalyzed reductive carbonylation of the 6-iodoquinolone derivative to form the aldehyde directly, which was then attached to a solid support via reductive amination. Amino acids were coupled, and the resulting dipeptidyl-quinolone derivatives were screened, revealing retention of PHD inhibitory activity but an altered PHD1, 2, and 3 selectivity profile. The compounds were found to be ~10-fold more potent against PHD1 and PHD3 than against PHD2, whereas the specific parent compound had shown no appreciable selectivity among the different PHD isoforms.

Introduction

The cellular response to changes in the level of dioxygen is coordinated through the α/β heterodimeric hypoxia-inducible transcription factor (HIF). During normoxia, the presence of sufficient cellular pO_2 is "sensed" through increased activity of a small class of dioxygenases, the HIF prolyl hydroxylases (PHD1, 2, and 3), in catalyzing the *trans*-4-hydroxylation of HIF-1α residues Pro402 or Pro564 within the oxygen-dependent degradation domain (ODD).[1] This post-translational modification allows HIF-1α to be bound by the von Hippel Lindau protein (pVHL),[2] thus targeting HIF-1α for poly ubiquitination by an associated E3 ubiquitin ligase and subsequent degradation by the proteosome. However, under hypoxic conditions, HIF-1α is stabilized through reduced PHD action, leading to HIF-mediated transcription of an array of genes that contain the hypoxia-responsive element (HRE), including those coding for glycolytic enzymes, erythropoietin, and the vascular endothelial growth factor (VEGF).

Stabilization of HIF-1α through inhibition of PHD has been examined as a potential treatment for ischemic diseases including anemia, myocardial infarction, and stroke.[3] PHD enzymatic activity is dependent on Fe^{2+}, ascorbate, and 2-oxoglutarate (2OG), and the first small molecule PHD inhibitors were non-specific iron coordinators and/or analogues of 2OG.[4] Further investigation led to the discovery of a variety of iron-coordinating aromatic heterocycles whose activity was enhanced by appending a carboxylate to mimic 2OG.[5] Co-crystallization of PHD2 with an isoquinoline inhibitor, (((4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl)amino)acetic acid,[6] in the 2OG binding pocket has facilitated the structure-based design and discovery of several new chemical classes of potent PHD inhibitors,[7] including the newly identified quinolones (Figure 1).[8] Most PHD inhibitors reported to date are 2OG competitive, bind Fe^{2+} in a bidentate manner, have a carboxylate that forms a salt bridge with the guanadinium side chain of HIF-1α residue Arg383, and make favorable van der Waals contacts with the hydrophobic residues lining the PHD active site (e.g., Tyr310, Met 299, and Trp389).[6] The highly conserved 2OG binding pocket (all 14 hydrophobic residues lining the enzyme active site are completely conserved among the three PHD isoforms[6]) has hindered identification of a PHD-selective inhibitor, which would be valuable for elucidation of the

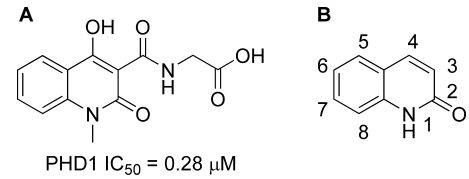

Figure 1. (A) Quinolone PHD inhibitor[8] and (B) numbering of positions around the quinolone ring.

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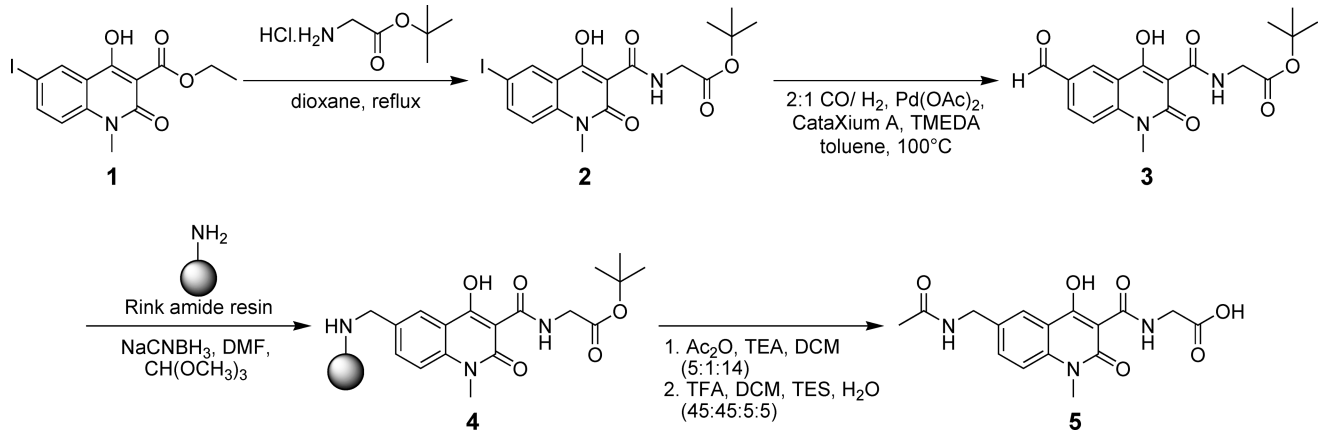
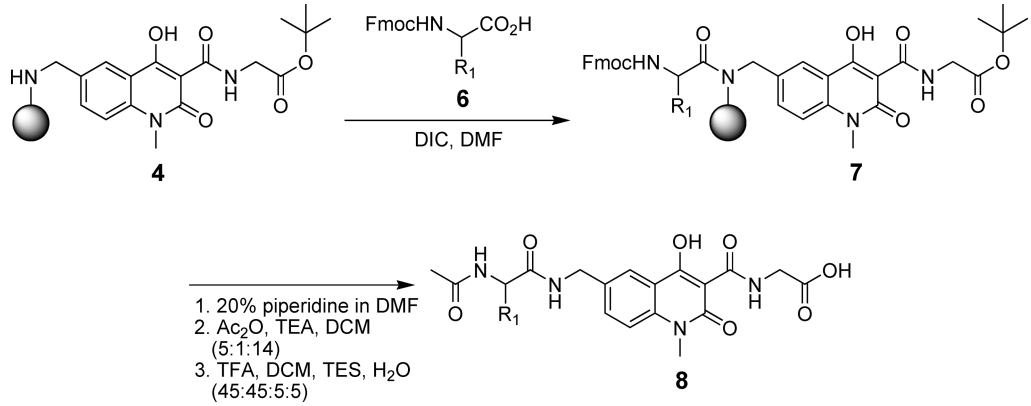
Scheme 1



Scheme 2



different roles of PHD1, 2, and 3 in vivo. To our knowledge, compounds with significant selectivity between the three PHD isoforms have yet to be reported in the literature.

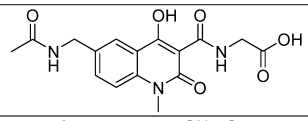
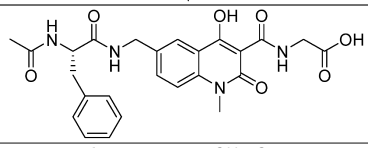
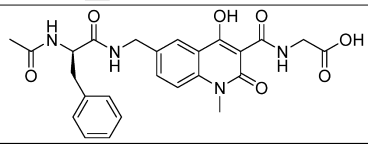
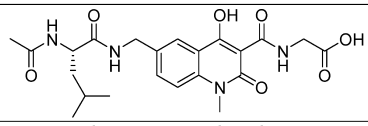
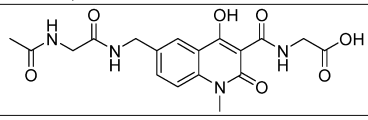
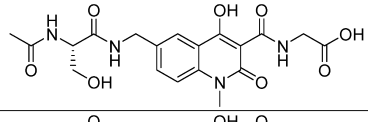
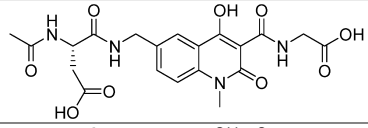
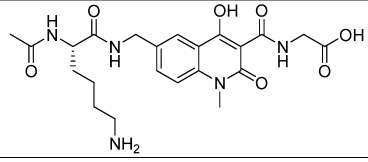
We sought to maintain the potency and alter the selectivity of the quinolone-based inhibitors by extending the molecule out of the 2OG binding site into a putative hydrophobic pocket within the HIF-1α (559−574) peptide binding site.[9] By analogy to the isoquinoline series, we assumed the amide carbonyl and phenolic oxygen of the quinolone coordinate the Fe^{2+} and the carboxylate of the attached glycine interacts with Arg383 to mimic the binding of 2OG in the bottom of the pocket, while the substituent at the 6-position of the heterocyclic ring is directed toward solvent at the mouth of the pocket.[6] We decided to derivatize the quinolone scaffold at this position via attachment of amino acids to explore the HIF-1α binding site on the surface of PHD. We designed and prepared a set of 96 dipeptidyl-quinolone analogues and screened for PHD inhibition. The compounds were initially found to be 10- to 30-fold more potent against PHD1 and PHD3 than against PHD2, whereas the parent compound had shown no appreciable selectivity among the different PHD isoforms. Several "hits" from the initial screen were resynthesized and validated as having half maximal inhibitory concentrations (IC_{50}) < 45 nM against PHD2 and IC_{50} values <10 nM for PHD1 and PHD3.

Results and Discussion

Initial Synthesis of Quinolone Derivatives. Our synthetic approach to the dipeptidyl-quinolone derivatives was designed to allow incorporation of amino acids via 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fmoc-SPPS). The initial steps of the synthesis involved the conversion of an aryl halide into a form suitable for immobilization on the solid-phase support (Scheme 1). Ethyl 4-hydroxy-6-iodo-1-methyl-quinolone-3-carboxylate (**1**), a derivative prepared during exploration of the structure−activity relationship of the quinolones,[8] was refluxed in dioxane with *tert*-butyl glycine hydrochloride to produce compound **2**. Using a slight modification of the conditions developed by Beller, Almena, and co-workers for the Pd-catalyzed reductive carbonylation of aryl bromides using CO/H_2 (synthesis gas), compound **2** was converted directly to the desired aldehyde, compound **3**.[10,11] Reductive amination of compound **3** with polystyrene Rink amide resin and sodium cyanoborohydride produced the resin-bound quinolone **4**. Acetylation of the quinolone-resin **4** was followed by trifluoroacetic acid (TFA) mediated cleavage from the solid support and removal of the *tert*-butyl protecting group to provide compound **5**.

Next, we prepared acylated amino acid-quinolone derivatives by following the above strategy but by introducing amino acids at the 6-position (Scheme 2). Our initial objective was to determine if derivatization at this site would significantly reduce PHD activity. Accordingly, we prepared and tested a relatively small number of compounds. Amino acids were chosen to maximize the diversity of size, hydrophobicity, polarity, and chirality within a very small set. Acylation of quinolone-resin **4** with N^{α}-Fmoc amino acid

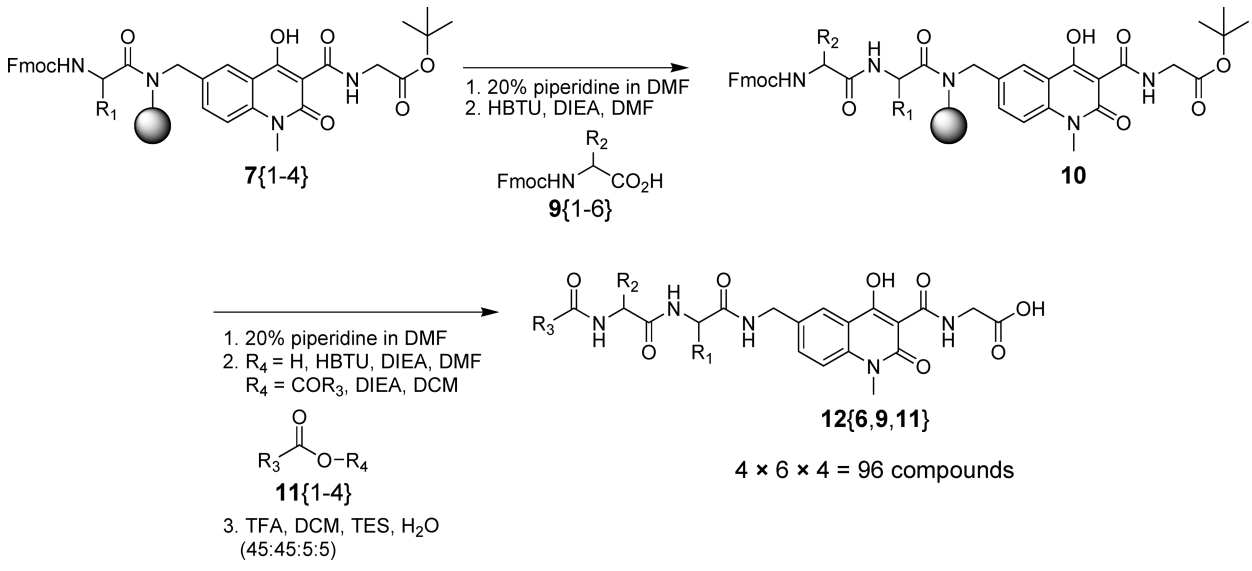
Table 1. PHD2 Inhibition by Quinolone-Amino Acid Conjugates

Compound	Structure	PHD2 IC_{50} (μM)
5		0.546
8{1}		0.482
8{2}		0.112
8{3}		0.521
8{4}		0.293
8{5}		0.265
8{6}		0.138
8{7}		0.409

reagent chemset **6** (Scheme 2, Figure 2) to produce chemset **7** proved challenging. Repeated (3−4 ×) 24 h treatments of the resin with a large excess (>10 equiv) of the preformed amino acid symmetrical anhydrides were necessary to achieve >90% conversion. Fmoc removal with 20% piperidine in *N*,*N*-dimethylformamide (DMF), followed by acetylation, and TFA cleavage with simultaneous global side chain deprotection of all members of chemset **7** afforded chemset **8**. Reversed phase (RP) HPLC purification produced compounds **8**{1−7} in sufficient quantity (1−2 mg) for screening in a representative PHD enzymatic assay. From the assay results (Table 1), we concluded that derivatization at position 6 of the quinolone did not abrogate PHD inhibition and that incorporation of an amino acid (D-phenylalanine or aspartic acid) could improve activity relative to acetylation (**8**{2} and **8**{6}). Further exploration of the series was justified, and we proceeded with preparation of additional analogues.

Synthesis of Dipeptidyl-Quinolone Derivatives. Dipeptidyl-quinolone derivatives were synthesized via Fmoc SPPS from the previously prepared quinolone-amino acid resins **7**{1−4} via the following reaction steps (Scheme 3).[12] Fmoc removal with 20% piperidine in DMF was followed by the coupling of N^{α}-Fmoc-amino acid reagent chemset **9** (Figure 3) with *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) to produce chemset **10**. Treatment with piperidine removed the Fmoc protecting group from chemset **10** members before capping the N-terminus of the dipeptide chain with reagent chemset **11** (Figure 4). In the final step, acylation with anhydrides **11**{1,2,4} was performed in dichloromethane (DCM) with *N*,*N*-diisopropylethylamine (DIEA), and coupling of carboxylic acid **11**{3} was achieved via preactivation with HBTU. TFA was added to the resin-bound compounds to cleave product chemset **12** from the solid support. The cleavage solution was collected, concentrated in a rotary evaporator, and dissolved in dimethylsulfoxide (DMSO) for analysis and purification. To ascertain the initial synthetic quality of the chemset **12** members, a small portion of the DMSO solution was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. The analytical data showed that the compounds in chemset **12** had an average initial purity of 70%, with 25% of the compounds being ≥80% pure (area percent of UV absor-

Scheme 3



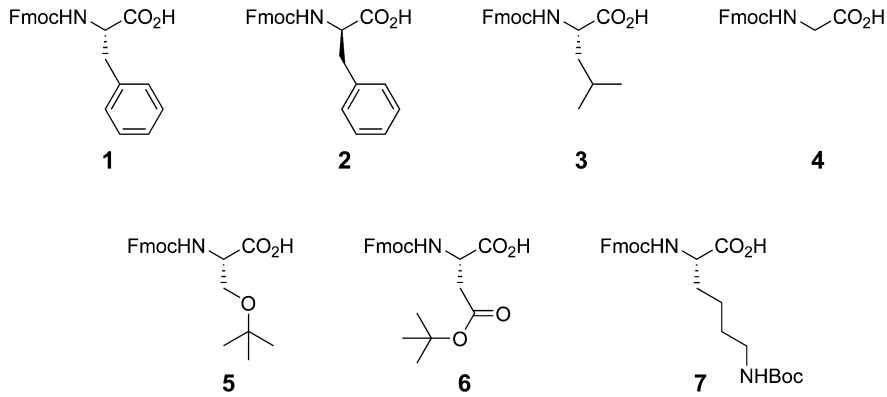


Figure 2. N^{α}-Fmoc amino acid diversity reagents **6**{1−7}.

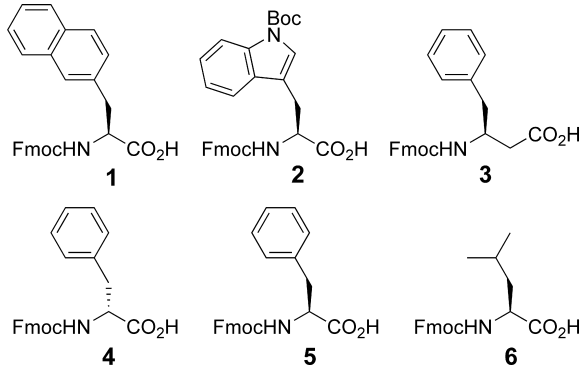


Figure 3. N^{α}-Fmoc amino acid diversity reagents **9**{1−6}.

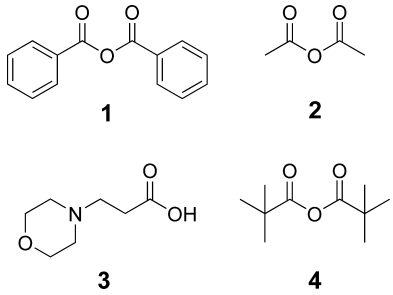


Figure 4. Diversity reagents **11**{1−4}.

bance at 214 nm of peak with molecular ion corresponding to target mass, see Figure 5).

Purification and Analysis. The 96 compounds generated by combining chemset **6**{1−4} (Figure 2), chemset **9**{1−6} (Figure 3), and chemset **11**{1−4} (Figure 4) to yield chemset **12** (nomenclature example: compound **12**[**6**{1},**9**{1},**11**{1}] represented as **12**{1,1,1}) were RP-HPLC purified and characterized before submission to the PHD assays. Figure 6 shows the HPLC trace before and after purification for compounds **12**{1,5,2}, **12**{1,5,1}, **12**{1,5,4}, and **12**{1,4,2}, and Figure 7 shows the purity profile of the 96-member library after purification. More than 90% of the compounds were 90% pure, but eight compounds had purities between 85% and 89% (see Supporting Information). One compound, **12**{1,3,1}, which had an initial purity of only 11% from prepurification analysis, had a final purity of 73% and was not included in the screen. The samples were submitted for screening in the PHD enzymatic assays. After the biochemical screening, the remaining sample material was characterized by ^{1}H NMR spectroscopic analysis (see Supporting Information).

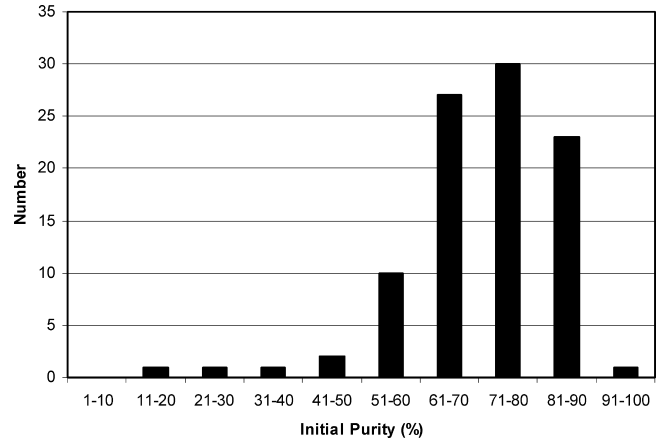


Figure 5. Histogram of initial compound purity from prepurification analytical LC-MS of chemset **12**.

Dipeptidyl-Quinolone Derivatives Have Altered PHD Selectivity Profiles. The compounds in chemset **12** were screened for inhibition of the PHD-mediated hydroxylation of Pro564 in the hHIF-1α(559−574) peptide.[13] All compounds showed complete inhibition of the three PHD isoforms at the highest concentration tested (see Supporting Information). The initial screening results (Figure 8) were analyzed for any potential structure/activity relationship (SAR) among the inhibitors. We found that chemset **12** compounds that had been capped with acetic anhydride (reagent **11**{2}) tended to be more active than compounds containing other chemset **11** reagents; products containing *N*-ethylmorpholine (reagent **11**{3}) showed the lowest activity. The incorporation of D-phenylalanine (reagent **6**{2}) into compounds in chemset **12** tended to produce more potent inhibitors, in good agreement with the result of **8**{2} being the most active compound in chemset **8**. No clear preferred member from reagent chemset **9** was discernible; however, compound **12**{4,1,2}, resulting from the sequential incorporation of glycine, L-2-naphthylalanine, and acetic anhydride, was identified as having a higher than expected potency based upon its constituents. Additionally, we found that compounds from chemset **12** tended to be selective toward the PHD1 and PHD3 isoforms (Figure 9 and Supporting Information), whereas the parent quinolone had not shown a bias toward any of the three isoforms (Figure 1). Two compounds with D-phenylalanine at the first diversity position and capped with a benzoyl group showed approximately 30-fold selectivity against PHD2 (see **12**{2,3,1}

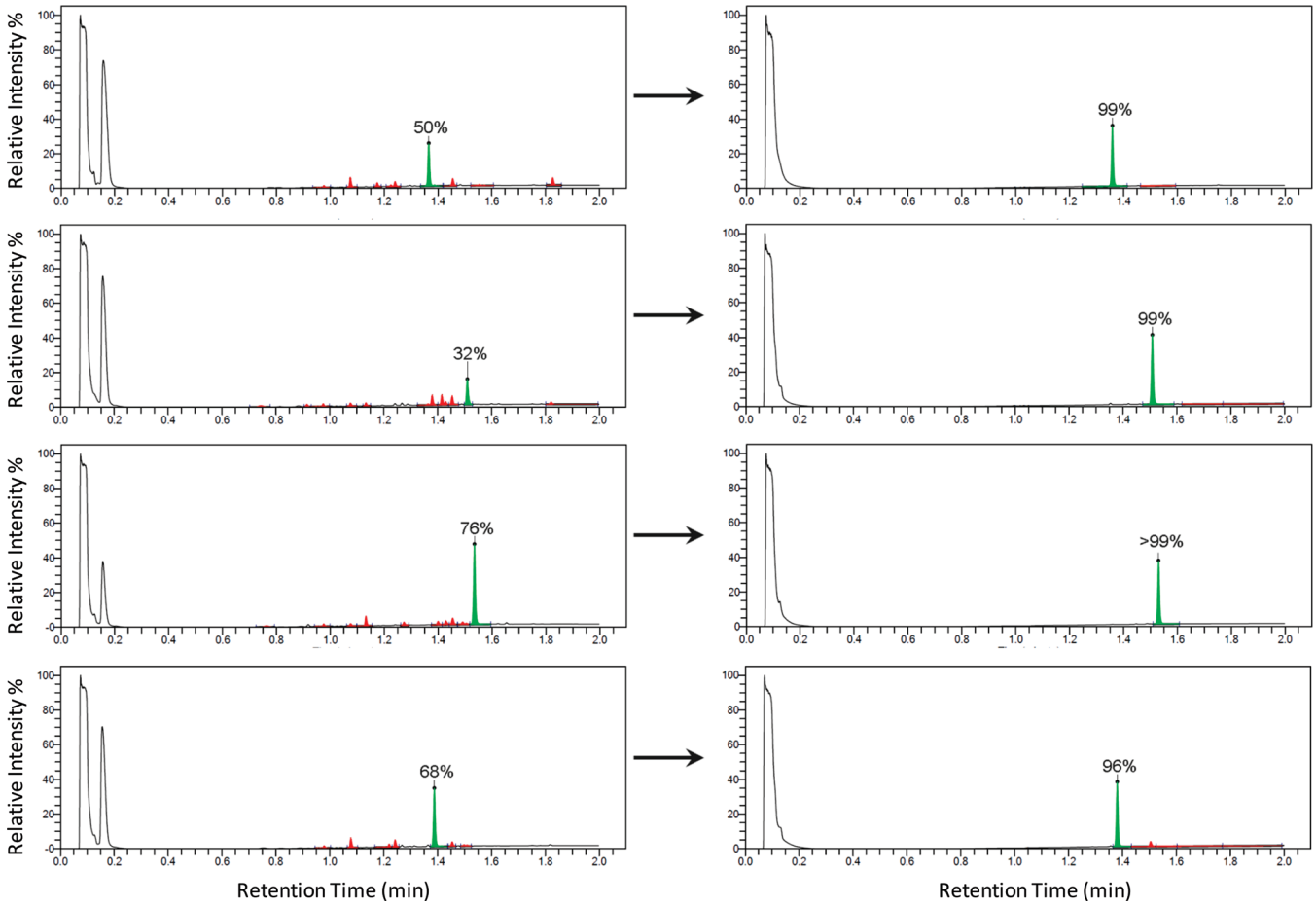


Figure 6. LC-MS chromatograms (UV absorbance at 214 nM) before (left) and after (right) purification of chemset compounds **12** (top to bottom) {1,5,2}, **12**{1,5,1}, **12**{1,5,4}, and **12**{1,4,2}. Peak with a molecular ion corresponding to the target mass indicated in green with area percent.

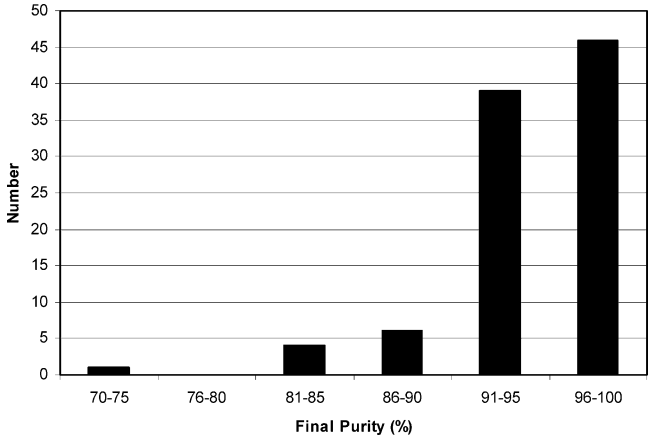


Figure 7. Final purity (area percent of UV absorbance at 214 nm of peak with molecular ion corresponding to target mass) of compounds in chemset **12** from post-purification analytical LC-MS. The compound with purity <80% was not submitted for assay.

and **12**{2,4,1} in Table 2). With further development, these PHD-selective inhibitors could be used to understand which isoform of the PHD enzyme is responsible for the different aspects of the hypoxia response in vivo.

To confirm the screening results and further validate the SAR, 12 compounds from the array (including the 3 most active "hits") were resynthesized, purified, and assayed (Table 3). We confirmed the activity of five potent PHD2 inhibitors with IC_{50} values <45 nM, including compounds **12**{2,4,2}, **12**{4,1,2}, and **12**{2,5,2}, which had been the most active "hits" from the initial screen. These same compounds were also low nanomolar inhibitors of PHD1 and PHD3 with 3- to 8-fold selectivity over PHD2. The most selective compound was **12**{2,1,2}, which was 7- and 16-fold selective for PHD1 and PHD3 over PHD2, respectively, and a significant improvement over the parent quinolone (Figure 1). The trends in potency and selectivity remained consistent for the products from the first and second syntheses; however, the activity values were somewhat shifted (more potent but less selective), possibly because of day-to-day assay variability and higher accuracy of compound quantification on the larger resynthesis scale. Modeling suggested that an aminoacyl- or dipeptidyl-sized group may be sufficient to present a hydrophobic side chain within close proximity of the hydrophobic pocket created by PHD2 residues Trp258 and Met299 that is normally occupied by HIF-1α (Figure 10).[14] Structural characterization of this additional interaction will be required to understand the altered selectivity and/or potency of this series of compounds as the residues surrounding the immediate opening of the PHD active site are completely conserved among the three

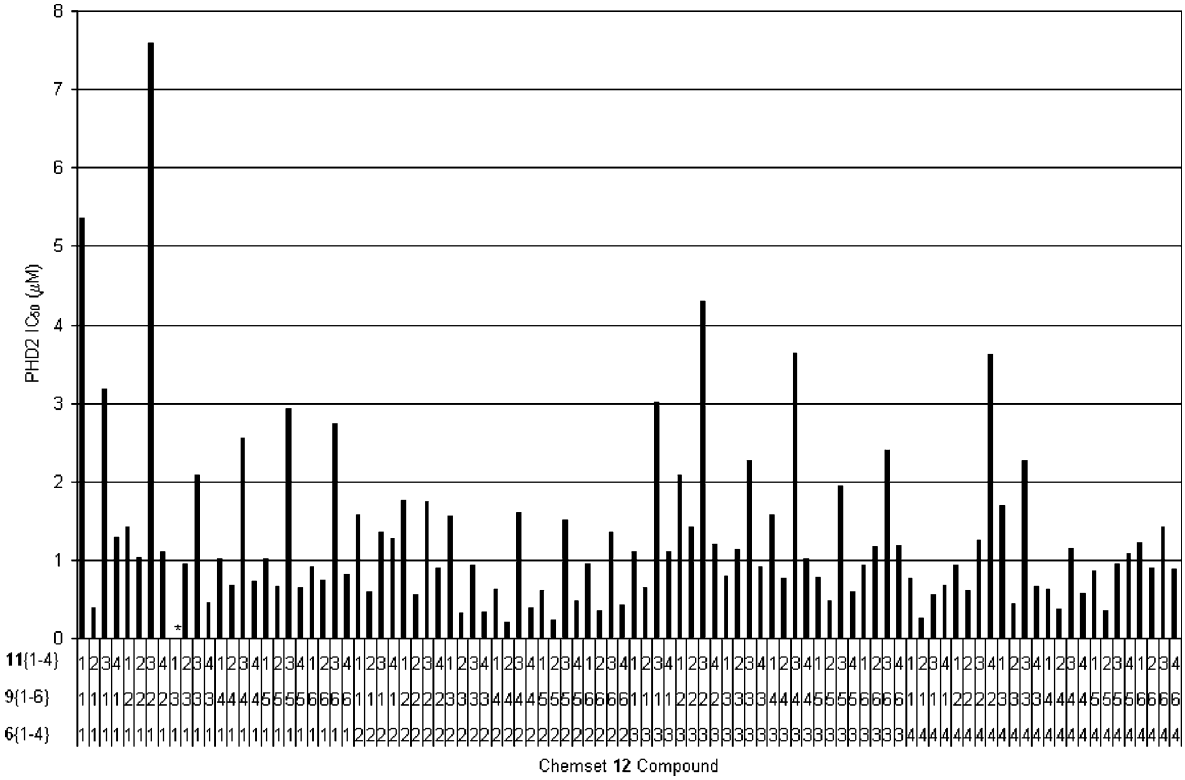


Figure 8. Screening of compounds in chemset **12** for inhibition of PHD2. * - Activity for compound **12**{1,3,1} was not determined because of its low purity.

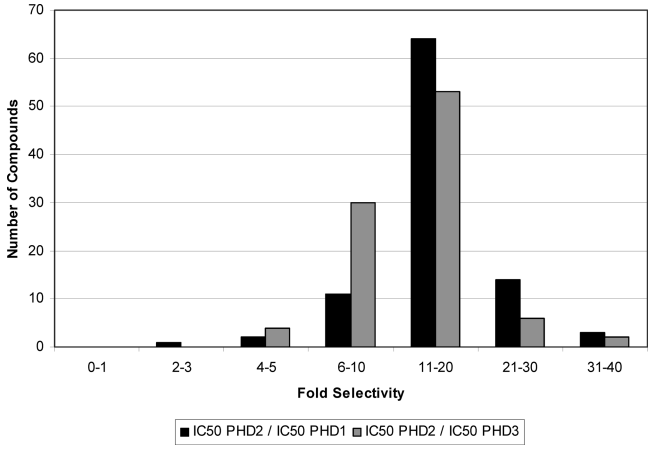


Figure 9. Selectivity of quinolone-dipeptides among PHD1, 2, and 3 from initial screening data.

isoforms (Asp254, Ile256, Trp258, Met299, Tyr310, His313, Asp315, His 374, Trp389).[6]

Conclusions

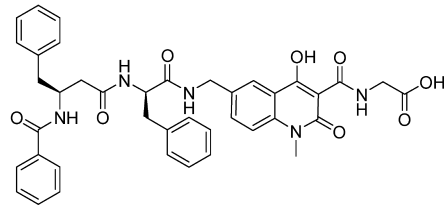
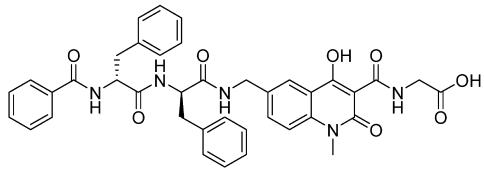
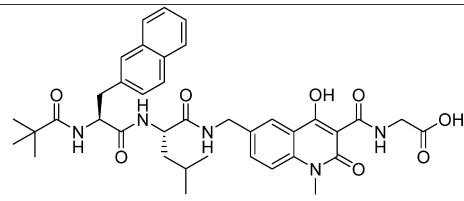
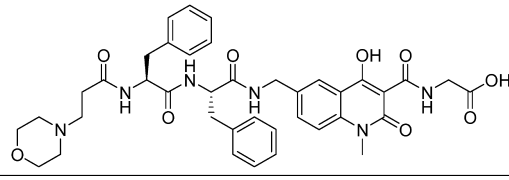
Modulating HIF transcriptional activity via HIF-1α stabilization by inhibiting PHD activity may have therapeutic application for anemia and ischemic diseases. We have investigated modification at the 6-position of the quinolone series of PHD inhibitors with a dipeptide group. After developing our synthetic approach with a small set of compounds, we prepared and screened a number of dipeptidyl-quinolone derivatives in the PHD1, 2, and 3 enzymatic assays, identifying several "hits." Resynthesis, purification, and testing of a subset of the original compounds confirmed that five of the compounds had PHD2 IC_{50} values <45 nM. Also, the dipeptidyl-quinolones showed increased potency (IC_{50} values <10 nM) and selectivity (~10-fold) toward PHD1 and PHD3, which could potentially be developed into useful compounds for the further understanding of HIF biology.

Experimental Methods

Preparation of Compound 5. In a 100 mL round-bottom flask were combined ethyl 4-hydroxy-6-iodo-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylate[8] (**1**, 4.135 g, 11.52 mmol), glycine *tert*-butyl ester hydrochloride (2.23 g, 13.3 mmol), triethylamine (3.08 mL, 22.14 mmol), and 1,4-dioxane (22 mL). The reaction mixture was refluxed for 24 h. The solvent was removed by rotary evaporation. The residue was dissolved in dichloromethane (50 mL) and was washed with water (2 × 100 mL) and brine (1 × 100 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. The product was purified by flash chromatography (10−50% ethyl acetate in hexanes over 20 min). The combined fractions were concentrated under vacuum to give **2** (3.7 g, 70% yield). LC-MS: 20−100% B (water and acetonitrile with 0.1% TFA in each as solvents A and B, respectively) over 2 min at 2.0 mL/min on a Phenomenex SB-C18, 4.6 × 50 mm, 1.8u column at 45 °C, rt = 1.79 min, m/z = 403.2 (-*t*Bu $C_{13}H_{11}IN_2O_5$ MW = 402.14). ^{1}H NMR (400 MHz, chloroform-*d*) δ ppm 10.67 (br. s., 1 H) 8.48 (d, J = 2.15 Hz, 1 H) 7.91 (dd, J = 8.90, 2.05 Hz, 1 H) 7.10 (d, J = 9.00 Hz, 1 H) 4.09−4.15 (m, 2 H) 3.65 (s, 3 H) 1.51 (s, 9 H).

To a 160 cc Parr bomb with a stir bar were added *tert*-butyl 2-(4-hydroxy-6-iodo-methyl-2-oxo-1,2-dihydroquinoline-3-carboxamido)acetate (**2**, 3.07 g, 6.70 mmol), CataXium

Table 2. Quinolone-Dipeptides with Greater than 30-Fold Selectivity against PHD2

Compound	Structure	PHD2 IC_{50} (μM)	PHD1 IC_{50} (μM)	PHD3 IC_{50} (μM)	IC_{50} PHD2/ IC_{50} PHD1	IC_{50} PHD2/ IC_{50} PHD3
12{2,3,1}		1.561	0.049	0.040	31.6	39.1
12{2,4,1}		0.622	0.022	0.020	28.2	30.5
12{3,1,4}		1.096	0.032	0.077	33.9	14.3
12{1,5,3}		2.924	0.095	0.165	30.9	17.7

A (0.137 g, 0.382 mmol), $Pd(OAc)_2$ (0.0292 g, 0.13 mmol), tetramethylethylenediamine (0.74 mL, 4.91 mmol), and toluene (50 mL). The reactor was assembled and purged with Ar (5 × 40 psig) and CO (3 × 20 psig). The reactor was pressurized with CO to 20 psig and sealed. The manifold was purged with syngas (1:1 CO/H_2). The reactor was charged with syngas to a total pressure of 60 psig to give a final CO/H_2 ratio of 2:1. The vessel was heated to 100 °C for 48 h. The reactor was cooled, vented, and purged with Ar. The reactor contents were washed into a round-bottom flask with ethyl acetate and concentrated under vacuum. The product was purified by flash chromatography (10−50% ethyl acetate in hexanes over 20 min) to give **3** (0.9 g, 37% yield). LC-MS: 20−100% B over 2 min at 2.0 mL/min on a Phenomenex SB-C18, 4.6 × 50 mm, 1.8u column at 45 °C, rt = 1.47 min, m/z = 305.2 (-*t*Bu $C_{14}H_{12}N_2O_6$ MW = 304.25). ^{1}H NMR (400 MHz, chloroform-*d*) δ ppm 10.07 (s, 1 H) 10.56 (br. s., 1 H) 8.69 (d, J = 1.51 Hz, 1 H) 8.21 (dd, J = 8.78, 1.76 Hz, 1 H) 7.49 (d, J = 8.53 Hz, 1 H) 4.14 (d, J = 5.52 Hz, 2 H) 3.74 (s, 3 H) 1.52 (s, 9 H).

To polystyrene Rink amide resin (3.33 g, 0.6 mmol/g, Novabiochem) and *tert*-butyl 2-(6-formyl-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxamido)acetate (**3**, 0.801 g, 2 mmol) in *N*-methylpyrrolidinone (50 mL) was added trimethyl orthoformate (0.2 mL, 2 mmol) and glacial acetic acid (0.2 mL). The reaction mixture was stirred at room temperature for 1 h followed by the addition of sodium cyanoborohydride (0.4 g, 7 mmol). The reaction mixture was stirred at room temperature overnight, filtered, washed with dichloromethane, methanol, and dichloromethane to give **4**. A small amount of resin was treated with triisopropylsilane (TIS, 0.05 mL), water (0.05 mL), dichloromethane (0.45 mL), and trifluoroacetic acid (TFA, 0.45 mL). The mixture was shaken for 1 h. The solution was drained and concentrated under vacuum to afford (2-(6-(aminomethyl)-4-hydroxy-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxamido)acetic acid. LC-MS: 10−100% B water over 3 min at 0.8 mL/min on a Phenomenex Synergi MAX-RP, 2 × 50 mm, 4u column at 40 °C, rt = 1.48 min, m/z = 306.0 ($C_{14}H_{15}N_3O_5$ MW = 305.29) ^{1}H NMR (400 MHz, chloroform-*d* + methanol-d_3) δ ppm 7.98 (s, 1 H) 7.80 (dd, J = 8.78, 1.76 Hz, 1 H) 7.55 (br. d, J = 8.80 Hz, 1 H) 4.22 (s, 2 H) 4.20 (s, 2 H) 3.72 (s, 3 H) 1.05 (s, 9 H).

To the resin (**4**, 42 mg, 0.025 mmol) was added a solution of acetic anhydride and triethylamine in dichloromethane (5:1:14, 2 mL). The mixture was shaken for 15 min. The solution was drained, and the resin was washed (5 × DCM). To the resin was added triisopropylsilane (0.2 mL), water (0.2 mL), dichloromethane (1.0 mL), and trifluoroacetic acid (TFA, 1.0 mL). The mixture was shaken for 1 h. The solution was drained and concentrated under vacuum. The residue was dissolved in DMSO (1 mL) and purified by RP-HPLC 5−50%B over 20 min on a Phenomenex Jupiter 4u Proteo 90A, 100 × 7.80 mm column, 3.0 mL/min. The collected fractions were pooled and concentrated under vacuum to afford **5** (3.0 mg, 35% yield). LC-MS analysis: 20−100% B over 3 min at 2.0 mL/min Phenomenex SB-C18, 4.6 × 50 mm, 1.8u, 45 °C rt = 2.28 min. m/z = 348.1 ($C_{16}H_{17}N_3O_6$

Table 3. PHD2 Inhibition by Selected Quinolone-Dipeptide Conjugates

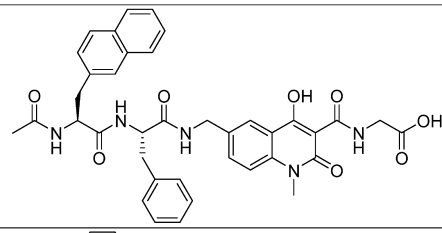
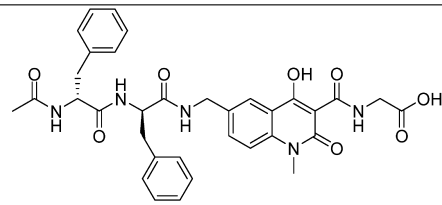
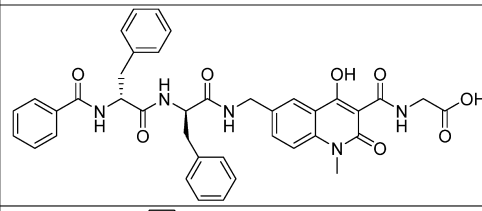
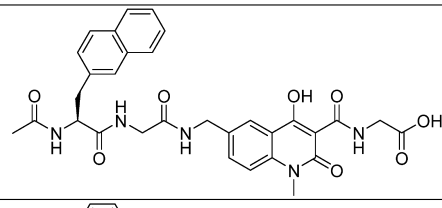
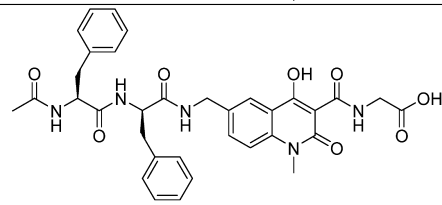
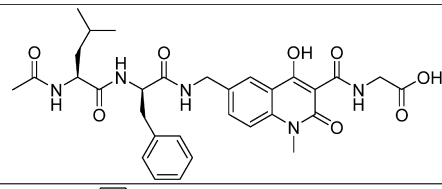
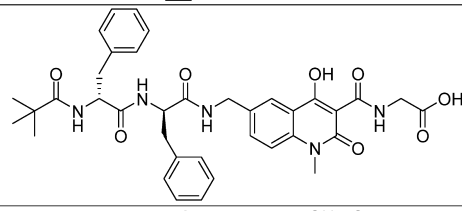
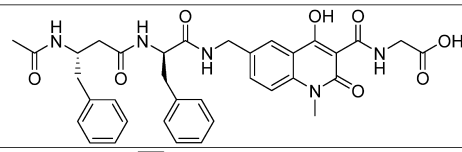
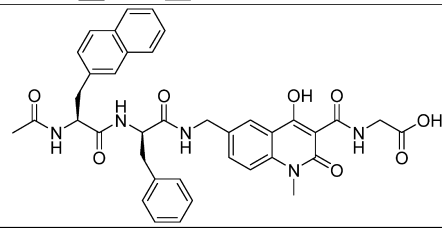
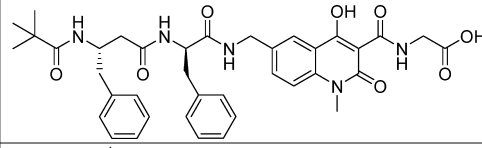
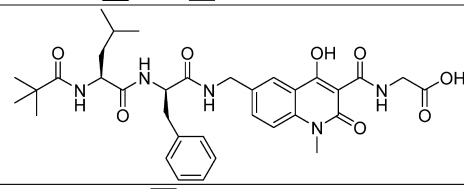
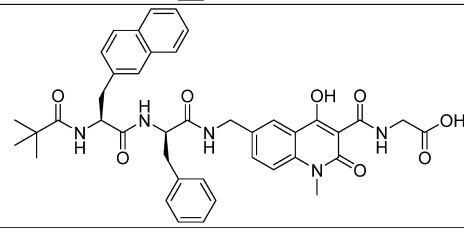
Compound	Structure	PHD2 IC_{50} (nM)	PHD1 IC_{50} (nM)	PHD3 IC_{50} (nM)
12{1,1,2}		35.0	4.3	4.5
12{2,4,2}		37.4	7.2	11.7
12{2,4,1}		38.3	8.5	5.3
12{4,1,2}		41.9	5.1	13.5
12{2,5,2}		44.8	7.6	8.9
12{2,6,2}		67.5	11.0	16.2
12{2,4,4}		86.4	12.9	12.6
12{2,3,2}		90.2	12.2	13.5
12{2,1,2}		93.8	13.5	6.4

Table 3. Continued

Compound	Structure	PHD2 IC_{50} (nM)	PHD1 IC_{50} (nM)	PHD3 IC_{50} (nM)
12{2,3,4}		103.6	14.6	13.2
12{2,6,4}		112.6	14.5	16.8
12{2,1,4}		307.5	60.8	18.8

MW = 347.32). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.58 (t, J = 5.52 Hz, 1 H) 7.97 (s, 1 H) 8.45 (t, J = 5.77 Hz, 1 H) 7.70 (dd, J = 8.53, 1.51 Hz, 1 H) 7.60 (d, J = 8.50 Hz, 1 H) 4.35 (d, J = 6.02 Hz, 1 H) 4.14 (d, J = 5.52 Hz, 1 H) 3.63 (s, 3 H)1.88 (s, 3 H).

Preparation of Chemset 8. Fmoc-amino acid (**6**{1−7}, 0.25 mmol, Novabiochem) was dissolved in dichloromethane (10 mL) in a round-bottom flask. To the solution was added diisopropylcarbodiimide (DIC, 0.125 mmol, 0.02 mL). The mixture was stirred for 20 min, during which time a white precipitate formed. The solvent was removed under vacuum to afford the symmetrical anhydride as a waxy solid. The solid was dissolved in DMF (2 mL) and added to the resin (**4**, 42 mg, 0.025 mmol). The mixture was shaken for 24 h. The solution was drained, and the resin was washed (3 × DCM). The acylation was repeated twice more to achieve

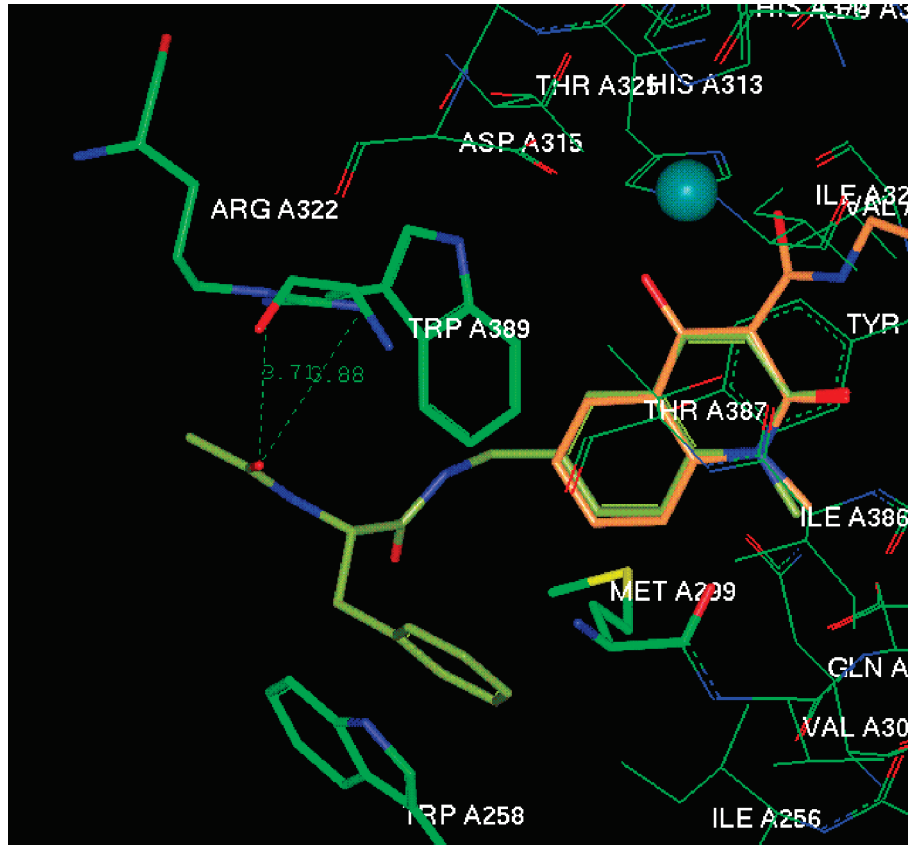


Figure 10. Predicted binding of quinolone and acetylaminoacyl-quinolone **8**{1} to PHD2.

90% conversion to resin **7**. Fmoc removal was accomplished by addition of a solution of 20% piperidine in DMF (2 mL) to the resin. The mixture was shaken for 5 min, the solution was drained, and the process was repeated. After draining, the resin was washed (5 × DMF, 5 × DCM). Acetylation, cleavage from the solid support, and LC-MS purification were accomplished as described for compound **5** to afford **8**{1−7}. LC-MS: 20−100% B over 2 min at 2.0 mL/min on a Phenomenex SB-C18, 4.6 × 50 mm, 1.8u column at 45 °C **8**{1}: 1.0 mg, rt = 1.30 min, *m/z* = 495.2 ($C_{25}H_{26}N_4O_7$ MW = 494.50), ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.58 (t, *J* = 5.58 Hz, 1 H) 8.41 (t, *J* = 5.77 Hz, 1 H) 8.33 (t, *J* = 6.06 Hz, 1 H) 8.23 (d, *J* = 7.83 Hz, 1 H) 7.99 (d, *J* = 1.96 Hz, 1 H) 7.83−7.87 (m, 1 H) 7.80 (d, *J* = 8.02 Hz, 2 H) 7.73 (s, 1 H) 7.59 (d, *J* = 8.80 Hz, 1 H) 7.38−7.51 (m, 3 H) 7.70 (dd, *J* = 8.80, 1.96 Hz, 1 H) 4.58 (ddd, *J* = 9.29, 8.02, 4.79 Hz, 1 H) 4.37 (d, *J* = 5.87 Hz, 2 H) 4.14 (d, *J* = 5.48 Hz, 2 H) 3.79 (dd, *J* = 16.60, 6.10 Hz, 1 H) 3.70 (dd, *J* = 16.60, 5.90 Hz, 1 H) 3.63 (s, 3 H) 3.19 (dd, *J* = 13.89, 4.89 Hz, 1 H) 2.93 (dd, *J* = 13.79, 9.68 Hz, 1 H) 1.74 (s, 3 H); **8**{2}: rt = 1.30 min, 5.6 mg, *m/z* = 495.2 ($C_{25}H_{26}N_4O_7$ MW = 494.50); **8**{3}: 2.0 mg, rt = 1.27 min, *m/z* = 461.2 ($C_{22}H_{28}N_4O_7$ MW = 460.48); **8**{4}: 1.6 mg, rt = 1.03 min, *m/z* = 405.2 ($C_{18}H_{20}N_4O_7$ MW = 404.38); **8**{5}: 1.8 mg, rt = 0.99 min, *m/z* = 435.2 ($C_{19}H_{22}N_4O_8$ MW = 434.40); **8**{6}: 2.3 mg, rt = 1.02 min, *m/z* = 463.2 ($C_{20}H_{22}N_4O_9$ MW = 462.41); **8**{7}: 2.1 mg, rt = 0.98 min, *m/z* = 476.2 ($C_{22}H_{29}N_5O_7$ MW = 475.50).

Preparation of Chemset 12. Resins **7**{1−4} were prepared on large scale from resin **4** (0.5 mmol, 830 mg each) by repeated treatment with the preformed symmetrical anhydrides of Fmoc-amino acids (**6**{1−4}, 4 × 5 mmol each). Resins **7**{1−4} were distributed into each well of a 96-well filter plate (1.0 mL of a 3:2 DCM/DMF resin slurry was delivered to each well using a pipet, 24 wells per derivative, 0.02 mmol per well). The resin was washed (8 × DMF). To each well was added 120 μL of 20% piperidine in DMF and allowed to stand for 5 min. The solution was drained, and the process was repeated. After draining, the resin was washed (8 × DMF). In separate vials, Fmoc amino acids **9**{1−6} (0.2 mL of a 0.5 M solution in DMF, 0.1 mmol per well) were activated with *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU, 0.2 mL of a 0.5 M solution in DMF) and *N*-methylmorpholine (NMM, 0.1 mL of 1.0 M solution in DMF), added to the wells, and allowed to stand for 20 min. The solution was drained, and this process was repeated. After draining, the resin was washed (8 × DMF). To each well was added 120 μL of 20% piperidine in DMF and allowed to stand for 5 min. The solution was drained, and the process was repeated. After draining, the resin was washed (8 × DMF, 8 × DCM). To the resin was added 200 μL of acylation cocktail (1.4:0.1:0.5 DCM/TEA/acetic, pivalic, benzoic anhydride) or preactivated 3-morpholinopropanoic acid (**11**{1−4}) and allowed to stand for 15 min. The solution was drained, and the process was repeated. The resin was washed (5 × DCM). To the resin was added 1 mL of cleavage cocktail (90:5:5 TFA/TIS/water). The cleavage solution was drained into a solid-bottom 96-well plate and concentrated under vacuum. The residue in each well was dissolved in DMSO (0.5 mL).

A portion (10 μL) of the DMSO solution of the crude peptide product mixtures was transferred to a shallow-well 96-well plate and diluted with MeOH (30 μL). The LC-MS analysis showed that the crude compounds were initially 70% pure on average. The remainder of the DMSO stock solution was purified by preparative mass-triggered RP-HPLC (250 μL injection on a Synergi 4u MAX-RP 80A, 150 × 21.20 mm column, 5−95%B over 10 min, 20 mL/min). The collected fractions were concentrated under vacuum in tared vials and subjected to final characterization by analytical LC-MS and ^{1}H NMR to provide Chemset **12** (see Supporting Information). Compounds were resynthesized from resins **7**{1−4} on a 0.05 mmol scale and purified to >95% purity by LC-MS.

PHD Enzymatic Assay. PHD1, 2, and 3 activity was measured utilizing homogeneous time-resolved fluorescence energy transfer technology by detecting the *trans*-4-hydroxylation of HIF-1α residue Pro564 in Biotin-hHIF-1α(558−574) (Biotin-DLEMLAPYIPMDDDFQL) peptide substrate resulting in recognition by the Europium-tagged Von Hippel-Lindau, Elongin B, and Elongin C heterotrimeric complex (VCB-Eu complex). Compound inhibitor potency was determined using 1 nM PHD1, 2, or 3, 100 nM Biotin-hHIF-1α(558−574), 0.25 μM 2-OG, 100 μM $FeCl_2$, and 2 mM ascorbic acid in reaction buffer (30 mM MES, pH 6, 10 mM NaCl, 10 mM $CaCl_2$, 0.25% Brij-35). The reaction was terminated after 1 h with 50 mM succinic acid in detection buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween 20, 0.5% NaN_3) containing a final concentration of 25 nM streptavidin-APC and 2.5 nM VCB-Eu. The POC (percentage of control) was determined by comparing the signal from hydroxylated peptide substrate, Biotin-[Hyp564]hHIF-1α(558−574), in the enzyme reaction containing inhibitor compound with that from PHD2 enzyme with DMSO vehicle alone, and no enzyme. The data were fit to the 4-parameter model using a Levenberg−Marquardt non-linear regression algorithm.[8]

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Supporting Information Available. ^{1}H NMR spectra, LC-MS chromatograms before and after purification, and PHD screening data for Chemset **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Molecular modeling studies were performed as described in ref 7f.

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