JOURNAL OF NATURAL PRODUCTS

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Total Synthesis of Hispidulin and the Structural Basis for Its Inhibition of Proto-oncogene Kinase Pim-1

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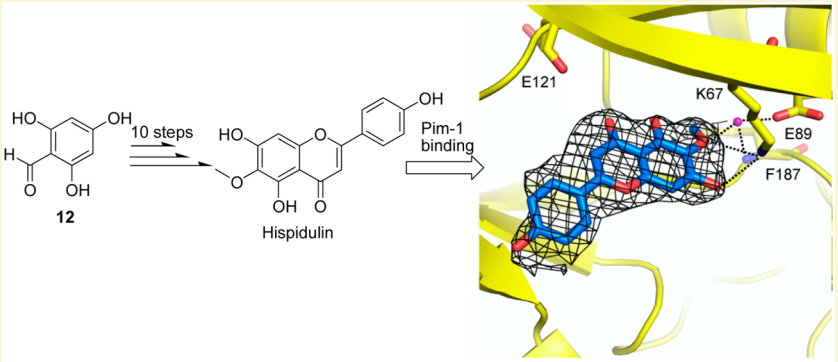
Supporting Information



ABSTRACT: A new method is applied to synthesize hispidulin, a natural flavone with a broad spectrum of biological activities. Hispidulin exhibits inhibitory activity against the oncogenic protein kinase Pim-1. Crystallographic analysis of Pim-1 bound to hispidulin reveals a binding mode distinct from that of quercetin, suggesting that the binding potency of flavonoids is determined by their hydrogen-bonding interactions with the hinge region of the kinase. Overall, this work may facilitate construction of a library of hispidulin-derived compounds for investigating the structure−activity relationship of flavone-based Pim-1 inhibitors.

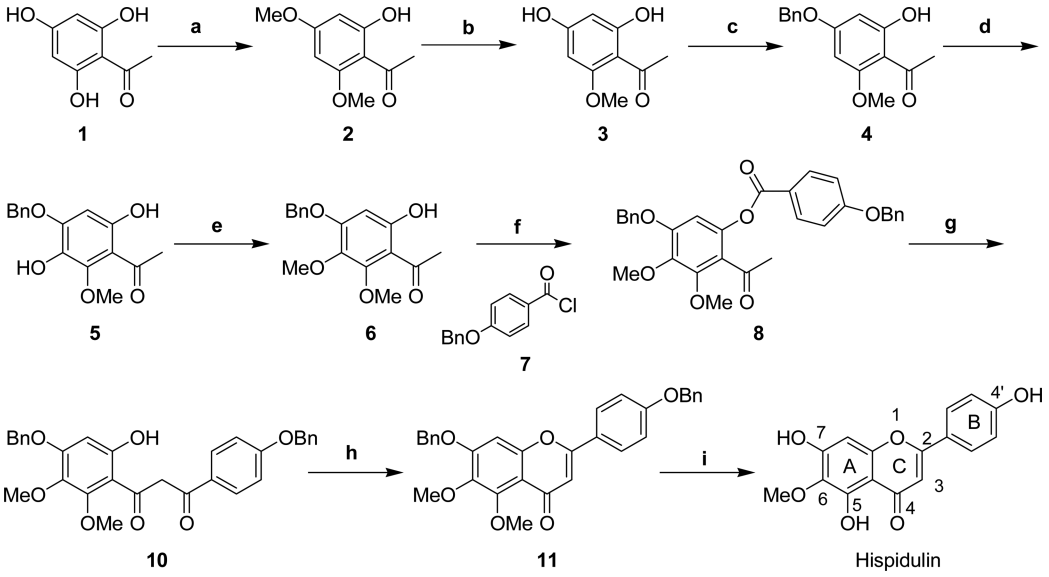
Hispidulin, a naturally occurring flavone, is widely distributed in plants of the Asteraceae.[1−5] It reportedly has antifungal,[6] antiplatelet,[7] anti-inflammatory,[8] antiosteoporotic,[9,10] anticonvulsant,[11,12] and, in particular, anticancer activity.[1,13−17] Studies indicated that hispidulin induces growth inhibition and apoptosis in various human cancer cell lines by stimulating AMP-activated protein kinase (AMPK) to inhibit the mammalian target of rapamycin (mTOR).[16] Hispidulin also triggers the vascular endothelial growth factor (VEGF)-related PI3K/Akt/mTOR signaling pathway and suppresses angiogenesis and cell growth in pancreatic cancer cells as well as in an in vivo model.[14] Additionally, hispidulin permeates the blood−brain barrier (BBB) and acts at a benzodiazepine-binding site of the human γ-aminobutyric acid $(GABA)_A$ receptor, thus enhancing the GABA-binding affinity in a positive allosteric manner.[12] Recently, we used a bioassay-guided approach to identify hispidulin as the active compound in *Clerodendrum inerme* that can alleviate methamphetamine-induced hyperlocomotion in mice, an animal model mimicking tic disorders.[18] Despite its various pharmacological properties, few studies have attempted to synthesize hispidulin. Previously, Kavvadias and co-workers developed a nine-step synthesis toward hispidulin (Scheme 1).[12] In our hands, the synthesis of compounds **5** and **6** failed (Scheme 1).[12] Additionally, this approach indicated that the yields of the debenzylation (C-7, C-4′) and selective demethylation (C-5) of compound **11** using BCl_3 were poor.

Shen and co-workers also reported a seven-step semisynthesis of hispidulin (Scheme 2).[19] This approach starts with the flavonoid glucuronide scutellarin isolated from Chinese herbs.[20−22] Despite a satisfactory overall yield, the ^{1}H NMR data for the synthesized compound using this method were not consistent with the reported data for hispidulin,[22,23] suggesting an incorrect chemical structure for the synthesized compound. Furthermore, commercial scutellarin is available only in

Received: April 12, 2015

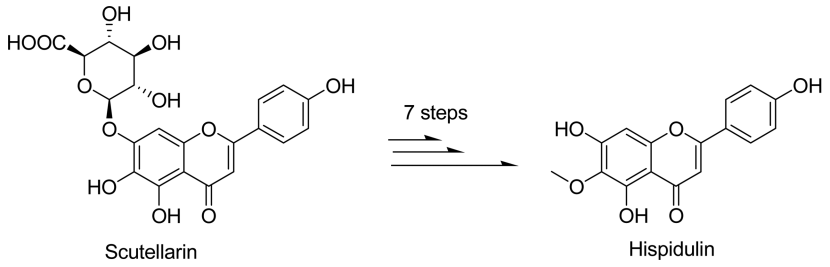
DOI: 10.1021/acs.jnatprod.5b00324
J. Nat. Prod. XXXX, XXX, XXX−XXX

Scheme 1. Synthesis of Hispidulin by Kavvadias and Co-workers[a]



[a]Reagents and conditions: (a) Me_2SO_4, K_2CO_3, acetone, Δ; (b) $AlCl_3$, chlorobenzene, Δ; (c) BnBr, K_2CO_3, acetone, Δ; (d) (1) $K_2S_2O_8$ pyridine, 10% $NaOH_{(aq)}$, RT; (2) 37% $HCl_{(aq)}$, Δ; (e) Me_2SO_4, K_2CO_3, acetone, Δ; (f) pyridine, RT; (g) KOH, pyridine, 60 °C; (h) HOAc, H_2SO_4, 60 °C; (i) BCl_3, CH_2Cl_2, −65 °C.

Scheme 2. Synthetic Route for Hispidulin by Shen and Co-workers



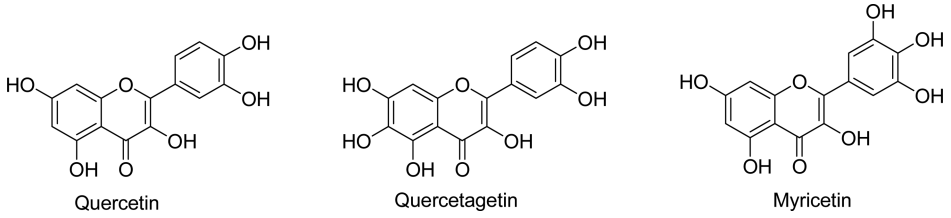


Figure 1. Examples of flavonols as Pim-1 kinase inhibitors.

milligram quantities, which hampers large-scale production of hispidulin. These issues motivated us to develop a feasible synthesis route toward hispidulin.

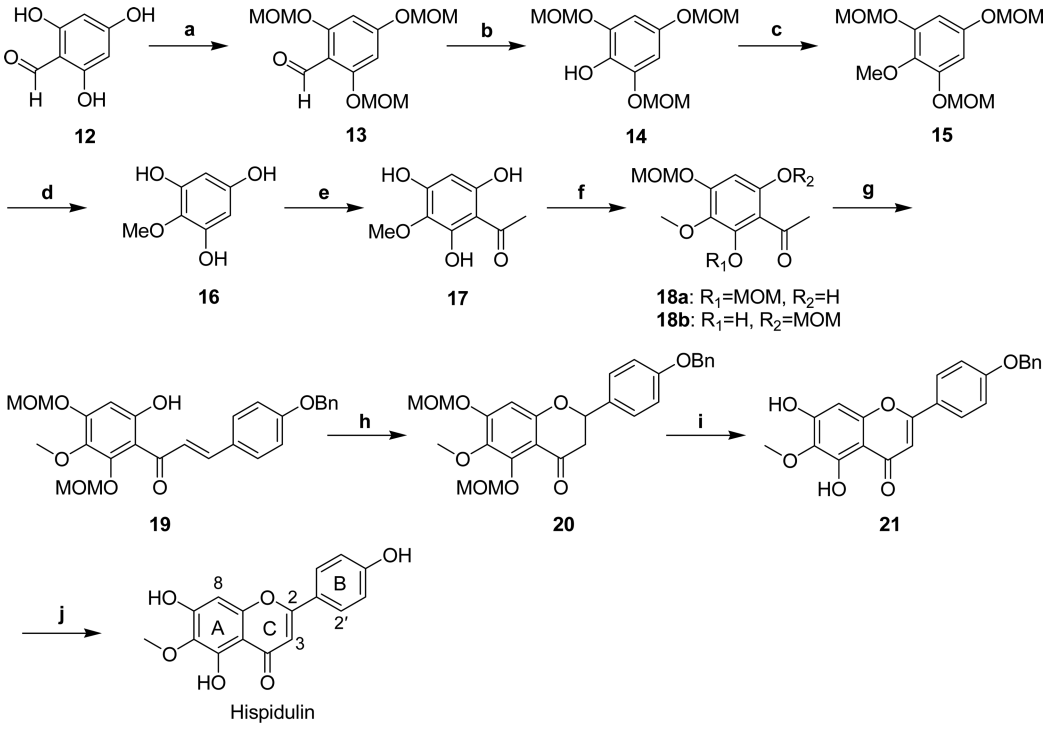
The A-, B-, and C-rings of hispidulin adopt a planar conformation; such a structure is compatible with one of the known chemical features of ATP-competitive kinase inhibitors. Indeed, several flavonols such as quercetin, quercetagetin, and myricetin have been identified to inhibit proto-oncogene kinase Pim-1 (Figure 1).[23] Among those flavonols, quercetagetin displayed a high degree of selectivity for Pim-1 over a panel of kinases. However, analysis of the crystal structure of Pim-1 in complex with these flavonols revealed two different binding poses,[23] which have made prediction of the binding mode of hispidulin to Pim-1 difficult. In this study, a new feasible method was developed for synthesizing hispidulin. The structure of the synthesized product was determined using 2D NMR spectroscopic techniques. An assay of enzyme inhibitory activity against the Pim-1 kinase shows an IC_{50} value of 2.71 μM. The specific binding mode of hispidulin was determined using a cocrystal structure analysis of hispidulin complexed to Pim-1. The results from this study would be helpful for developing Pim-1-specific inhibitors based on a flavonoid scaffold.

RESULTS AND DISCUSSION

Synthesis of Hispidulin. 2,4,6-Trihydroxybenzaldehyde (**12**) was chosen as the starting material with a view to transform the formyl group into a hydroxy group (Scheme 3). Treatment of 2,4,6-trihydroxybenzaldehyde (**12**) with chlorodimethyl ether (MOMCl) gave the tris(methoxymethoxy)-protected compound **13**.[24] Baeyer−Villiger oxidation followed by basic hydrolysis[25] yielded phenol **14**. Methylation of

DOI: 10.1021/acs.jnatprod.5b00324
J. Nat. Prod. XXXX, XXX, XXX−XXX

Scheme 3. Synthesis of Hispidulin[a]



[a]Reagents and conditions: (a) MOMCl, DIPEA, CH_2Cl_2, 0 °C, 82%; (b) (1) MCPBA, dry CH_2Cl_2; (2) K_2CO_3, MeOH, 65%; (c) MeI, K_2CO_3, acetone, Δ, 93%; (d) HCl, MeOH, RT, 92%; (e) BF_3−Et_2O, Ac_2O, RT, 32%; (f) MOMCl, K_2CO_3, acetone, Δ, **18a**: 30%, **18b**: 29%; (g) **18a**, 4-BnOPhCHO, KOH, EtOH, H_2O, 0 °C, 86%; (h) NaOAc, EtOH, Δ, 83%; (i) I_2, pyridine, 90 °C, 65%; (j) BCl_3, CH_2Cl_2, 0 °C, 80%.

compound **14** with MeI afforded methyl ether **15** in a quantitative yield. Demethoxymethylation of compound **15** with HCl gave triphenol **16**.[26] Friedel−Crafts acetylation of compound **16** using Ac_2O and BF_3−Et_2O provided compound **17**.[27] At room temperature, the reaction of compound **17** with MOMCl preferentially afforded 4,6-bis(methoxymethoxy)-protected **18b** over the desired 2,4-bis(methoxymethoxy)-protected **18a** due to steric hindrance. The two compounds **18a** and **18b** could be distinguished based on NOESY correlations of H-5 and C3-OMe (Figure 2) and their

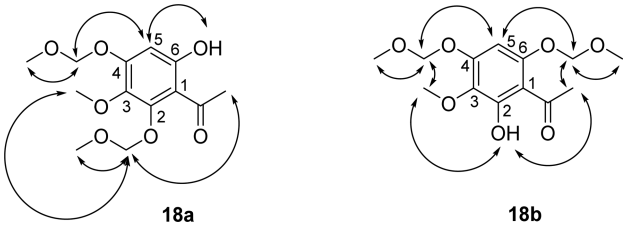


Figure 2. NOESY correlations of compounds **18a** and **18b**.

HMBC data (Supporting Information). The yield of compound **18a**, however, was improved by increasing the reaction temperature and reducing the quantity of MOMCl to 2.5 equiv of compound **17**. Aldol condensation of compound **18a** with 4-benzyloxybenzaldehyde yielded chalcone **19**. Oxidative cyclization[28] of compound **19** using catalytic I_2 did not provide flavone **21**, but afforded product **18a** presumably via a retro-Aldol-type reaction. Base-catalyzed cyclization of compound **19** with NaOAc[29] gave flavanone **20**. Compound **20** was converted to flavone **21** through oxidative dehydrogenation with I_2 and simultaneous methoxymethoxy deprotection via the release of HI. Debenzylation of compound **21** with BCl_3 yielded hispidulin. The ^1H and ^{13}C NMR spectra of hispidulin resembled the reported data.[30,31] Its structure was also confirmed by 2D NMR techniques such as NOESY, HSQC, and HMBC (Supporting Information). The purity of all synthesized compounds was estimated to be at least 97% as determined by HPLC analyses (Supporting Information).

Structure of Hispidulin Bound to Pim-1. The enzyme inhibitory activity of hispidulin against Pim-1 was assessed using quercetin as the reference (Figure 3). Hispidulin

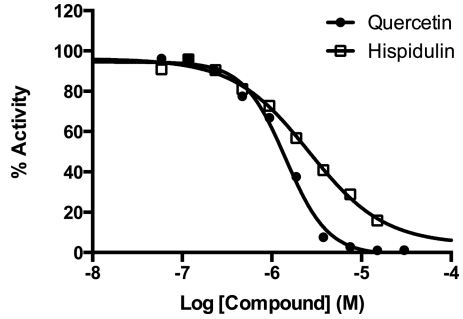


Figure 3. Dose−response curve of hispidulin and quercetin for the Pim-1 kinase.

exhibited activity with an IC_{50} value of 2.71 μM. There are two reported binding poses of a flavonoid to Pim-1. In order to establish the correct binding pose, the crystal structure of Pim-1 in complex with hispidulin at a 2.04 Å resolution (Table 1) was generated. In the hispidulin-bound Pim-1 structure, the binding orientation of hispidulin resembles that of myricetin and 5,7,3′,4′,5′-pentahydroxyflavone in Pim-1 (PDB code: 2O63

DOI: 10.1021/acs.jnatprod.5b00324
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. Data Collection and Structural Refinement Statistics

	Pim-1 bound to hispidulin
Data Collection	
space group	$P6_5$
cell dimens	
a, *b*, *c* (Å)	98.00, 98.00, 80.60
α, *β*, *γ* (deg)	90.00, 90.00, 120.00
wavelength (Å)	0.9
resolution (Å)	50−2.04 (2.11−2.04)
total observns	160 436
unique reflns	28 099
multiplicity	5.7 (5.7)
$I/\sigma I$	25.07 (2.13)
R_{merge}	0.068 (0.819)
completeness (%)	100 (100)
Refinement	
resolution (Å)	50−2.04 (2.11−2.04)
no. reflns	26 911
R_{work}/R_{free}	0.200/0.226
no. of atoms	
protein	2224
compound	22
water	65
Wilson *B* factor (Å^2)	25.1
average *B* factor (Å^2)	
protein	31.53
compound	40.58
water	29.61
rms deviations	
bond lengths (Å)	0.009
bond angles (deg)	1.33
Ramachandran statistics (%)	
most favored	93.7
additionally allowed	6.3
generously allowed	0
disallowed	0
PDB code	4XH6

and 2O65) with the A-ring bound deeply inside the ATP-binding cleft, where the methoxy group makes a van der Waals contact with a hydrophobic pocket guarded by the gate keeper residue Leu120 of Pim-1 (Figure 4A). The activation loop for hispidulin-bound Pim-1 adopts an active "DFG-in" conformation. Both the 7-OH group and the oxygen of the 6-OMe group form hydrogen bonds with the side chain *ε*-amino group of the absolutely conserved residue Lys67. Additionally, the oxygen of the 6-OMe group indirectly interacts with the backbone of Phe187 and the side chain of Glu89 through a water molecule. Notably, no hydrogen-bonding interaction to the hinge backbone of the kinase is found, due to the absence of a C-ring hydroxy group.

The binding mode of hispidulin is significantly different from that of quercetin or quercetagetin.[23] The latter compounds have been shown to bind deeply to the ATP-binding cleft of Pim-1 via the B- rather than the A-ring. Comparing the contrasting binding poses between hispidulin and the two flavonols suggests that the hydrogen-bonding potential to the conserved Lys67 determines whether the A- or the B-ring of the compound binds to the interior portion of the ATP cleft. In hispidulin, the A-ring has more hydrogen donors than the B-ring; on the contrary, the B-ring of quercetin and quercetagetin would make stronger hydrogen bonding than their A-rings (Figure 4B and C). Moreover, the hinge interaction with Glu121 is mediated by the 3-OH groups of quercetin and quercetagetin, which is lacking in hispidulin (Figure 4B and C). Hence, the presence of more hydrogen bond donors in the C-ring of quercetin for interacting with the hinge may explain why it has stronger inhibitory potency toward Pim-1 than hispidulin.

Further insights were obtained based on a comparison of Pim-1 cocrystal structures with hispidulin and other reported flavone compounds.[23] Both hispidulin- and 5,7,3′,4′,5′-pentahydroxyflavone-bound Pim-1 show an almost identical binding mode (Figure 4D). Interestingly, the pentahydroxyflavone binds in the A-ring-in mode despite three hydroxy groups in its B-ring; it has been suggested that the trisubstituted B-ring may be too hydrophilic to be buried in the cleft.[23] Although hispidulin and myricetin bind in the same orientation, there are differences between the binding poses of these two inhibitors (Figure 4E). Hispidulin moves inward by ∼1.5 Å to form an interaction of its A-ring with Lys67. Its lack of a 3-OH group prevents strong hydrogen bonding to the hinge residue Glu121. An additional hydrogen-bonding interaction with the backbone carbonyl of the unique hinge residue Pro123 is absent for hispidulin due to the lack of a 5′-OH group. These two differences lead to hispidulin moving away from the hinge region by ∼2.5 Å.

Although hispidulin exists in many plants and has a wide range of biological activities, isolating the compound from plant sources needs tedious extraction, purification, and chromatography. These time-consuming processes limit its availability in large quantities for mechanistic evaluation of the in vivo activities and for preparation of hispidulin derivatives for structure−activity relationship (SAR) studies. The chemical synthesis of hispidulin was reported previously;[12,18] however, these methods have several drawbacks, including poor reproducibility and low yields in key reaction steps during scale-up synthesis. In the current study, we synthesized hispidulin via a different route and used the synthesized compound to determine the binding mode of hispidulin as a Pim-1 kinase inhibitor by a cocrystallographic analysis. Although the synthetic route has more steps than previous methods, it is highly feasible and reproducible, as confirmed by detailed 1D NMR and 2D NMR analyses. Importantly, this new synthetic protocol can be applied to synthesize 6-OMe-retaining hispidulin-derived compounds with a modified B-ring, which may enrich the compound's diversity to permit identification of specific Pim-1 inhibitors by exploring the SAR of hispidulin derivatives.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were recorded with a Fisher-Johns apparatus (Fisher 12-144). IR spectra (KBr disk) were recorded using a Jasco Fourier transform infrared spectrometer (Jasco FT/IR-410). The NMR spectra (^{1}H and ^{13}C NMR, HMBC, HSQC, and NOESY) were obtained with a Bruker AV500 or AV600 using standard pulse programs. The MS data were obtained with a Finnigan Mat TSQ-7000 mass spectrometer (HRESIMS). HPLC was performed on an Ascentis C_{18} column (150 × 4.6 mm) by using an L-2130 pump (Hitachi) and a UV/vis L-2420 detector (Hitachi). All TLC analyses were performed on silica gel plates (KG60-F254, Merck). Reagents and materials were used without further purification, and chemicals were purchased from ACROS (Geel, Belgium). Dichloromethane was distilled from CaH_2 under a nitrogen atmosphere. MOMCl was purchased from TCI

DOI: 10.1021/acs.jnatprod.5b00324
J. Nat. Prod. XXXX, XXX, XXX−XXX

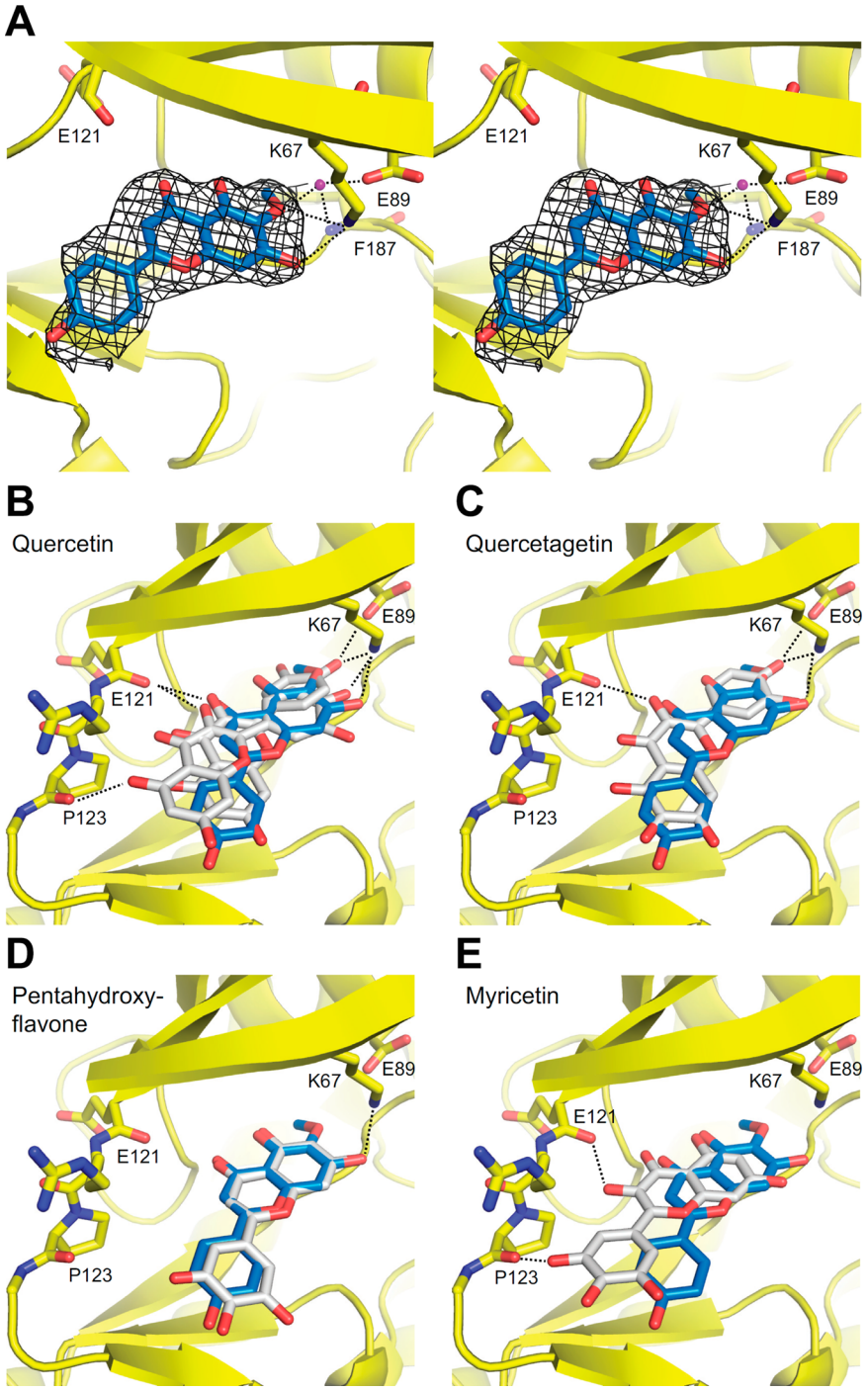


Figure 4. Structural comparison of Pim-1 bound to hispidulin and other flavone compounds. (A) Stereoview of hispidulin bound to the ATP-binding site of Pim-1 (this work). Bound hispidulin is shown as stick model with a simulated annealing $2F_o-F_c$ composite omit map contoured at the 1σ level. The compound is colored by atom type: green, carbon atoms; red, oxygen atoms. Hydrogen bonds are indicated by gray dashed lines, and the water molecule is shown as a purple sphere. Binding residues are shown as red sticks and labeled. (B–E) Comparison of hispidulin (blue sticks) with quercetin (B), quercetagetin (C), 5,7,3′,4′,5′-pentahydroxyflavone (D), and myricetin (E). The protein ribbon model is Pim-1 in the hispidulin-bound structure. Residues that form hydrogen bonds (in dashed lines) with each of the superimposed compounds (in gray sticks) are shown in sticks and labeled.

(Tokyo, Japan), and 2,4,6-trihydroxybenzaldehyde was acquired from Alfa Aesar (Heysham, UK).

*Synthesis of 2,4,6-Tris(methoxymethoxy)benzaldehyde (**13**).* To a solution of **12** (10 g, 64.9 mmol) in CH_2Cl_2 (100 mL) was added DIPEA (56.5 mL, 324.4 mmol). The resulting mixture was stirred for 10 min in an ice-bath under N_2. MOMCl (19.7 mL, 324.4 mmol) was added dropwise to the reaction mixture. The mixture was warmed to RT and stirred overnight. The solution was diluted with CH_2Cl_2 (100 mL) and washed with 0.5 N HCl (2 × 50 mL) and 10% NaOH (2 × 50 mL). The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc–*n*-hexane, 1:2) to give **13** (15.23 g, 82%) as a yellow, microcrystalline powder: mp 55–57 °C; IR (KBr) ν_{max} 2907, 2826, 1678, 1604, 1573, 1442, 1386, 1213, 1144, 1045, 1020 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 10.39 (1H, s), 6.49 (2H, s), 5.22 (4H, s), 5.17 (2H, s), 3.49 (6H, s), 3.46 (3H, s); ^{13}C NMR ($CDCl_3$, 150 MHz)

DOI: 10.1021/acs.jnatprod.5b00324
J. Nat. Prod. XXXX, XXX, XXX–XXX

δ 187.7, 163.3, 161.2, 111.1, 96.8, 94.9, 94.2, 56.5; HRESIMS *m/z* 309.0973 $[M + Na]^+$ (calcd for $C_{13}H_{18}NaO_7$, 309.0950).

*Synthesis of 2,4,6-Tris(methoxymethoxy)phenol (**14**).* To a solution of **13** (15 g, 52.4 mmol) in CH_2Cl_2 (100 mL) was added 70% MCPBA (19.37 g, 78.6 mmol) in portions. The resulting solution was stirred at RT overnight, and then 10% $NaOH_{(aq)}$ (60 mL) and MeOH (60 mL) were added. The mixture was stirred at RT for 3 h, acidified with 2 N $HCl_{(aq)}$, and extracted with EtOAc (3 × 100 mL). The combined organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:4) to give **14** (9.33 g, 65%) as a brown oil: IR (KBr) ν_{max} 3385, 2895, 2820, 1640, 1498, 1436, 1393, 1206, 1144, 1076, 1020 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.15 (1H, s), 6.45 (2H, s), 5.10 (4H, s), 5.03 (2H, s), 3.39 (6H, s), 3.35 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 149.2, 145.9, 133.4, 100.6, 95.4, 94.9, 55.9, 55.7; HRESIMS *m/z* 297.0968 $[M + Na]^+$ (calcd for $C_{12}H_{18}NaO_7$, 297.0950).

*Synthesis of 2-Methoxy-1,3,5-tris(methoxymethoxy)benzene (**15**).* To a mixture of **14** (8.5 g, 31 mmol) and K_2CO_3 (34.25 g, 248.2 mmol) in acetone (60 mL) was added CH_3I (7.7 mL, 124.1 mmol). The mixture was heated to 56 °C under N_2. After filtration to remove K_2CO_3, the filtrate was concentrated in vacuo, diluted with H_2O (30 mL), and extracted with EtOAc (3 × 60 mL). The combined organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:5) to give **15** (8.31 g, 93%) as a yellow oil; IR (KBr) ν_{max} 2938, 2901, 2826, 1591, 1492, 1430, 1393, 1225, 1039, 1020 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.54 (2H, s), 5.17 (4H, s), 5.07 (2H, s), 3.80 (3H, s), 3.49 (6H, s), 3.45 (3H, s); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 153.6, 151.2, 135.2, 99.5, 95.4, 94.9, 61.1, 56.2, 56.0; HRESIMS *m/z* 311.1124 $[M + Na]^+$ (calcd for $C_{13}H_{20}NaO_7$, 311.1107).

*Synthesis of 1,3,5-Trihydroxy-2-methoxybenzene (**16**).* To a solution of **15** (8.32 g, 28.9 mmol) in MeOH (300 mL) was added 12 N $HCl_{(aq)}$ (21.6 mL) dropwise by addition funnel. The reaction mixture was stirred at RT overnight. The mixture was concentrated in vacuo, diluted with H_2O (30 mL), and extracted with EtOAc (3 × 60 mL). The combined organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:4) to give **16** (4.15g, 92%) as a brown, microcrystalline powder: mp 180−184 °C; IR (KBr) ν_{max} 3342, 2938, 2839, 1616, 1517, 1480, 1374, 1275, 1144, 1051 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.76 (2H, s), 8.69 (1H, s), 5.72 (2H, s), 3.55 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 153.5, 151.1, 128.8, 94.8, 60.1; HRESIMS *m/z* 157.0507 $[M + H]^+$ (calcd for $C_7H_9O_4$, 157.0501).

*Synthesis of 2,4,6-Trihydroxy-3-methoxyacetophenone (**17**).* To a solution of **16** (3 g, 19.2 mmol) in Ac_2O (30 mL) was added BF_3-Et_2O (2.6 mL, 1.1 mmol) dropwise by syringe in an ice-bath. The reaction mixture was warmed to RT and stirred under N_2 overnight. The mixture was cooled to 0 °C, adjusted to pH 3−4 with 10% $NaOH_{(aq)}$, and extracted with EtOAc (3 × 50 mL). The combined organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:4) to give **17** (1.22 g, 32%) as a yellow, microcrystalline powder: mp 156−159 °C; IR (KBr) ν_{max} 3329, 2920, 2845, 1728, 1629, 1591, 1504, 1442, 1355, 1293 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 12.43 (1H, s), 11.51 (1H, s), 10.31 (1H, s), 5.89 (1H, s), 3.59 (3H, s), 2.55 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 203.1, 159.0, 157.8, 156.7, 128.1, 104.1, 94.5, 60.1, 32.5; HRESIMS *m/z* 221.0422 $[M + Na]^+$ (calcd for $C_9H_{10}NaO_5$, 221.0426).

Preparation of Compounds 18a and 18b. To a mixture of **17** (1.1 g, 5.6 mmol) and K_2CO_3 (5.37 g, 38.9 mmol) in acetone (30 mL) was added MOMCl (1.1 mL, 13.9 mmol) dropwise by syringe. The reaction mixture was heated to 56 °C under N_2 overnight. After filtration to remove K_2CO_3, the filtrate was concentrated in vacuo, diluted with distilled H_2O (30 mL), and extracted with EtOAc (3 × 60 mL). The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:8) to give **18a** (476 mg, 30%) and **18b** (471 mg, 29%).

*6-Hydroxy-3-methoxy-2,4-bis(methoxymethoxy)acetophenone (**18a**):* yellow oil; IR (KBr) ν_{max} 3410, 2926, 2833, 1741, 1622, 1598, 1362, 1306, 1281, 1157, 1051 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 11.89 (1H, s), 6.40 (1H, s), 5.22 (2H, s), 5.12 (2H, s), 3.66 (3H, s), 3.41 (3H, s), 3.39 (3H, s), 2.54 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 203.0, 157.0, 155.5, 150.6, 135.1, 113.1, 100.0, 99.5, 94.8, 61.1, 57.8, 56.5, 32.5; HRESIMS *m/z* 309.0949 $[M + Na]^+$ (calcd for $C_{13}H_{18}NaO_7$, 309.0950).

*2-Hydroxy-3-methoxy-4,6-bis(methoxymethoxy)acetophenone (**18b**):* light yellow oil; IR (KBr) ν_{max} 3410, 2926, 2833, 1736, 1697, 1616, 1591, 1486, 1411, 1275, 1151, 1070 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 12.96 (1H, s), 6.39 (1H, s), 5.26 (2H, s), 5.25 (2H, s), 3.65 (3H, s), 3.41 (3H, s), 3.40 (3H, s), 2.58 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 203.8, 156.7, 155.4, 155.0, 131.7, 108.4, 95.0, 94.6, 93.3, 60.4, 56.7, 56.4, 33.1; HRESIMS *m/z* 309.0951 $[M + Na]^+$ (calcd for $C_{13}H_{18}NaO_7$, 309.0950).

*Synthesis of (E)-1-(6-Hydroxy-3-methoxy-2,4-bis-(methoxymethoxy)phenyl)-3-(4-benzyloxyphenyl)prop-2-en-1-one (**19**).* To a mixture of **18** (450 mg, 1.57 mmol) and 4-benzyloxybenzaldehyde (367 mg, 1.73 mmol) in EtOH (4 mL) was added a solution of KOH (1.09 g) in EtOH−H_2O (2 mL:1 mL) dropwise by syringe. The resulting solution was cooled to 0 °C and stirred for 3 h under N_2. The mixture was warmed to RT overnight. The reaction mixture was poured into ice water, acidified to pH 3−4 with 1 N $HCl_{(aq)}$, and extracted with EtOAc (3 × 30 mL). The combined organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:6) to give **19** (650 mg, 86%) as a yellow, microcrystalline powder; mp 61−63 °C; IR (KBr) ν_{max} 3398, 2920, 2851, 1736, 1622, 1554, 1504, 1411, 1374, 1343 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 13.25 (1H, s), 7.91 (1H, d, *J* = 15.5 Hz), 7.81 (1H, d, *J* = 15.5 Hz), 7.60 (2H, d, *J* = 8.7 Hz), 7.42 (2H, d, *J* = 7.2 Hz), 7.37 (2H, t, *J* = 7.2 Hz), 7.32 (1H, t, *J* = 7.2 Hz), 6.99 (2H, d, *J* = 8.7 Hz), 6.52 (1H, s), 5.25 (2H, s), 5.19 (2H, s), 5.10 (2H, s), 3.81 (3H, s), 3.50 (3H, s), 3.47 (3H, s); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 192.9, 161.7, 160.8, 157.1, 151.9, 143.3, 136.5, 135.2, 130.4, 128.7, 128.3, 128.2, 127.5, 124.6, 115.3, 110.5, 100.8, 99.7, 94.7, 70.1, 61.2, 58.2, 56.6; HRESIMS *m/z* 503.1681 $[M + Na]^+$ (calcd for $C_{27}H_{28}NaO_8$, 503.1682).

*Synthesis of 4′-Benzyloxy-6-methoxy-5,7-bis(methoxymethoxy)-flavanone (**20**).* To a solution of **19** (600 mg, 1.25 mmol) in EtOH−H_2O (6 mL: 0.5 mL) was added NaOAc (410 mg, 5.00 mmol). The reaction mixture was heated to 78 °C under N_2 overnight. The mixture was diluted with EtOAc (60 mL) and washed with distilled H_2O (3 × 30 mL). The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:5) to give **20** (498 mg, 83%) as a yellow, microcrystalline powder; mp 121−123 °C; IR (KBr) ν_{max} 3404, 2920, 2845, 1734, 1684, 1604, 1517, 1467, 1418, 1380 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.41 (2H, d, *J* = 7.2 Hz), 7.38 (2H, t, *J* = 7.2 Hz), 7.35 (2H, d, *J* = 8.6 Hz), 7.31 (1H, t, *J* = 7.2 Hz), 7.00 (2H, d, *J* = 8.6 Hz), 6.60 (1H, s), 5.32 (1H, dd, *J* = 2.7, 13.4 Hz), 5.22 (2H, s), 5.20 (1H, d, *J* = 6.1 Hz), 5.16 (1H, d, *J* = 6.1 Hz), 5.07 (2H, s), 3.82 (3H, s), 3.62 (3H, s), 3.48 (3H, s), 2.99 (1H, dd, *J* = 13.4, 16.6 Hz), 2.73 (1H, dd, *J* = 2.6, 16.6 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 189.7, 159.7, 159.2, 157.1, 151.3, 138.1, 136.8, 130.9, 128.6, 128.0, 127.7, 127.4, 115.1, 109.8, 100.3, 99.9, 94.7, 78.9, 70.1, 61.3, 57.5, 56.6, 45.3; HRESIMS *m/z* 503.1697 $[M + Na]^+$ (calcd for $C_{27}H_{28}NaO_8$, 503.1682).

*Synthesis of 4′-Benzyloxy-6-methoxy-5,7-dihydroxyflavone (**21**).* To a solution of **20** (300 mg, 0.63 mmol) in pyridine (10 mL) was added I_2 (159 mg, 0.63 mmol). The mixture was heated at 90 °C for 3 h. The reaction mixture was diluted with EtOAc (30 mL) and washed with distilled H_2O (3 × 30 mL). The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−*n*-hexane, 1:2) to give **21** (159 mg, 65%) as a yellow, microcrystalline powder; mp 152−153 °C; IR (KBr) ν_{max} 3360, 2920, 2845, 1728, 1647, 1610,

DOI: 10.1021/acs.jnatprod.5b00324
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1498, 1455, 1368 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 13.01 (1H, s), 10.71 (1H, s), 8.02 (2H, dd, J = 2.0, 9.0 Hz), 7.46 (2H, d, J = 7.3 Hz), 7.40 (2H, t, J = 7.3 Hz), 7.34 (1H, t, J = 7.3 Hz), 7.17 (2H, dd, J = 2.0, 9.0 Hz), 6.85 (1H, s), 6.60 (1H, s), 5.22 (2H, s), 3.74 (3H, s); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 182.3, 163.5, 161.5, 157.5, 152.9, 152.6, 136.7, 131.6, 128.7, 128.5, 128.2, 127.9, 123.2, 115.6, 104.3, 103.3, 94.5, 69.7, 60.1; HRESIMS m/z 413.1002 $[M + Na]^+$ (calcd for $C_{23}H_{18}NaO_6$, 413.1001).

Synthesis of Hispidulin. To a solution of **21** (150 mg, 0.13 mmol) in dry CH_2Cl_2 (10 mL) at −78 °C was added 1 M BCl_3 (69 μL) in CH_2Cl_2 (2 mL) dropwise by syringe under N_2. The resulting solution was warmed to RT over 1 h. The reaction mixture was adjusted to pH 2−3 with saturated $NaHCO_{3(aq)}$ and extracted with EtOAc (3 × 30 mL). The combined organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (EtOAc−n-hexane, 1:1) to give hispidulin (89 mg, 80%) as a yellow microcrystalline powder; mp 275−277 °C (lit.[32] mp 271−273 °C); IR (KBr) ν_{max} 3323, 2920, 2851, 1734, 1647, 1566, 1486, 1461, 1362 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 13.07 (1H, s, OH), 7.92 (2H, d, J = 8.8 Hz, H-2′, H-6′), 6.93 (2H, d, J = 8.8 Hz, H-3′, H-5′), 6.77 (1H, s, H-3), 6.59 (1H, s, H-8), 3.75 (3H, s, 6-OMe); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 182.1 (C-4), 163.8 (C-2), 161.2 (C-4′), 157.3 (C-7), 152.8 (C-5), 152.4 (C-9), 131.4 (C-6), 128.5 (C-2′, C-6′), 121.2 (C-1′), 116.0 (C-3′, C-5′), 104.1 (C-10), 102.4 (C-3), 94.3 (C-8), 60.0 (6-OCH_3); HRESIMS m/z 323.0515 $[M + Na]^+$ (calcd for $C_{16}H_{12}NaO_6$, 323.0532).

Pim-1 Kinase Inhibition Assay. The Pim-1 kinase reaction was performed in 20 mmol/L HEPES (pH 7.5), 10 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.02% Brij 35, 0.02 mg/mL bovine serum albumin, 2 mmol/L DTT, and 1% DMSO. The final ATP concentration was 10 μm/L. The IC_{50} values of the tested compounds were determined by Reaction Biology Corp. The purified recombinant Pim-1 kinase was incubated with serial 2-fold dilutions of the tested compounds starting at a concentration of 30 μM. The dose−response curves were obtained using Prism 5.0 from GraphPad software.

Expression and Purification of Pim-1. The production and purification of the recombinant His_6-tagged Pim-1 for crystallography have been described previously.[33] The cDNA fragment encoding Pim-1 (residues 29−313) was synthesized (GenScript) and cloned into an expression vector, in frame with a carboxyl-terminal His_6 tag. The recombinant protein was expressed in *Escherichia coli* BL21 (DE3) cells using Terrific-Broth medium. Cells were cultured to an OD_{600} of 1.2 and induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 15 °C for 16 h. Harvested bacteria were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, and Roche EDTA-free protease inhibitor cocktail) and ruptured in a high-pressure homogenizer (Avestin). The crude lysate was centrifuged at 35000 g for 45 min, and the supernatant was applied to a nickel-nitrilotriacetic acid (Qiagen) column and washed with 20−100 mM imidazole. The protein fraction, eluted with 250 mM imidazole, was loaded onto a Mono Q column (GE Healthcare) with 25 mM Tris-HCl pH 8.0, 100 mM NaCl, and 2 mM DTT and eluted with a linear gradient of 0.1−1 M NaCl. The pooled Pim-1 fractions were combined and passed on a Superdex 75 column (GE Healthcare) equilibrated in 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 4 mM DTT.

Protein Crystallization. Pim-1 was crystallized under the reported conditions.[34] The protein was crystallized by the hanging-drop vapor-diffusion method in which equal volumes of protein (7 mg/mL) and reservoir solution (0.4−1.4 M NaOAc, 0.1 M imidazole, pH 6.5−7.1) were mixed and allowed to equilibrate against the reservoir at 4 °C. The rod-shaped crystals grew in about 2 days. Complexes of Pim-1 with hispidulin were obtained by soaking apo crystals in mother liquid solutions with the compound, dissolved in DMSO, at a concentration of 1 mM overnight. The crystals were harvested and cryoprotected in a well solution containing 20% glycerol prior to data collection. The X-ray diffraction data for the Pim-1−hispidulin complex were collected at the SP44XU beamline at SPring-8, Japan.

Structure Determination. All images were processed using the HKL-2000 package. The crystal belongs to the hexagonal space group $P6_5$. The structure was solved by the molecular replacement using the program Phaser in the CCP4 suite by employing a Pim-1 model (PDB code 1XWS) for searches. The models were manually rebuilt using the program Coot and refined using the program refmac5. The model of hispidulin was built using the program PRODRG and fitted to the electron density map after the protein model has been completed. The atomic coordinates and structure factors for the complex were deposited in the Protein Data Bank (http://www.rcsb.org/) with the accession number 4XH6.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00324.

^1H and ^{13}C NMR spectra and HPLC chromatogram of all compounds synthesized; comparison of experimental and reported hispidulin data; and NOESY, HMQC, and HMBC spectra of compounds **18a,b** and final product hispidulin (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge the financial support from the Ministry of Science and Technology (NSC102-2320-B-001-016 to C.-I.C. and NSC102-2320-B-038-019-MY3 to W.-J.H.) and from the Academia Sinica in Taiwan (C.-I.C.).

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