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Total Synthesis of the Bicyclic Depsipeptide HDAC Inhibitors Spiruchostatins A and B, 5″-*epi*-Spiruchostatin B, FK228 (FR901228) and Preliminary Evaluation of Their Biological Activity

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Dedicated to Dr. Shiro Terashima on the occasion of his 70th birthday

Abstract: The bicyclic depsipeptide histone deacetylase (HDAC) inhibitors spiruchostatins A and B, 5″-*epi*-spiruchostatin B and FK228 were efficiently synthesized in a convergent and unified manner. The synthetic method involved the following crucial steps: i) a Julia–Kocienski olefination of a 1,3-propanediol-derived sulfone and a L- or D-malic acid-derived aldehyde to access the most synthetically challenging unit, (3*S* or 3*R*,4*E*)-3-hydroxy-7-mercapto-hept-4-enoic acid, present in a D-alanine- or D-valine-containing segment; ii) a condensation of a D-valine-D-cysteine- or D-*allo*-isoleucine-D-cysteine-containing segment with a D-alanine- or D-valine-containing segment to directly assemble the corresponding *seco*-acids; and iii) a macrocyclization of a *seco*-acid using the Shiina method or the Mitsunobu method to construct the requisite 15- or 16-membered macrolactone. The present synthesis has established the C5″ stereochemistry of spiruchostatin B. In addition, HDAC inhibitory assay and the cell-growth inhibition analysis of the synthesized depsipeptides determined the order of their potency and revealed some novel aspects of structure–activity relationships. It was also found that unnatural 5″-*epi*-spiruchostatin B shows extremely high selectivity (ca. 1600-fold) for class I HDAC1 ($IC_{50}=2.4$ nM) over class II HDAC6 ($IC_{50}=3900$ nM) with potent cell-growth-inhibitory activity at nanomolar levels of IC_{50} values.

Keywords: FK228 · histone deacetylase inhibitors · natural products · spiruchostatins · total synthesis

Introduction

Recently, histone deacetylase (HDAC) inhibitors have received considerable attention because of their biological and pharmaceutical attributes.[1] HDAC enzymes are zinc metalloenzymes that catalyze the hydrolysis of acetylated lysine residues on proteins, particularly on histones.[1] 18 HDACs have been identified in humans; they have been grouped into four classes according to their homology with the yeast deacetylase.[2] Class I (HDACs 1–3 and 8), class II (HDACs 4–7, 9 and 10) and class IV (HDAC 11) enzymes are Zn^+-binding enzymes, while class III HDAC enzymes have an NAD^+-dependent mechanism of deacetylation.[1,2] It has been reported that HDAC inhibitors can arrest the growth of a wide range of transformed cells and can inhibit the growth of human tumor xenografts.[1] Potent and selective HDAC inhibitors, therefore, are anticipated to be promising candidates for novel molecular-targeted anticancer agents.[1]

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Spiruchostatins A (**1**) and B (**2**) (Figure 1), isolated from a culture broth of *Pseudomonas* sp. by Shin-ya et al. in 2001,[3] were found to exhibit potent HDAC inhibitory activity.[4] Structurally, these compounds are 15-membered bicyclic depsipeptides consisting of (3*S*,4*R*)-statine (in blue), D-cysteine (in orange), D-alanine (in green), (3*R*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid (in red) and a characteristic disulfide bond linkage.[3] Structurally similar 16-membered bicyclic depsipeptides FK228 (**3**) (previously known as FR901228) and FR901375 (**4**), isolated from a culture broth of *Chromobacterium violaceum* (No. 968) and *Psuedomonas chloroaphis* (No. 2552), respectively, by Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc.) in 1994,[5] also exhibit potent HDAC inhibitory activity.[6] Yoshida et al. proposed a molecular mechanism by which FK228 inhibits HDAC.[7] In that mechanism, the depsipeptide FK228, which itself serves as a stable prodrug, is activated by reductive cleavage of the disulfide bond after incorporation into the cells, and the released sulfhydryl group in the four-carbon side chain binds to the zinc cation located at the active site of the HDACs, resulting in a potent inhibitory effect. It has also been shown that FK228 exhibits prominent in vivo antitumor activity against human tumor xenograft models.[5b,6] This activity has led to its evaluation as an anticancer agent in advanced clinical trials (phase II) in the United States.[8] However, the phase II study was recently terminated due to serious adverse cardiac events;[9] the undesirable side effects seem to arise from insufficient class selectivity of this agent.[10]

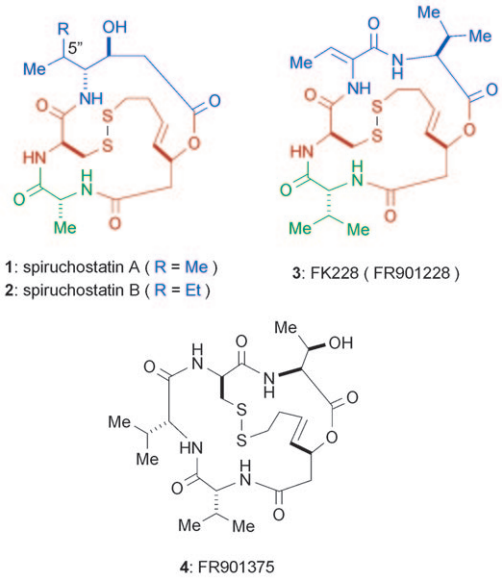


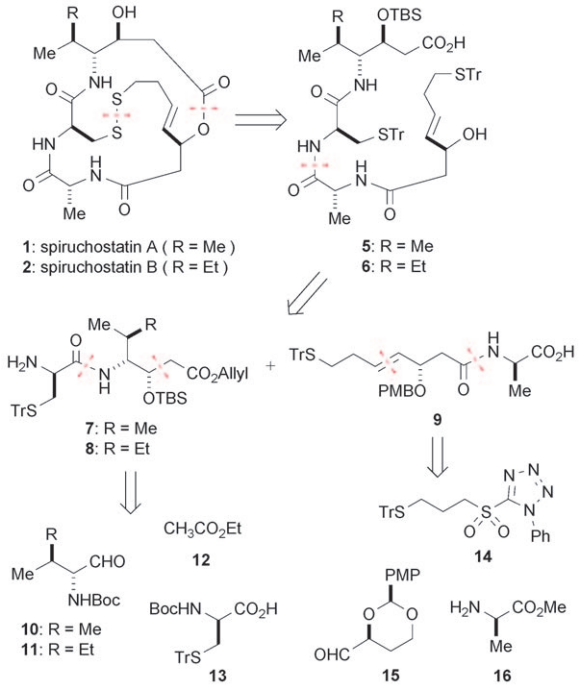
Figure 1. Structures of spiruchostatins A (**1**), B (**2**), FK228 (FR901228) (**3**) and FR901375 (**4**).

The significant biological properties and unique structural features have made **1**–**4** exceptionally intriguing and timely targets for the total synthesis. To date, three total syntheses of FK228 (**3**) have been reported: first by Simon et al.[11] in 1996 followed by Williams et al.[12] and Ganesan et al.[13] in 2008. One total synthesis of FR901375 (**4**) has been reported by Wentworth, Janda et al.[14] in 2003. In addition, three total syntheses of spiruchostatin A (**1**) have been reported by Ganesan et al.[15] in 2004, Doi, Takahashi et al.[16] in 2006 and Miller et al.[17] in 2009. However, the total synthesis of spiruchostatin B (**2**) has not been mentioned in the literature, and the stereochemistry at C5″ (spiruchostatin numbering) of **2** has not been clearly assigned. We have previously reported our own preliminary results on the total synthesis of spiruchostatins A and B that led to the establishment of the stereochemistry at the C5″ position of **2**.[18] In this paper, we describe in complete detail our total synthesis of **1**–**3** and 5″-*epi*-spiruchostatin B (**52**) (cf. Scheme 9), using a highly convergent and unified scheme. In addition, HDAC inhibition assay and the cell-growth inhibition analysis of the synthesized compounds **1**–**3** and **52** are also described.

Results and Discussion

Synthesis of spiruchostatins A (1), B (2) and 5″-*epi*-spiruchostatin B (52)

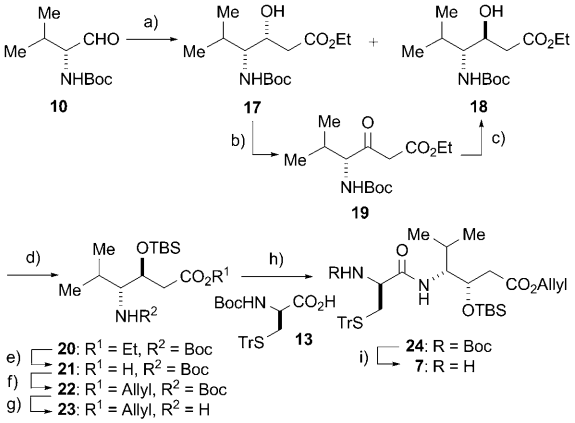
Synthetic plan: In our synthetic plan for spiruchostatins A (**1**) and B (**2**), we envisioned that the target molecules **1** and **2** could be synthesized by macrolactonization of the corresponding *seco*-acids **5** and **6**, followed by a disulfide bond formation according to the protocols described by previous studies (Scheme 1).[15,16] The key feature of this Scheme was



Scheme 1. Synthetic plan for spiruchostatins A (**1**) and B (**2**). TBS = *tert*-butyldimethylsilyl, Tr (trityl) = triphenylmethyl, Boc = *tert*-butoxycarbonyl, PMB = 4-methoxybenzyl, PMP = 4-methoxyphenyl.

expected to be a highly convergent assembly of **5** and **6** by direct condensation of segment **7** or **8** with segment **9**. Segments **7** and **8** would be prepared via an aldol coupling of *N*-Boc-D-valinal (**10**)[19] or D-allo-isoleucinal (**11**)[20] with ethyl acetate (**12**), and subsequent condensation with D-cysteine dervative **13**.[21] Segment **9**, which contains the most synthetically challenging unit, (3*S*,4*E*)-3-hydroxy-7-mercaptohept-4-enoic acid, would be produced via Julia–Kocienski olefination[22] of sulfone **14** (accessible from 1,3-propanediol) with aldehyde **15**[23] (accessible from L-malic acid), followed by condensation with D-alanine methyl ester (**16**).

Synthesis of segment 7: We initially pursued the synthesis of segment **7** (Scheme 2). Aldol coupling of the lithium enolate of ethyl acetate (**12**) (generated in situ by reaction with LDA) with the known *N*-Boc-D-valinal (**10**)[19] provided the

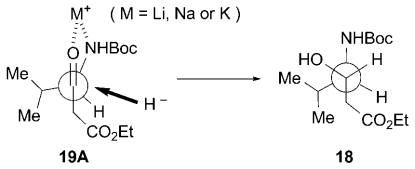


Scheme 2. Synthesis of segment **7**. a) LDA, CH_3CO_2Et (**12**), THF, −78 °C; at −78 °C, add **10**, 63% for **17**, 30% for **18** (**17**/**18** ca. 2:1); b) Jones reagent, acetone, 0 °C to RT, 80%; c) KBH_4, MeOH, −40 °C, 82% for **18**, 5% for **17** (**18**/**17** 16:1) (see entry 4 in Table 1); d) TBSCl, imidazole, DMF, RT, 84%; e) 1 M NaOH, EtOH, RT, 82%; f) allyl bromide, K_2CO_3, DMF, RT, 91%; g) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; MeOH, RT; MeOH, RT, 90%; h) **13**, PyBOP, *i*Pr_2NEt, MeCN, RT, 88%; i) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT, 99%. LDA = lithium diisopropylamide, TMSOTf = trimethylsilyl trifluoromethanesulfonate, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

desired coupling product **18** (30%) and the undesired stereoisomer **17** (63%). Next, conversion of **17** to **18** by inversion of the hydroxy group was investigated. The sequence involved Jones oxidation[24] and subsequent stereoselective reduction of the resulting ketone **19**. In the reduction step, several reducing agents, such as $LiBH_4$, $NaBH_4$ and KBH_4, were examined (Table 1). Among them, the use of KBH_4 gave the best result (entry 3) and the desired alcohol **18** was produced in 80% yield with high stereoselectivity (**18**/**17** 10:1). When the reaction was performed at a lower temperature (−40 °C), the stereoselectivity was apparently increased (entry 4, 82% yield of **18** with **18**/**17** 16:1). With the use of $LiBH_4$ or $NaBH_4$ (entries 1 and 2), a lower stereoselectivity of **18**/**17** was observed (4:1 and 7:1, respectively). The remarkable stereoselectivity in the reduction of **19** can be explained by the well-known Cram's chelation model (Scheme 3);[25] the stereoselectivity was consistent with the chelation ability of alkali metal ions ($K^+ > Na^+ > Li^+$) to form a chelate in the proposed transition state **19A**.

Table 1. Stereoselective reduction of ketone **19**.

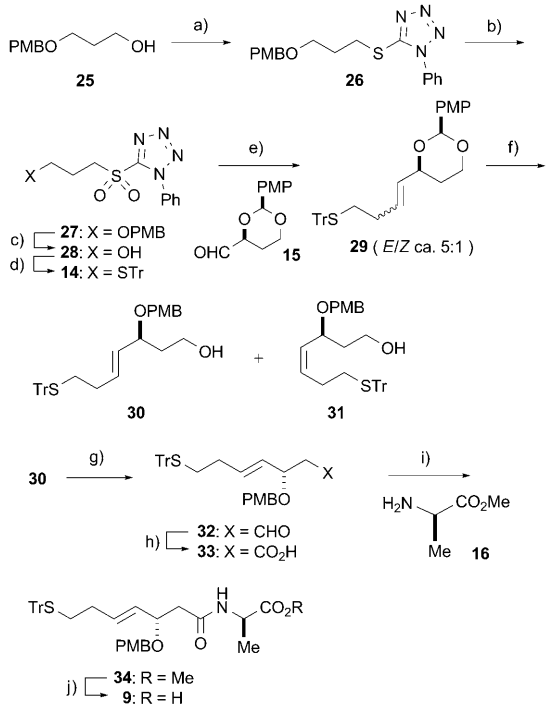
Entry	Reducing agent	T [°C]	t [h]	Yield [%]		Ratio
				18	**17**	**18**/**17**
1	$LiBH_4$	0	1	52	13	4:1
2	$NaBH_4$	0	1	65	9	7:1
3	KBH_4	0	1	80	8	10:1
4	KBH_4	−40	1	82	5	16:1



Scheme 3. Cram's chelation model for the stereoselective reduction of ketone **19A**.

To continue the synthesis (cf. Scheme 2), ethyl ester **18** was then transformed to allyl ester **23** via a four-step sequence involving TBS protection of the hydroxy group in **18** (84%), saponification of the ester moiety in the resulting TBS ether **20** (82%), formation of an allyl ester from the liberated carboxylic acid **21** (91%) and deprotection of the *N*-Boc group in **22** (90%). Subsequent condensation of amine **23** with *N*-Boc-*S*-trityl-D-cysteine (**13**)[21] proceeded smoothly to produce amide **24** in 88% yield. Finally, deprotection of the *N*-Boc group in **24** furnished the desired segment **7** in quantitative yield.

Synthesis of segment 9: Next, we performed the synthesis of segment **9**, the condensation partner of **7**, starting from the known 3-(4-methoxybenzyloxy)propan-1-ol (**25**)[26] (Scheme 4). Sulfone **14**, a substrate for the Julia–Kocienski olefination,[22] was efficiently prepared from **25** via a four-step operation involving the formation of tetrazole **26** (95%), molybdenum-mediated oxidation[27] of **26**, deprotection of the PMB group in sulfone **27** (86% in two steps), and substitution of the primary hydroxy group in **28** with a *S*-trityl group (96%). The crucial Julia–Kocienski olefination[22] of **14** with the known aldehyde **15**[23] (readily prepared from L-malic acid) proceeded smoothly to form the desired product **29** as an inseparable mixture of *E*/*Z* stereoisomers (*E*/*Z* ca. 5:1 by 400 MHz ^{1}H NMR) in 66% yield. Subsequent reductive opening of **29** with DIBAL[28] at 0 °C provided the desired *E*-olefinic primary alcohol **30** as a major product (60%) along with the undesired *Z*-olefinic



Scheme 4. Synthesis of segment **9**. a) 1-phenyl-1*H*-tetrazole-5-thiol, DEAD, PPh_3, THF, RT, 95%; b) $[Mo_7O_{24}(NH_4)_6]\cdot 4H_2O$, 30% H_2O_2, EtOH, RT; c) DDQ, CH_2Cl_2/H_2O, RT, 86% (2 steps); d) TrSH, DEAD, PPh_3, CH_2Cl_2, reflux, 96%; e) $LiN(SiMe_3)_2$, DMF, −60°C; at −60°C, add. **15**, −60 to 0°C, 66% (*E*/*Z* ca. 5:1); f) DIBAL, toluene, 0°C, 60% for **30**, 12% for **31**; g) Dess–Martin periodinane, $NaHCO_3$, CH_2Cl_2, 0°C to RT, 88%; h) $NaClO_2$, NaH_2PO_4, $DMSO/H_2O$, 0°C to RT, 75%; i) D-alanine methyl ester (**16**), PyBOP, iPr_2NEt, CH_2Cl_2, 0°C to RT, 90%; j) 1 M LiOH, MeOH, RT, 98%. DEAD = diethyl azodicarboxylate, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, DIBAL = diisobutylaluminium hydride.

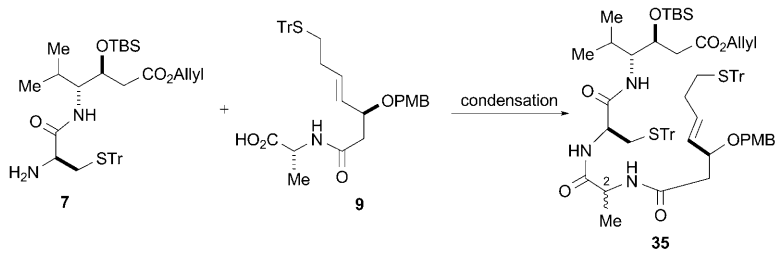
isomer **31** (12%); at this stage, the *E*/*Z* isomers could be separated by silica gel column chromatography. Dess–Martin oxidation[29] of **30** (86%) followed by Pinnick oxidation[30] of the resulting aldehyde **32** yielded the corresponding carboxylic acid **33**. Finally, condensation of **33** with D-alanine methyl ester (**16**) followed by saponification of the ethyl ester moiety of the resulting amide **34**, afforded the requisite segment **9** in 66% overall yield from **32**.

Completion of the total synthesis of spiruchostatin A (1): With the key segments **7** and **9** synthesized, we next investigated the synthesis of the first target spiruchostatin A (**1**) by assembling the two segments (Tables 2 and 3, Schemes 5 and 6). Initial attempts to achieve the crucial condensation of **7** and **9** using standard condensing agents[31] (e.g., PyBOP, PyBOP/HOBt, EDCI/HOBt or HATU in CH_2Cl_2 at −30°C) resulted in partial epimerization at the C2 stereogenic center (D-alanine part in **35**) (entries 1–4, Table 2), while the condensation product **35** was obtained in high yield (82–91%).

Eventually, we overcame this epimerization problem by using a combination of HATU and HOAt at a low temperature (entry 5). Thus, treating a mixture of **7** and **9** in CH_2Cl_2 with HATU (1.3 equiv) in the presence of iPr_2NEt (2.6 equiv) at −30°C for 3 h resulted in the desired product **35** in 94% yield without appreciable epimerization at the C2 position. In this condensation, the temperature was critical: when the reaction was performed at room temperature, a small degree of epimerization (α-Me/β-Me 10:1) was observed (entry 6). The condensation product **35** was then converted to *seco*-acid **5**, a substrate for macrolactonization, in 88% overall yield via alcohol **36** by successive deprotection of the PMB and allyl groups (Scheme 5).

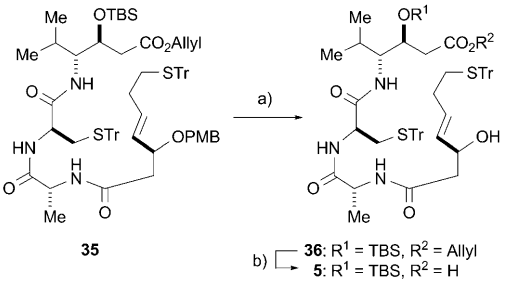
Having obtained *seco*-acid **5** in a convergent way, we next examined the crucial macrolactonization (Table 3). In the previous two total syntheses of spiruchostatin A (**1**), Ganesan et al.[15] successfully achieved macrolactonization of the *O*-triisopropylsilyl (TIPS) variant of **5** (R = TIPS) using the Yamaguchi method (2,4,6-$Cl_3C_6H_2COCl$, Et_3N, MeCN/THF, 0 to 20°C; DMAP, toluene, 50°C, 53%); on the other hand, Doi, Takahashi et al.[16] efficiently performed macrolactonization of *O*-non-protected variant of **5** (R = H) using the Shiina method [2-methyl-6-nitrobenzoic acid (MNBA),

Table 2. Condensation of segment **7** with segment **9**.

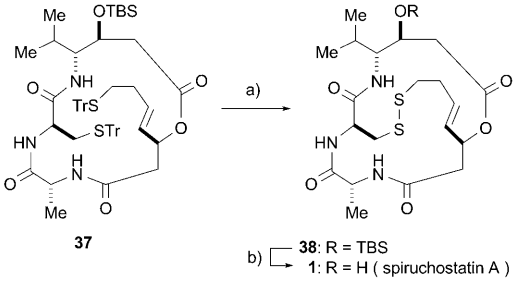


Entry	Conditions	Yield [%][a] **35**	Ratio[b] α-Me/β-Me
1	PyBOP (1.3 equiv), iPr_2NEt (2.6 equiv), CH_2Cl_2, −30°C, 3 h	82	3:1
2	PyBOP (1.3 equiv), HOBt (1.3 equiv), iPr_2NEt (2.6 equiv), CH_2Cl_2, −30°C, 3 h	85	5:1
3	EDCI (1.3 equiv), HOBt (1.3 equiv), iPr_2NEt (2.6 equiv), CH_2Cl_2, −30°C, 3 h	91	5:1
4	HATU (1.3 equiv), iPr_2NEt (2.6 equiv), CH_2Cl_2, −30°C, 3 h	89	6:1
5	HATU (1.3 equiv), HOAt (1.3 equiv), iPr_2NEt (2.6 equiv), CH_2Cl_2, −30°C, 3 h	94	1:0
6	HATU (1.3 equiv), HOAt (1.3 equiv), iPr_2NEt (2.6 equiv), CH_2Cl_2, RT, 1 h	91	10:1

[a] Isolated yield. [b] Determined by 400 MHz ^1H NMR analysis. HOBt = 1-hydroxybenzotriazole, EDC = 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole.



Scheme 5. Synthesis of *seco*-acid **5**. a) DDQ, CH_2Cl_2/H_2O, RT, 89%; b) $[Pd(PPh_3)_4]$, morpholine, THF, RT, 99%.



Scheme 6. Synthesis of spiruchostatin A (**1**). a) I_2, $MeOH/CH_2Cl_2$, RT, 80%; b) HF·pyridine, pyridine, RT, 92%.

DMAP, CH_2Cl_2, RT, 67%]. Given those previous results, we investigated the macrolactonization of **5** under several conditions (Table 3). The best result was obtained using the Shiina's standard method (entry 1).[32] Thus, treatment of a dilute solution of **5** in CH_2Cl_2 (0.001 M) with MNBA (1.3 equiv) and DMAP (3.0 equiv) at room temperature for 15 h produced the desired macrocyclic compound **37** in 90% yield. The use of other Shiina's protocols[33] (entries 2 and 3) resulted in almost the same yields (87% each). In contrast, when macrolactonization was performed as per the Yamaguchi method[34] (entry 4) or the Mukaiyama–Corey–Nicolaou method[35] (entry 5), the yield of **37** was lower (67% and 36%, respectively).

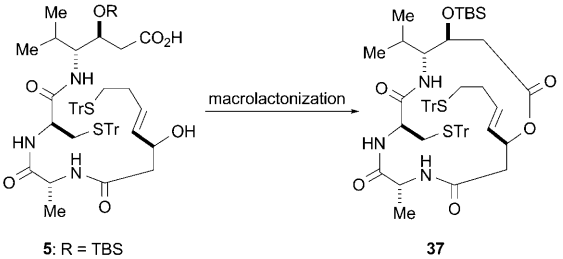
The final route that led to the completion of the total synthesis of **1** is depicted in Scheme 6. Simultaneous *S*-Tr deprotection and disulfide bond formation of **37** was performed by brief exposure to iodine in dilute $MeOH/CH_2Cl_2$ solution (0.5 mM) at ambient temperature, as described in previous studies,[11–16,36] producing the desired disulfide **38** in 80% yield. Finally, deprotection of the *O*-TBS group in **38** delivered **1**, $[\alpha]_D^{24} = -62.8°$ ($c = 0.14$ in MeOH) {lit.[3] $[\alpha]_D^{26} = -63.6°$ ($c = 0.14$ in MeOH)}, in 92% yield. The spectroscopic properties (IR, ^1H and ^{13}C NMR, HRMS) of the synthetic sample **1** were identical with those reported for natural **1**.[3]

Synthesis of spiruchostatin B (2) and 5″-*epi*-spiruchostatin B (52): As mentioned previously, the stereochemistry at the C5″ position of spiruchostatin B (**2**) has not been clearly assigned; therefore, we decided to undertake the synthesis of two possible C5″ stereoisomers, namely, **2** (cf. Scheme 8) and its C5″-epimer **52** (cf. Scheme 9); the successful synthesis revealed that natural **2** possesses the (5″*S*)-configuration as depicted in Scheme 8 (see below).

Synthesis of segment 8: As shown in Scheme 7, the synthesis of segment **8** was conducted starting from *N*-Boc-D-allo-isoleucinal (**11**)[20] by employing a reaction sequence similar to that described in the section on the synthesis of segment **7** (cf. Scheme 2). Thus, aldol coupling of **11** with the lithium enolate of ethyl acetate (**12**) afforded the desired coupled product **40** (31%) and the undesired stereoisomer **39** (62%). After inversion of the hydroxy group in **39** (85% in two steps), the resulting alcohol **40** was further converted to amine **44** in four steps via TBS ether **41**, carboxylic acid **42** and allyl ester **43** (69% overall yield). Subsequent condensation of **44** with D-cysteine derivative **13**[21] provided the requisite segment **8** (86% overall yield) after *N*-Boc deprotection of amide **45**.

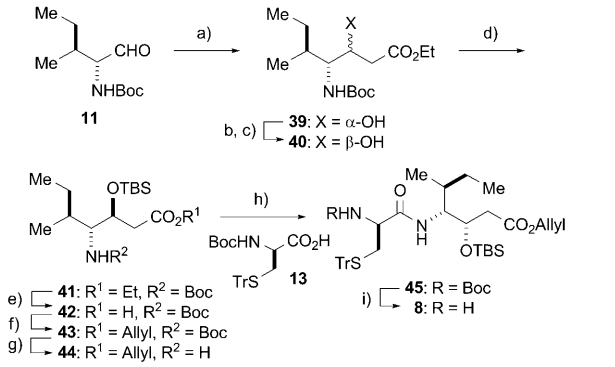
Completion of the total synthesis of spiruchostatin B (2): After obtaining the key segment **8**, we performed the synthesis of the second target spiruchostatin B (**2**) (Scheme 8). Condensation of **8** with the common intermediate **9** proceeded smoothly and cleanly under the optimized conditions described previously (cf. entry 5, Table 2); the desired product **46** was obtained in

Table 3. Macrolactonization of *seco*-acid **5** leading to macrocyclic compound **37**.[a]

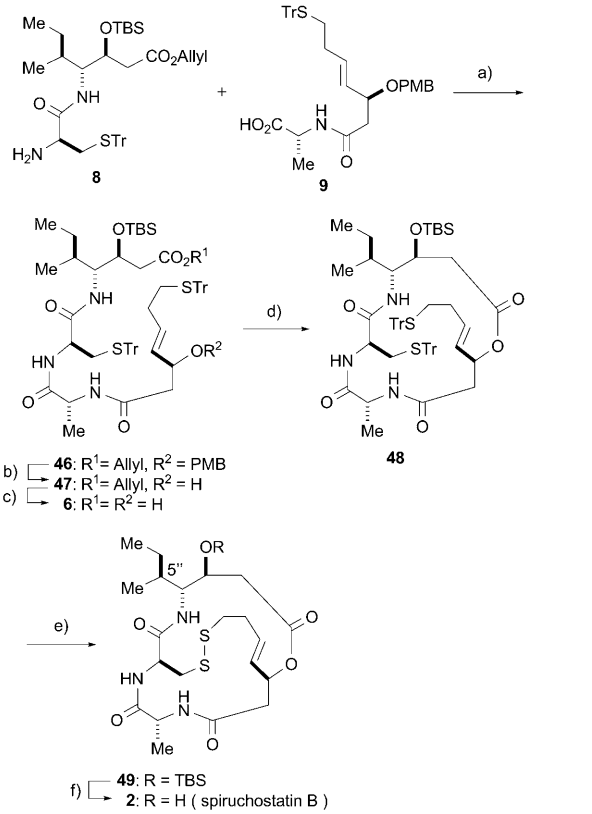


Entry	Conditions	Yield **37** [%]
1	MNBA (1.3 equiv), DMAP (3.0 equiv), CH_2Cl_2, RT, 15 h	90
2	MNBA (1.3 equiv), 4-PPY (3.0 equiv), CH_2Cl_2, RT, 15 h	87
3	DMNBA (1.3 equiv), DMAP (3.0 equiv), CH_2Cl_2, RT, 15 h	87
4	2,4,6-trichlorobenzoyl chloride (5.0 equiv), Et_3N (5.0 equiv), THF, 0°C to RT; DMAP (3.0 equiv), toluene, 60°C, 16 h	67
5	2,2-dipyridyl disulfide (3.0 equiv), Ph_3P (1.5 equiv), toluene, 80°C, 10 h	36

[a] DMAP = 4-dimethylaminopyridine, MNBA = 2-methyl-6-nitrobenzoic anhydride, 4-PPY = 4-piperidinopyridine, DMNBA = 2,6-dimethyl-4-nitrobenzoic anhydride.



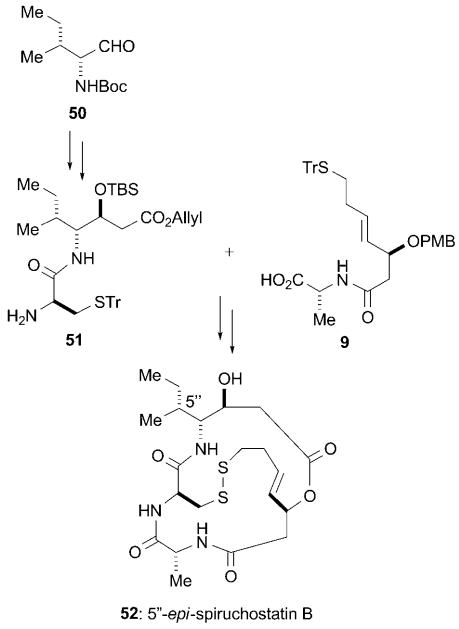
Scheme 7. Synthesis of segment **8**. a) LDA, CH_3CO_2Et (**12**), THF, −78°C; at −78°C, add **11**, 62% for **39**, 31% for **40** (**39**/**40** 2:1); b) Jones reagent, acetone, 0°C to RT, 95%; c) KBH_4, MeOH, −40°C, 90% for **40**, 6% for **39** (**40**/**39** 15:1); d) TBSCl, imidazole, DMF, RT, 96%; e) 1M NaOH, EtOH, RT, 80%; f) allyl bromide, K_2CO_3, DMF, RT, 96%; g) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; MeOH, RT, 92%; h) **13**, PyBOP, iPr_2NEt, MeCN, RT, 86%; i) TMSOTf, 2,6-lutidine, CH_2Cl_2, RT; MeOH, RT, 99%.



Scheme 8. Synthesis of spiruchostatin B (**2**). a) HATU, HOAt, iPr_2NEt, CH_2Cl_2, −30°C, 94%; b) DDQ, CH_2Cl_2/H_2O, RT, 85%; c) $[Pd(PPh_3)_4]$, morpholine, THF, RT, 91%; d) MNBA, DMAP, CH_2Cl_2, RT, 89%; e) I_2, $MeOH/CH_2Cl_2$, RT, 94%; f) HF·pyridine, pyridine, RT, 93%.

94% yield. Subsequent removal of the PMB and allyl protecting groups from **46** furnished the *seco*-acid **6** in 77% overall yield via allyl alcohol **47**. The crucial macrolactonization of **6** was also efficiently achieved under the same conditions as explored for the preparation of **37** (cf. entry 1, Table 3) resulting in the desired cyclization product **48** in 89% yield. By employing a reaction sequence similar to that described for the synthesis of **1** (cf. Scheme 6), compound **48** was transformed to **2**, $[\alpha]_D^{20} = -59.8°$ ($c = 1.02$ in MeOH) {lit.[3] $[\alpha]_D^{26} = -58.6°$ ($c = 0.11$ in MeOH)}, via *O*-TBS ether **49**. The ^1H and ^{13}C NMR spectra of the synthetic sample **2** were essentially identical to those reported for natural spiruchostatin B.[3] To assure the C5″ stereochemistry in **2**, we also pursued the synthesis of the other possible stereoisomer, namely, 5″-*epi*-spiruchostatin B (cf. **52**, Scheme 9). We describe this in the following section.

Completion of the total synthesis of 5″-*epi*-spiruchostatin B (52): As shown in Scheme 9, using (2*R*,3*R*)-*N*-Boc-D-isoleucinal (**50**)[20] instead of (2*R*,3*S*)-*N*-Boc-D-allo-isoleucinal (**11**)



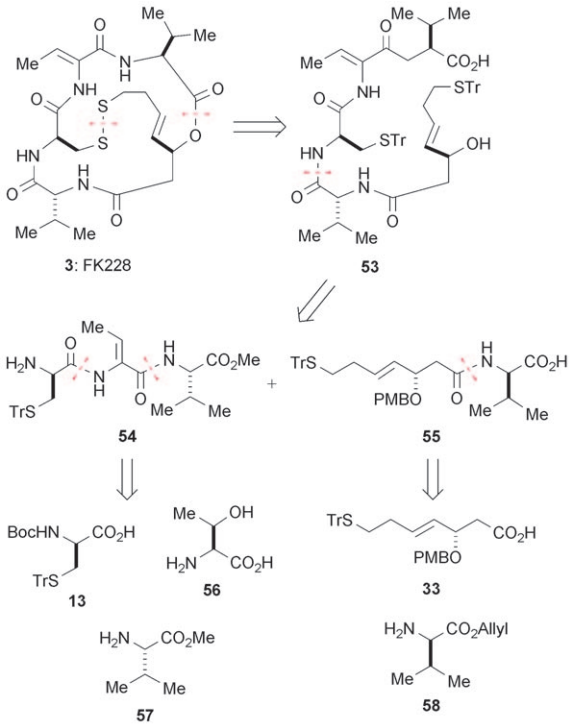
52: 5″-*epi*-spiruchostatin B

Scheme 9. Synthesis of 5″-*epi*-spiruchostatin B (**52**).

as the starting material, 5″-*epi*-spiruchostatin B (**52**) was synthesized through condensation of segments **51** and **9** in the same manner as described for the synthesis of spiruchostatin B (**2**) (cf. Schemes 7 and 8). The ^1H and ^{13}C NMR spectra of the synthesized **52** did not match those of natural **2**. From these results, the C5″ stereochemistry of **2** was unambiguously determined to be *S* configuration as depicted in Scheme 8.

Synthesis of FK228 (3): Having successfully synthesized spiruchostatins A (**1**) and B (**2**) as well as 5″-*epi*-spiruchostatin B (**52**) in a convergent and unified manner, we further investigated the synthesis of the final target FK228 (**3**) using the same strategy as explored earlier.

Primary synthetic plan: In our primary synthetic plan shown in Scheme 10, we envisaged that the target molecule **3** could

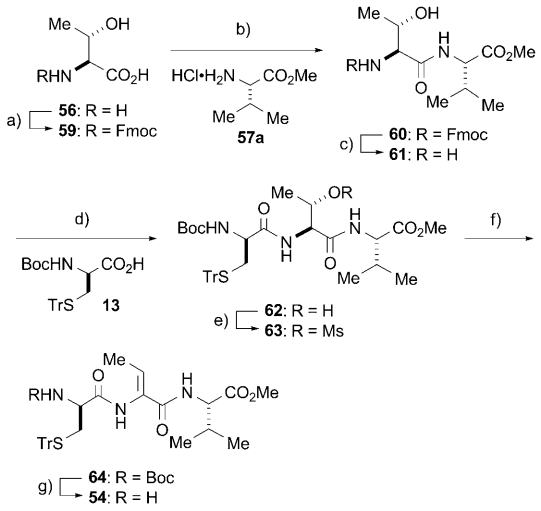


Scheme 10. Primary synthetic plan for FK228 (**3**).

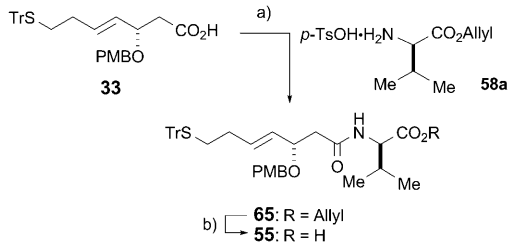
be synthesized by macrolactonization of *seco*-acid **53**, accessible by condensation of segments **54** and **55**, followed by a disulfide bond formation. Segment **54** would be formed by successive condensation of D-cysteine derivative **13**, L-threonine (**56**) and L-valine methyl ester (**57**). Segment **55** would, in turn, be prepared through a condensation of the common intermediate **33** and D-valine allyl ester (**58**).

Synthesis of segment 54: First, the synthesis of segment **54** was performed starting from L-threonine (**56**) (Scheme 11). After protection of the amino function in **56** with a 9-fluorenylmethoxycarbonyl (Fmoc) group, the resulting carbamate **59** was subjected to condensation with L-valine methyl ester hydrochloride (**57a**), producing the desired amide **60** in 96% overall yield. Subsequent deprotection of the *N*-Fmoc group in **60**, followed by condensation of the liberated amine **61** with D-cysteine derivative **13**, provided tripeptide **62** in 91% overall yield. This was further converted to the requisite segment **54** via a three-step sequence involving mesylation of the hydroxy group in **62** (quantitative yield), elimination of the resulting mesylate **63** (96%) and deprotection of the *N*-Boc group in **64** (73%).

Synthesis of segment 55: Segment **55**, the condensation partner of **54**, was readily synthesized in 69% overall yield by amide formation between the common intermediate **33** and D-valine allyl ester *p*-toluenesulfonate (**58a**) followed by deprotection of the allyl ester moiety in the resulting amide **65** (Scheme 12).



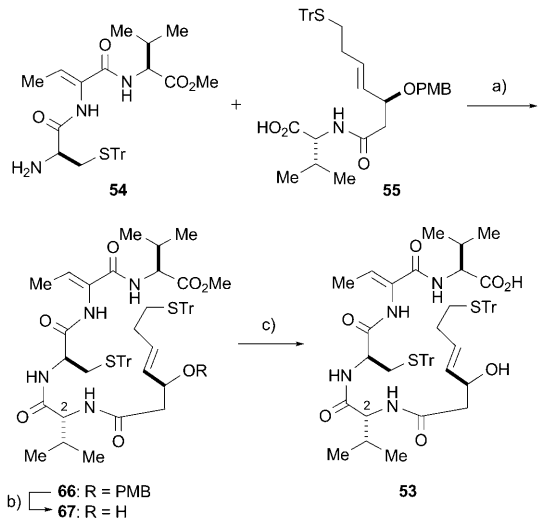
Scheme 11. Synthesis of segment **54**. a) FmocCl, 10% aq. Na_2CO_3, dioxane, RT; b) **57a**, PyBOP, *i*Pr_2NEt, MeCN, RT, 96% (2 steps); c) Et_2NH, MeCN, RT; d) **13**, PyBOP, *i*Pr_2NEt, MeCN, RT, 91% (2 steps); e) MsCl, Et_3N, CH_2Cl_2, 0°C, quant.; f) DABCO, THF, 0°C to RT, 90%; g) $BF_3{\cdot}Et_2O$, CH_2Cl_2, RT, 73%. FmocCl = 9-fluorenylmethoxycarbonyl chloride, MsCl = methanesulfonyl chloride, DABCO = 1,4-diazabicyclo-[2.2.2]octane.



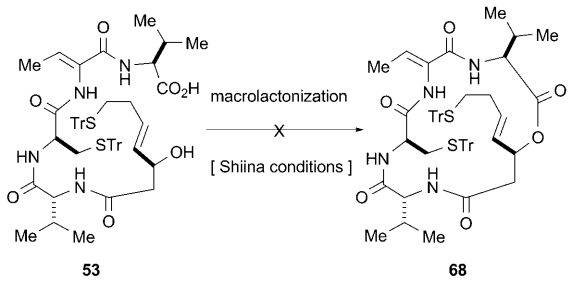
Scheme 12. Synthesis of segment **55**. a) **58a**, PyBOP, *i*Pr_2NEt, MeCN, RT, 73%; b) $[Pd(PPh_3)_4]$, morpholine, THF, RT, 94%.

Synthesis of *seco*-acid 53: Having obtained the requisite segments **54** and **55**, we next pursued the synthesis of *seco*-acid **53** by assembling the two segments (Scheme 13). Thus, condensation of **54** with **55** proceeded efficiently under the same conditions as mentioned previously (cf. entry 5, Table 2), providing the desired product **66** in 81% yield. This was further transformed to the requisite *seco*-acid **53** (77% yield in two steps) by removal of the two protecting groups via alcohol **67**.

Initial attempts to achieve the macrolactonization of *seco*-acid 53: With the key precursor in hand, we next attempted the macrolactonization of *seco*-acid **53** (Scheme 14). We had originally intended to achieve the macrolactonization by using the powerful and reliable Shiina reagents that had been very effective in the synthesis of spiruchostatins (cf. **5**→**37**, entries 1–3, Table 3; **6**→**48**, Scheme 8). However, to our chagrin, all attempts to realize this macrolactonization under the Shiina conditions [MNBA or DMNBA, DMAP or 4-PPY, CH_2Cl_2, RT] proved unsuccessful; none of the de-



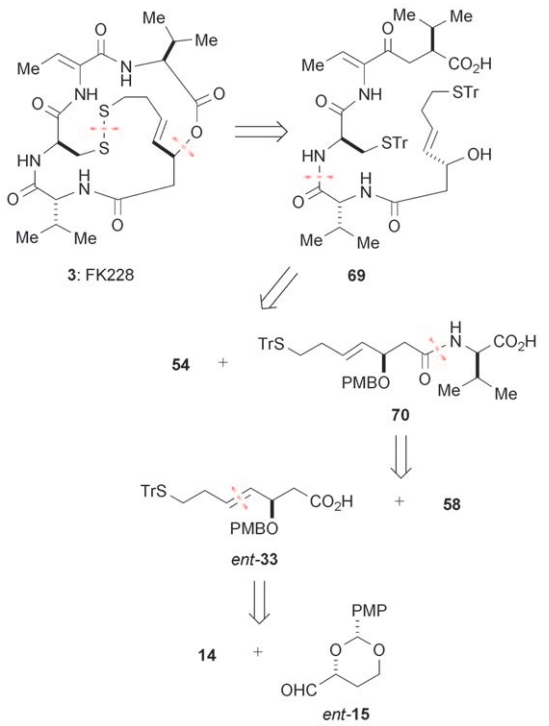
Scheme 13. Synthesis of *seco*-acid **53**. a) HATU, HOAt, iPr$_2$NEt, CH_2Cl_2, −30 °C, 81 %; b) DDQ, CH_2Cl_2/H_2O, RT, 83 %; c) LiOH, THF/H_2O, 0 °C, 93 %.



Scheme 14. Attempts on the macrolactonization of *seco*-acid **53**.

sired macrocyclization products **68** were obtained, and the starting material **53** was always recovered with a small amount of unidentified byproducts. We assumed that these failures resulted from the very low reactivity of the activated carboxylic acid, which we attributed to the presence of the sterically hindered isopropyl substituent adjacent to the carbonyl reaction site. Incidentally, similar findings were also reported recently by Ganesan et al.[13] in their total synthesis of FK228. These unsuccessful results forced us to modify our original synthetic plan. We describe this in the following section.

Modified synthetic plan: As mentioned in the preceding section, our initial attempts to realize the macrolactonization of *seco*-acid **53** resulted in failure; therefore, we settled on modifying our original synthetic plan based on the Simon's pioneering work[11] (Scheme 15). The modified synthetic plan involved Mitsunobu-based macrolactonization[14,37] of *seco*-acid **69**, which possesses the opposite stereochemistry at the hydroxy group in the mercaptoheptenoic acid part. The macrolactonization precursor **69** should be prepared starting from aldehyde *ent*-**15** (available from D-malic acid)

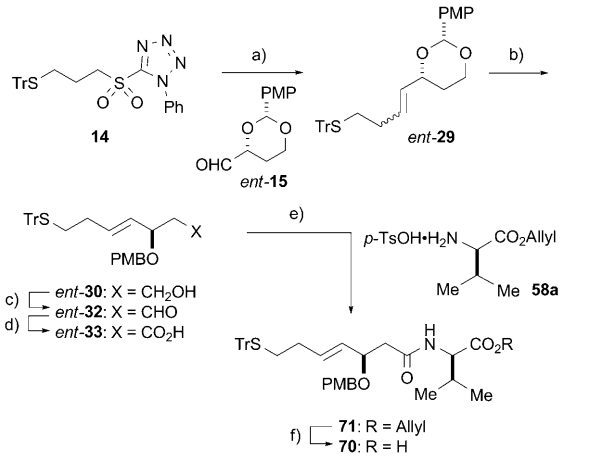


Scheme 15. Modified synthetic plan for FK228 (**3**).

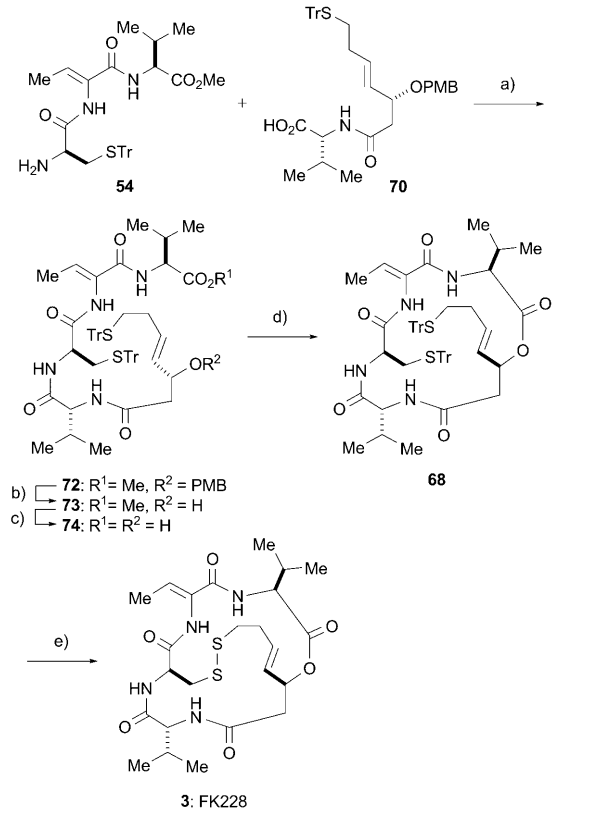
employing the same reaction sequence (cf. **14** + *ent*-**15** → *ent*-**33**, *ent*-**33** + **58** → **70**, **54** + **70** → **69**) as explored earlier.

Synthesis of segment 70: Segment **70**, which corresponds to the epimer of **55** (cf. Scheme 13) at the *O*-PMB group, was efficiently synthesized via the same reaction sequence as described for the preparation of **55** (Scheme 16). Thus, condensation of sulfone **14** with aldehyde *ent*-**15**[22] (readily prepared from D-malic acid) (66 %) followed by reductive acetal opening of the resulting olefin *ent*-**29** provided alcohol *ent*-**30** (57 %). After twofold oxidation of *ent*-**30**, the resulting carboxylic acid *ent*-**33** was subjected to condensation with D-valine derivative **58a** to afford amide **71** (67 % overall yield from *ent*-**30**). Finally, removal of the allyl protecting group from **71** furnished the requisite segment **70** (92 %).

Completion of the total synthesis of FK228 (3): With segment **70** synthesized, we next performed the synthesis of FK228 (**3**) (Scheme 17). Thus, condensation of segments **54** and **70**, under the same conditions as described previously, proceeded smoothly to deliver the desired product **72** (76 %), which was further converted to the requisite *seco*-acid **74** (85 % yield in two steps) via alcohol **73**. Following Simon's procedure,[11] the macrocyclization of **74** under Mitsunobu conditions was next examined. Thus, treatment of *seco*-acid **74** (~60 mg scale) with a large excess of Ph_3P (25 equiv) and diisopropyl azodicarboxylate (DIAD) (20 equiv) in the presence of *p*TsOH (5 equiv) in dry THF at 0 °C to room temperature for 4 h, and after purification



Scheme 16. Synthesis of segment **70**. a) $LiN(SiMe_3)_2$, DMF, −60 °C; at −60 °C, add *ent*-**15**, −60 to 0 °C, 66 % (*E*/*Z* ca. 5:1); b) DIBAL, toluene, 0 °C, 57 %; c) Dess–Martin periodinane, $NaHCO_3$, CH_2Cl_2, 0 °C to RT, 92 %; d) $NaClO_2$, NaH_2PO_4, DMSO/H_2O, 0 °C to RT; e) **58a**, PyBOP, iPr_2NEt, MeCN, RT, 73 % (2 steps); f) $[Pd(PPh_3)_4]$, morpholine, THF, RT, 92 %.



Scheme 17. Synthesis of FK228 (**3**). a) HATU, HOAt, iPr_2NEt, CH_2Cl_2, −20 °C, 76 %; b) DDQ, CH_2Cl_2/H_2O, RT, 91 %; c) LiOH, THF/H_2O, 0 °C, 93 %; d) DIAD, PPh_3, *p*TsOH, THF, 0 °C to RT, 62 %; e) I_2, MeOH/CH_2Cl_2, RT, 75 %. DIAD = diisopropyl azodicarboxylate.

by silica gel column chromatography, the desired macrolactone **68** was obtained in 62 % yield. Recently, Williams et al.[12] and Ganesan et al.[13] noted independently that they had encountered difficulties reproducing the 62 % yield reported by Simon and coworkers for this cyclization event in their FK228 syntheses, whereas our results were in accordance with the reported yield. Ultimately, disulfide bond formation accompanied by oxidative *S*-Tr deprotection of **68** resulted in the production of **3**, $[\alpha]_D^{20} = +37.6°$ ($c = 0.27$ in $CHCl_3$) {lit.[5b] $[\alpha]_D^{20} = +39°$ ($c = 1.0$ in $CHCl_3$)}, in 75 % yield. The spectroscopic properties (IR, ^1H and ^{13}C NMR, HRMS) of the synthetic sample **3** were identical with those reported for natural **3**.[5b]

Biological evaluation: The synthesized spiruchostatins A (**1**) and B (**2**), 5″-*epi*-spiruchostatin B (**52**) and FK228 (**3**) were evaluated for their HDAC inhibitory activity and their cell-growth inhibitory activity to determine the order of the potency and to reveal some novel aspects of structure–activity relationships (SAR).

HDAC inhibition assay: It has been reported that specific inhibition of class I HDACs is a useful mechanism for anticancer agents,[38] and that inhibition of class II HDACs may cause undesirable side effects such as the promotion of cardiac hypertrophy.[10,39] Spiruchostatin A (**1**) and FK228 (**3**) have been shown to display high selectivity for HDAC1 (class I) over HDAC6 (class II).[4a,7] Based on this information, compounds **1**–**3** and **52** were tested for their HDAC inhibitory activity against HDAC1 and HDAC6 to determine the degree of the potency and the isoform selectivity. Trichostatin A (TSA) was used as a reference compound. As summarized in Table 4, all compounds tested exhibited extremely potent inhibitory activity against HDAC1 in the low nanomolar range ($IC_{50} = 2.2$–3.6 nM). The order of the potency was estimated to be **2** ($IC_{50} = 2.2$ nM) ≈ **52** ($IC_{50} = 2.4$ nM) ≥ **1** ($IC_{50} = 3.3$ nM) ≈ **3** ($IC_{50} = 3.6$ nM) ⩾ TSA ($IC_{50} = 20$ nM). Interestingly, unnatural **52** showed almost the same potency as compared with **2**, highlighting that the C5″ stereochemistry in **2** has no influence on HDAC1 inhibition efficacy. As for HDAC6 inhibitory activity, all compounds tested were essentially inactive ($IC_{50} = 390$–3900 nM), as expected. Among the compounds tested, compound **52** showed the highest isoform selectivity, that is, 1625-fold differential for HDAC1 over HDAC6. Isoform selectivity

Table 4. HDAC Inhibitory activity of spiruchostatins A (**1**), B (**2**), 5″-*epi*-spiruchostatin B (**52**), and FK228 (**3**).

Compound	IC_{50}[a] [nM] HDAC1[b]	HDAC6[b]	SI[d]	EC_{1000}[e] [nM] cell HDAC
1	3.3	1600	485	8.8
2	2.2	1400	636	2.5
52	2.4	3900	1625	3.4
3	3.6	390	108	3.1
TSA[c]	20	63	3	18

[a] The concentration that induces 50 % inhibition against HDACs. [b] The enzyme assay was performed in the presence of 100 mM dithiothreitol (DTT). [c] Positive control as a representative HDAC inhibitor. [d] Selectivity index (HDAC6 IC_{50}/HDAC1 IC_{50}) as the selectivity toward class I HDAC1 over class II HDAC6. [e] The concentration that induces the luciferase activity 10-fold higher than the basal level.

(class I/II) is expressed, for convenience, as a selectivity index (SI) value. The order of the selectivity was estimated to be **52** (SI = 1625) > **2** (SI = 636) > **1** (SI = 485) > **3** (SI = 108) ≫ TSA (SI = 3). Notably, the isoform selectivity of **52** is 15-fold higher than that of FK228, which represents, to the best of our knowledge, the highest level of the class I/II selectivity among the natural depsipeptides and the synthesized depsipeptide analogues known to date. In addition, a good correlation between enzymatic (HDAC1) and cellular HDAC inhibitory activity was found for all of compounds tested. From these results, it was revealed that minor structural modification of the side chain on the statine part in spiruchostatins is quite effective in improving the isoform selectivity without loss of the potent HDAC inhibitory activity.

Cell-growth inhibition assay: The growth-inhibitory activity of **1**–**3** and **52** was evaluated using a panel of 39 human cancer cell lines in the Japanese Foundation for Cancer Research.[41] The number of cell lines and their origin (organ) are as follows: five breast, six central nervous system (brain), one melanoma, five ovary, two kidney, six stomach and two prostate cancers. Dose-response curves were measured at five different concentrations (10^{-10}–10^{-6} M) for each compound, and the concentration causing 50% cell-growth inhibition (GI_{50}) was compared with that of the control (Table 5). It is evident that all compounds tested exhibited extremely potent growth-inhibitory activity against almost all 39 cell lines in the nanomolar range, which closely corresponded to their ability to inhibit HDAC1. Although no distinct differences of the growth-inhibitory activity were observed among the test compounds, the order of the potency was estimated by the MG-MID value (mean value of GI_{50} over all cell lines tested) to be **2** (5.6 nM) ≈ **3** (6.2 nM) ≥ **52** (7.6 nM) > **1** (15 nM). Considering the HDAC inhibitory activity and the antiproliferative activity together, compound **52**, a novel congener of spiruchostatins, seems to be a promising candidate or a new possibility for the development of novel anticancer agents targeting class I HDAC1.

Table 5. Growth inhibition of spiruchostatins A (**1**), B (**2**), 5″-*epi*-spiruchostatin B (**52**) and FK228 (**3**) against a panel of 39 human cancer cell lines.

		GI_{50}[a] [nM]			
Origin of cancer	Cell line	**1**	**2**	**52**	**3**
breast	HBC-4	8.5	5.6	6.9	6.9
	BSY-1	19	8.3	15	8.5
	HBC-5	20	12	17	13
	MCF-7	7.1	4.1	4.8	4.2
	MDA-MB-231	6.8	4.5	4.6	5.5
central nervous system (brain)	U-251	7.1	3.6	4.5	3.9
	SF-268	16	4.5	5.9	4.9
	SF-295	22	4.8	7.1	4.0
	SF-539	6.3	3.3	4.5	3.6
	SNB-75	10	4.4	8.1	7.2
	SNB-78	36	6.3	9.6	9.6
colon	HCC2998	8.9	2.2	4.0	3.1
	KM-12	5.9	3.7	3.9	3.4
	HT-29	5.8	3.2	3.4	3.3
	HCT-15	660[b]	380[b]	390[b]	450[b]
	HCT-116	5.1	2.6	3.2	3.1
lung	NCI-H23	13	4.8	4.9	4.6
	NCI-H226	55	9.3	17	8.9
	NCI-H522	3.0[c]	1.3[c]	1.5[c]	1.8[c]
	NCI-H460	8.5	3.3	4.7	3.0
	A549	4.6	1.9	2.5	2.6
	DMS273	38	8.5	14	5.8
	DMS114	7.1	3.3	4.6	3.6
melanoma	LOX-IMVI	4.0	2.4	2.3	2.5
ovary	OVCAR-3	11	3.0	4.5	4.6
	OVCAR-4	47	13	28	20
	OVCAR-5	4.3	2.5	2.1	2.8
	OVCAR-8	16	4.6	5.9	5.5
	SK-OV-3	5.4	2.2	3.6	3.3
kidney	RXF-631 L	23	8.7	14	6.6
	ACHN	32	17	23	20
stomach	St-4	48	26	46	22
	MKN1	6.8	2.6	3.6	3.2
	MKN7	25	10	11	4.9
	MKN28	69	22	58	17
	MKN45	45	15	31	14
	MKN74	10	2.4	3.6	3.0
prostate	DU-145	21	4.7	5.5	6.0
	PC-3	29	8.7	7.8	18
	MG-MID[d]	15	5.6	7.6	6.2

[a] The concentration that induces 50% inhibition of cell growth compared to control. [b] The least sensitive cell. [c] The most sensitive cell. [d] Mean value of GI_{50} over all cell lines tested.

Conclusion

We have established unified synthetic routes to the bicyclic depsipeptide HDAC inhibitors spiruchostatins A (**1**) and B (**2**), 5″-*epi*-spiruchostatin B (**52**) and FK228 (**3**). The method explored features i) Julia-Kocienski olefination of sulfone **14** and aldehyde **15** or *ent*-**15** to install the requisite *E* olefin unit present in the critical segment **9** or **55** (**14** + **15** → **29**, Scheme 4; **14** + *ent*-**15** → *ent*-**29**, Scheme 16); ii) condensation of segment **7** or **8** with the common intermediate **9**, and segment **54** with segment **70** to directly assemble the crucial *seco*-acid **5**, **6** or **69** (**7** + **9** → **35**, Table 2; **8** + **9** → **46**, Scheme 8; **54** + **70** → **72**, Scheme 17); and iii) macrocyclization of **5**, **6**, or **69** using the Shiina method or the Mitsunobu method to construct the desired macrolactone **37**, **48** or **68** (**5** → **37**, Table 3; **6** → **48**, Scheme 8; **74** → **68**, Scheme 17). The C5″ stereochemistry of **2** was determined by the present synthesis. Preliminary biological evaluation of the synthesized compounds **1**–**3** and **52** determined the order of their efficacy and some novel aspects of the SAR study which would be useful for designing and development of anticancer agents with therapeutic potential that target isoform-selective inhibition of HDACs. On the basis of the present study further investigations concerning the synthesis of spiruchostatin analogs and the SAR study are currently underway and will be reported in due course.

Experimental Section

General techniques: All reactions involving air- and moisture-sensitive reagents were carried out using oven dried glassware and standard syringe-septum cap techniques. Routine monitoring of reaction were carried out using glass-supported Merck silica gel 60 F_{254} TLC plates. Flash column chromatography was performed on Kanto Chemical Silica Gel 60N (spherical, neutral 40–50 mm) with the solvents indicated.

All solvents and reagents were used as supplied with following exceptions. Tetrahydrofuran (THF), Et_2O and dioxane were freshly distilled from Na metal/benzophenone under argon. Toluene was distilled from Na metal under argon. *N*,*N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), CH_2Cl_2, MeCN, pyridine, iPr$_2$NH and iPr$_2$NEt were distilled from calcium hydride under argon. Measurements of optical rotations were performed with a JASCO DIP-370 automatic digital polarimeter. Melting points were taken on a Yanaco MP-3 micro melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were measured with a JEOL AL-400 (400 MHz) spectrometer. Chemical shifts were expressed in ppm using Me_4Si ($\delta=0$) as an internal standard. The following abbreviations are used: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br). Infrared (IR) spectral measurements were carried out with a JASCO FT/IR-4100 spectrometer. Low- and High-resolution mass (HRMS) spectra were measured on a JEOL JMS-DX 303/JMA-DA 5000 SYSTEM high resolution mass spectrometer.

Spiruchostatin A (1): HF·pyridine (0.20 mL) was added to a stirred solution of **38** (19.5 mg, 33 mmol) in pyridine (1.5 mL) at room temperature. After 14 h, the reaction mixture was diluted with EtOAc (60 mL), and the organic layer was washed successively with 3% aqueous HCl (3× 15 mL), saturated aqueous $NaHCO_3$ (2×15 mL) and brine (2×15 mL), then dried over Na_2SO_4. Concentration of the solvent in vacuo afforded a residue, which was purified by column chromatography ($CHCl_3$/MeOH 10:1) to give **1** (spiruchostatin A) (14.4 mg, 92%) as a white amorphous solid. $[\alpha]_D^{25}=-69.9°$ ($c=0.14$ in MeOH) {lit.[3] $[\alpha]_D^{26}=-63.6°$ ($c=0.14$ in MeOH)}; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.93$ (d, $J=6.8$ Hz, 3H), 1.03 (d, $J=6.8$ Hz, 3H), 1.51 (d, $J=7.3$ Hz, 3H), 2.38–2.47 (m, 2H), 2.56 (d, $J=13.2$ Hz, 1H), 2.70–2.77 (m, 5H), 3.09–3.28 (m, 4H), 4.25 (dq, $J=3.9$, 7.3 Hz, 1H), 4.54–4.59 (m, 1H), 4.89 (dt, $J=3.9$, 9.3 Hz, 1H), 5.50 (s, 1H), 5.65 (d, $J=15.1$ Hz, 1H), 5.92 (s, 1H), 6.28–6.31 (m, 4H), 6.71 (d, $J=9.3$ Hz, 1H), 7.40 ppm (d, $J=6.8$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $d=16.7$, 19.6, 20.6, 29.6, 33.3, 39.6, 40.7, 40.9, 52.3, 54.3, 63.8, 69.3, 70.5, 77.2, 128.6, 133.6, 169.0, 170.6, 170.9, 171.9 ppm; IR (neat): $\tilde{\nu}=$ 3375, 2933, 1633, 1542, 1160, 755 cm^{-1}; HRMS (FAB): m/z: calcd for $C_{20}H_{32}N_3O_6S_2$: 474.1732, found 474.1750 $[M+H]^+$. The IR, ^{1}H and ^{13}C NMR, and HRMS spectrum are essentially identical with those reported for natural spiruchostatin A.[3]

Spiruchostatin B (2): HF·pyridine (1.0 mL) was added to a stirring solution of **49** (68.3 mg, 0.11 mmol) in pyridine (2.0 mL) at room temperature. After 14 h, the reaction mixture was diluted with EtOAc (40 mL), and the organic layer was washed successively with 3% aqueous HCl (3× 10 mL), saturated aqueous $NaHCO_3$ (2×10 mL) and brine (2×10 mL), then dried over Na_2SO_4. Concentration of the solvent in vacuo afforded a residue, which was purified by column chromatography ($CHCl_3$/MeOH 20:1) to give **2** (spiruchostatin B) (51.3 mg, 93%) as a white amorphous solid. $[\alpha]_D^{25}=-59.8°$ ($c=1.02$ in MeOH) {lit.[3] $[\alpha]_D^{26}=-58.6°$ ($c=0.11$ in MeOH)}; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.89$ (t, $J=7.5$ Hz, 3H), 0.90 (d, $J=7.0$ Hz, 3H), 1.18–1.29 (m, 2H), 1.50 (d, $J=7.3$ Hz, 3H), 1.54–1.59 (m, 1H), 2.04–2.11 (m, 1H), 2.42–2.51 (m, 1H), 2.61 (d, $J=13.2$ Hz, 1H), 2.69–2.78 (m, 4H), 2.92–2.95 (m, 1H), 2.94 (ddd, $J=4.0$, 7.0, 9.0 Hz, 1H), 3.11–3.24 (m, 2H), 3.33 (dd, $J=7.3$, 13.1 Hz, 2H), 4.22 (dq, $J=3.9$, 7.3 Hz, 1H), 4.60–4.65 (m, 1H), 4.87 (dt, $J=3.4$, 9.2 Hz, 1H), 5.50–5.51 (m, 1H), 5.68 (d, $J=15.6$ Hz, 1H), 6.27 (s, 1H), 6.37–6.42 (m, 1H), 6.78 (d, $J=9.7$ Hz, 1H), 7.29 ppm (d, $J=9.3$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=11.5$, 15.4, 16.6, 27.1, 33.3, 36.3, 39.5, 40.5, 40.7, 41.3, 52.2, 54.5, 61.7, 68.2, 70.6, 128.6, 133.4, 169.2, 170.6, 171.2, 171.8 ppm; IR (neat): $\tilde{\nu}=3374$, 3332, 1731, 1660, 1539, 1273, 990 cm^{-1}; HRMS (FAB): m/z: calcd for $C_{21}H_{34}N_3O_6S_2$: 488.1889, found 488.1900 $[M+H]^+$. The IR, ^{1}H and ^{13}C NMR, and HRMS spectrum are essentially identical with those reported for natural spiruchostatin B.[3]

FK228 (3): A solution of **68** (35.9 mg, 35 mmol) in MeOH (8 mL) was added dropwise to a vigorously stirring solution of I_2 (175 mg, 0.69 mmol) in CH_2Cl_2 (63 mL, 0.5 mM concentration) over 10 min at room temperature. After 10 min, the reaction was quenched with 0.2 M ascorbic acid/citric acid buffer (7 mL, adjusted to pH 4.0) at room temperature, and the resulting mixture was extracted with EtOAc (3× 70 mL). The combined extracts were washed with brine (2×30 mL) and dried over Na_2SO_4. Concentration of the solvent in vacuo afforded a residue, which was purified by column chromatography (EtOAc) to give **3** (14.2 mg, 75%) as a white solid. $[\alpha]_D^{20}=+35.6°$ ($c=0.27$ in $CHCl_3$) {lit.[5b] $[\alpha]_D^{20}=+39°$ ($c=1.0$ in $CHCl_3$)}; ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 10:1): $\delta=1.00$ (d, $J=6.8$ Hz, 3H), 1.02 (d, $J=6.8$ Hz, 3H), 1.10 (d, $J=$ 6.8 Hz, 3H), 1.12 (d, $J=6.8$ Hz, 3H), 1.75 (d, $J=6.8$ Hz, 3H), 2.19–2.27 (m, 1H), 2.35–2.41 (m, 1H), 2.60–2.72 (m, 3H), 2.79–2.83 (m, 1H), 2.94–3.01 (m, 1H), 3.11–3.21 (m, 3H), 4.00 (dd, $J=4.2$, 6.1 Hz, 1H), 4.55 (dd, $J=3.9$, 7.8 Hz, 1H), 4.69–4.75 (m, 1H), 5.67–5.72 (m, 2H), 5.75–5.82 (m, 1H), 6.35 (q, $J=6.8$ Hz, 1H), 7.64 (d, $J=7.8$ Hz, 1H), 7.81 (d, $J=6.1$ Hz, 1H), 8.19 (d, $J=3.4$ Hz, 1H), 8.40 ppm (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD 10:1): $\delta=13.1$, 18.1, 18.3, 19.0, 19.1, 28.9, 30.1, 31.7, 34.5, 37.7, 37.8, 56.6, 58.2, 62.4, 70.1, 129.2, 129.6, 129.7, 130.4, 165.7, 168.4, 169.4, 171.4, 172.3 ppm; IR (KBr): $\tilde{\nu}=3337$, 2963, 2926, 1724, 1664, 1637, 1522, 1465, 1438, 1254, 1218, 1111, 1028, 979, 753 cm^{-1}; HRMS (FAB): m/z: calcd for $C_{24}H_{37}N_4O_6S_2$: 541.2155, found 541.2153 $[M+H]^+$. The IR, ^{1}H and ^{13}C NMR, and HRMS spectrum are identical with those reported for natural FK228.[5b]

5"-*epi*-Spiruchostatin B (52): This compound was prepared through condensation of **51** and **9** in the same manner as described for the synthesis of spiruchostatin B (**2**). $[\alpha]_D^{25}=-51.9°$ ($c=0.10$ in MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.82$ (t, $J=7.3$ Hz, 3H), 0.97 (d, $J=6.8$ Hz, 3H), 1.02–1.11 (m, 1H), 1.46 (d, $J=7.3$ Hz, 3H), 1.50–1.56 (m, 1H), 2.07 (q, $J=7.3$ Hz, 1H), 2.41–2.49 (m, 1H), 2.68 (d, $J=12.6$ Hz, 1H), 2.64–2.78 (m, 4H), 2.95 (q, $J=7.8$ Hz, 1H), 3.24 (dd, $J=6.8$, 13.2 Hz, 2H), 4.13–4.19 (m, 1H), 4.45–4.47 (m, 1H), 4.72 (dd, $J=8.3$, 13.2 Hz, 1H), 5.50 (br s, 1H), 5.75 (d, $J=15.6$ Hz, 1H), 6.16–6.18 (m, 1H), 6.98 (d, $J=$ 8.7 Hz, 1H), 7.07 (s, 1H), 7.42 ppm (d, $J=7.3$ Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=10.8$, 16.3, 16.4, 25.8, 32.6, 35.8, 39.5, 39.7, 39.9, 40.9, 52.2, 55.8, 61.7, 69.1, 71.0, 129.5, 132.8, 169.2, 171.1, 171.4, 171.9 ppm; IR (neat): $\tilde{\nu}=3375$, 3320, 2964, 1731, 1660, 1652, 1539, 1040, 891, 753 cm^{-1}; HRMS (FAB): m/z: calcd for $C_{21}H_{34}N_3O_6S_2$: 488.1889, found 488.1886 $[M+H]^+$. The ^{1}H and ^{13}C NMR spectrum are not identical with those reported for natural spiruchostatin B.[3]

HDACs preparation and enzyme inhibition assay:[40] In a 100 mm dish, 293T cells ($1–2\times10^6$) were grown for 24 h and transiently transfected with 10 mg each of the vector pcDNA3-HDAC1 for human HDAC1 or pcDNA3-mHDA2/HDAC6 for mouse HDAC6, using the LipofectAMINE2000 reagent (Invitrogen). After successive cultivation in DMEM for 24 h, the cells were washed with PBS and lysed by sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% NP40. The soluble fraction collected by microcentrifugation was precleared by incubation with protein A/G agarose beads (Roche). After the cleared supernatant had been incubated for 1 h at 4°C with 4 mg of an anti-FLAG M2 antibody (Sigma–Aldrich Inc.) for HDAC1 and HDAC6, the agarose beads were washed three times with lysis buffer and once with histone deacetylase buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10% glycerol. The bound proteins were released from the immune complex by incubation for 1 h at 4°C with 40 mg of the FLAG peptide (Sigma–Aldrich Inc.) in histone deacetylase buffer (200 mL). The supernatant was collected by centrifugation. For the enzyme assay, 10 mL of the enzyme fraction was added to 1 mL of fluorescent substrate (2 mM Ac-KGLGK(Ac)-MCA) and 9 mL of histone deacetylase buffer, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 30 mL of tripsin (20 mg mL^{-1}) and incubated at 37°C for 15 min. The released amino methyl coumarin (AMC) was measured using a fluorescence plate reader. The 50% inhibitory concentrations (IC_{50}) were determined as the means with SD calculated from at least three independent dose-response curves.

Cell HDAC inhibition assay (p21 promoter assay):[40] A luciferase reporter plasmid (pGW-FL) was constructed by cloning the 2.4 kb genomic fragment containing the transcription start site into HindIII and SmaI sites of the pGL3-Basic plasmid (Promega Co., Madison, WI). Mv1Lu (mink lung epithelial cell line) cells were transfected with the pGW-FL and a phagemid expressing neomycin/kanamycin resistance gene (pBK-CMV, Stratagene, La Jolla, CA) with the Lipofectamine reagent (Life Technology, Rockville, MD, USA). After the transfected cells had been selected by 400 mg mL^{-1} Geneticin (G418, Life Technology), colonies formed were isolated. One of the clones was selected and named MFLL-9. MFLL-9 expressed a low level of luciferase, whose activity was enhanced by TSA in a dose-dependent manner. MFLL-9 cells (1×10^5) cultured in a 96-well multi-well plate for 24 h were incubated for 20 h in the medium containing various concentrations of drugs. The luciferase activity of each cell lysate was measured with a LucLite luciferase Reporter Gene Assay Kit (Packard Instrument Co., Meriden, CT) and recorded with a Luminescencer-JNR luminometer (ATTO, Tokyo, Japan). Data were normalized to the protein concentration in cell lysates. Concentrations at which a drug induces the luciferase activity 10-fold higher than the basal level are presented as the 1000% effective concentration 1000% (EC_{1000}). The human wild-type p21 promoter luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein.

Cell-growth inhibition assay:[41] This experiment was carried out at the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. The screening panel consisted of the following 39 human cancer cell lines (HCC panel): breast cancer HBC-4, BSY-1, HBC-5, MCF-7, and MDA-MB-231; brain cancer U-251, SF-268, SF-295, SF-539, SNB-75, and SNB-78; colon cancer HCC2998, KM-12, HT-29, HCT-15, and HCT-116; lung cancer NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273, and DMS114; melanoma LOX-IMVI; ovarian cancer OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; renal cancer RXF-631 L and ACHN; stomach cancer St-4, MKN1, MKN7, MKN28, MKN45, and MKN74; prostate cancer DU-145 and PC-3. The GI_{50} (50% cell-growth inhibition) value for these cell lines was determined by using the sulforhodamine B colorimetric method.

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