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Potent and Orally Efficacious Bisthiazole-Based Histone Deacetylase Inhibitors

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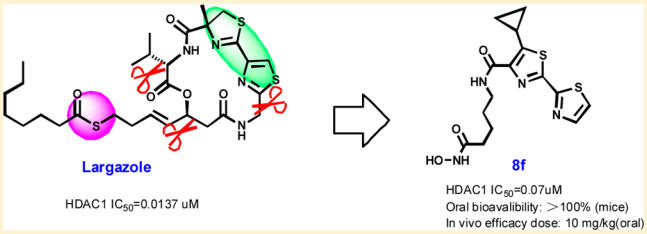
Supporting Information

ABSTRACT: Inspired by the thiazole−thiazoline cap group in natural product largazole, a series of structurally simplified bisthiazole-based histone deacetylase inhibitors were prepared and evaluated. Compound **8f** was evaluated in vivo in an experimental autoimmune encephalomyelitis (EAE) model and found to be orally efficacious in ameliorating clinical symptoms of EAE mice.



KEYWORDS: *Histone deacetylase inhibitors, largazole, bisthiazole*

Precise control of gene expression is important for many cellular processes including cell survival, apoptosis, and proliferation. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are two functionally opposing enzymes that control gene transcription via regulation of histone lysine acetylation. Histone acetylation neutralizes positive charges on lysine residues and disrupts nucleosome structure, allowing unfolding of associated DNA and binding of transcription factors, resulting in changes of gene expression.[1] HDACs have been validated as targets for cancer chemotherapy.[2−5] Of the 18 known human HDAC isoforms, which are divided into zinc-dependent classic HDACs (class I, II, and IV enzymes) and NAD^+-dependent class III enzymes (sirtuins),[6,7] evidence suggests that class I HDACs are most relevant to cancer growth.[8,9]

HDAC inhibitors (HDACis) block the deacetylation action of HDACs, resulting in hyperacetylation of histones and thereby affecting gene expression.[10] Currently reported small-molecule HDACis can be categorized into short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides/depsipeptides, electrophilic ketones, and benzamides.[11−13] A typical HDACi has a three-motif pharmacophoric model consisting of a recognition cap group, a hydrophobic linker, and a zinc-binding group (ZBG).[14] To date, two HDACis, vorinostat (SAHA) and romidepsin, have been approved by the FDA to treat cutaneous T cell lymphoma.[15,16] In addition, the pro-apoptotic activity of HDACis holds potential for treatment of inflammatory and autoimmune diseases.[17]

Multiple sclerosis (MS), one of the foremost causes of nontraumatic neurological disability in young adults, is a representative autoimmune disease, which induces inflammatory demyelination of the central nervous system (CNS).[18−20] Because of the limited understanding of the pathogenesis of MS, there remain many difficulties in treating MS. The development of novel therapeutic targets and agents is in high demand. HDACis have been shown to inhibit proinflammatory cytokine expression.[21,22] HDACi trichostatin (TSA) was reported to alleviate clinical symptoms of EAE, an animal model for MS.[23] In our previous work, HDACi valproic acid (VPA) was demonstrated to induce apoptosis in pathogenic T cells and ameliorate pathogenesis in EAE mice.[24] Accordingly, the idea of repurposing VPA for MS therapy has elicited interest.[25]

Largazole (**1a**, Figure 1) is a depsipeptide natural product isolated from the cyanobacterium *Symploca sp.* by Luesch and co-workers in 2008.[26] As a novel natural HDACi with potent antiproliferative activity and selectivity for cancer cells, it has attracted significant interest. To date more than ten groups have reported its total synthesis and SAR studies.[27−46]

We previously reported that C7-demethyl bisthiazole largazole analogue **2a** (Figure 1) has significantly decreased HDAC inhibitory activity and antiproliferative activity against HCT-116 colon cancer cells compared to largazole.[33] de Lera et al. have since reported that C7-demethyl largazole (**1c**) is equipotent to largazole,[34] indicating that the C7-methyl is unimportant for activity; they also reported the potent activity of **2a** against NB4 cells. Williams et al. have demonstrated that related thiazole-thiazole analogue **2b** displays intermediate HDAC inhibitory activity compared to largazole thiol.[31] These results inspired us to design a series of simplified analogues of **2a** that retains the bisthiazole cap group while cleaving across the β-hydroxy and thiazole fragments. The synthesis of ring-opened analogues **3a−3d** and **4a−4d** eventually culminated in the discovery of structurally simple bisthiazole-based HDACis **8a−8t** (Figure 1).

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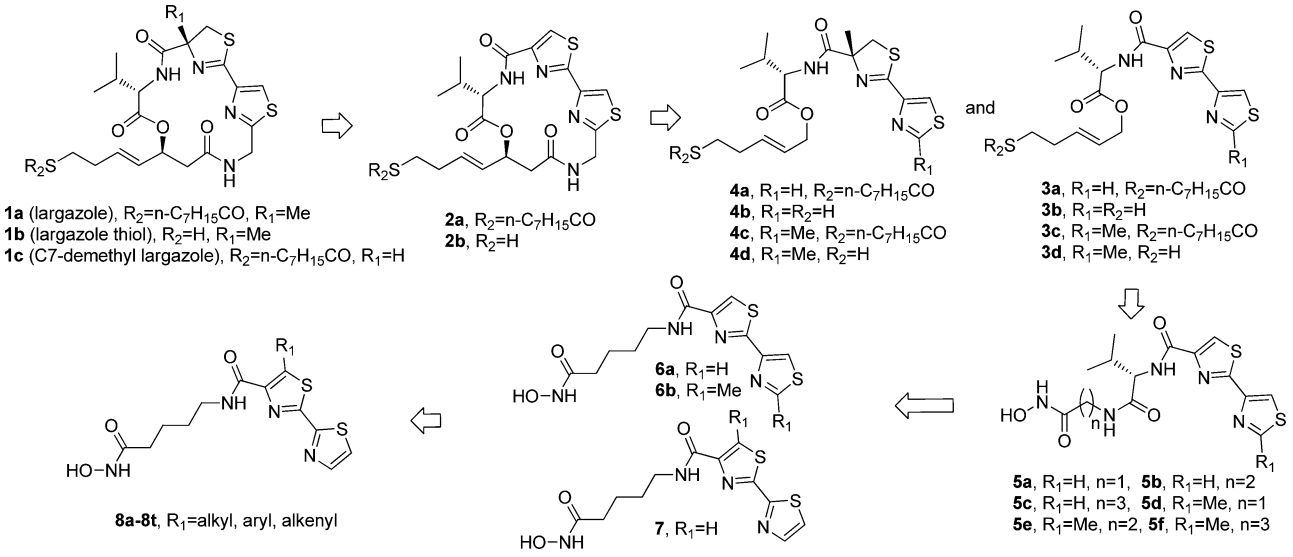
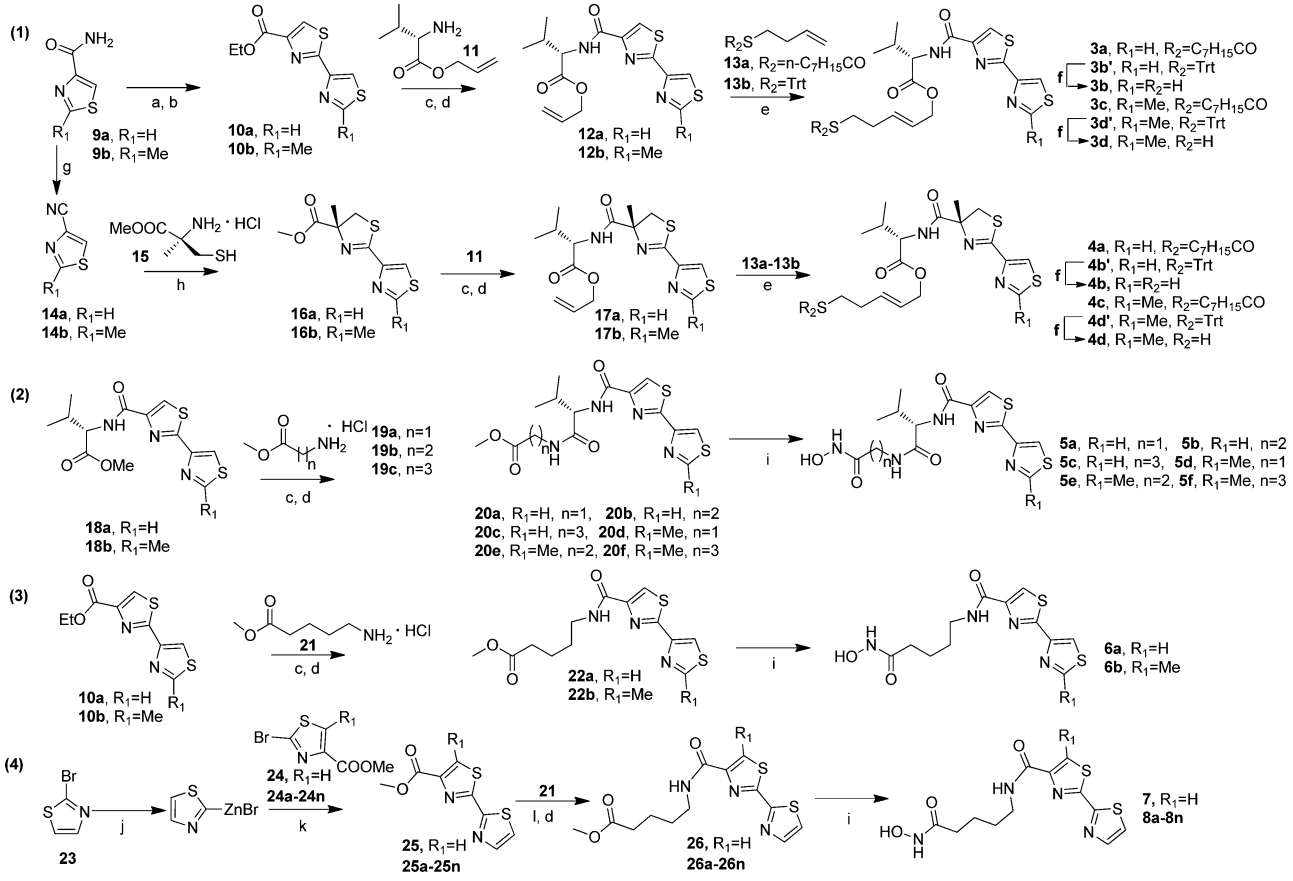


Figure 1. Structures of synthesized ring-opened analogues.

Scheme 1. Synthesis of Ring-Opened Analogues[a]



[a]Reagents and conditions: (a) Lawesson's reagent, DME, room temperature, 12 h. (b) $BrCH_2COCOOEt$, $KHCO_3$, TFAA, 2,6-lutidine, DME, room temperature, 10 h. (c) LiOH, $MeOH/H_2O$, room temperature, 8 h. (d) EDCI, HOBt, *i*-Pr_2NEt, DMF, room temperature, 12 h. (e) Grubbs' second generation catalyst, toluene, reflux, 8 h. (f) TFA, Et_3SiH, CH_2Cl_2, room temperature, 2 h. (g) Et_3N, TFAA, THF, room temperature, 2 h. (h) Et_3N, MeOH, 50 °C, 12 h. (i) $NH_2OH{\cdot}HCl$, KOH, MeOH, room temperature, 2 h. (j) Zn, dry THF, reflux, 2 h. (k) $Pd(OAc)_2$, PPh_3, toluene, reflux, 8 h. (l) NaOH, $MeOH/H_2O$, reflux, 1 h.

To access **3a**−**3d**, the thiazole−thiazole moiety was constructed by a modified Hantzsch procedure (route 1, Scheme 1).[47] The thiazoline−thiazole moiety in **4a**−**4d** was accessed via condensation of cyanothiazole with **15**.[48] Side chain installation via olefin cross-metathesis then afforded ring-opened analogues **3a**−**3d** and **4a**−**4d**.[27]

With ring-opened analogues **3a**−**3d** and **4a**−**4d** in hand, we assessed their inhibitory activities against human HDAC1, 3,

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Table 1. Inhibitory Activity against Human HDACs

			IC_{50} (μM)[d]		
compd	R_1	R_2	HDAC1	HDAC3	HDAC6
1a[a]		*n*-$C_7H_{15}CO$	0.0137	0.2450	11.50
1b[b]		H	0.0012	0.0034	0.049
2a[a]		*n*-$C_7H_{15}CO$	2.0	16.8	14.7
2b[b]		H	0.077	0.085	>30
3a	H	*n*-$C_7H_{15}CO$	1.90 ± 0.25	2.83 ± 0.34	NA[c]
3b	H	H	0.09 ± 0.02	0.06 ± 0.01	3.71 ± 0.27
3c	Me	*n*-$C_7H_{15}CO$	2.60 ± 0.45	3.56 ± 0.44	NA
3d	Me	H	0.12 ± 0.02	0.11 ± 0.02	4.32 ± 0.33
4a	H	*n*-$C_7H_{15}CO$	NA[c]	NA[c]	NA[c]
4b	H	H	16.82 ± 1.0	NA[c]	NA[c]
4c	Me	*n*-$C_7H_{15}CO$	NA[c]	NA[c]	NA[c]
4d	Me	H	13.86 ± 2.2	NA[c]	NA[c]

[a]Data reported in our previous work.[33] [b]Data reported by Williams et al.[31] [c]No inhibitory activity observed at 20 μg/mL. [d]Values are expressed as the mean ± SD, and the test was performed in triplicate.

and 6 (Table 1). Unexpectedly, the thiazoline−thiazole analogues (**4a**−**4d**) displayed significantly decreased inhibitory activity, while the thiazole−thiazole analogues (**3a**−**3d**) retained moderate activity. Compound **3b** in particular was nearly equipotent to the thiol form of C7-demethyl bisthiazole largazole **2b**, indicating that the thiazole−thiazole moiety might be a suitable cap group for a HDAC inhibitor.

A series of HDACis was then synthesized containing the thiazole−thiazole cap group and classic hydroxamic acid ZBG, as found in SAHA (**5a**−**5f**, Figure 1). To assess the potential impact of side chain length on activity,[30,49] amines **19** with variant linker length were condensed with acids prepared from esters **18a**−**18b** to afford respective amides **20a**−**20f**. Upon treatment with hydroxylamine, compounds **5a**−**5f** were rapidly accessed (route 2, Scheme 1). Disappointingly, most of these hydroxamic acid analogues showed significantly reduced inhibitory activity compared to compound **3b** (Table 2).

Table 2. Inhibitory Activity against Human HDACs

		IC_{50} (μM)[b]		
compd	R_1	HDAC1	HDAC3	HDAC6
5a	H	NA[a]	NA[a]	NA[a]
5b	H	9.69 ± 2.5	3.17 ± 0.45	NA[a]
5c	H	2.59 ± 0.49	1.64 ± 0.13	NA[a]
5d	Me	NA[a]	NA[a]	NA[a]
5e	Me	2.66 ± 0.71	2.22 ± 0.41	NA[a]
5f	Me	2.45 ± 0.31	1.58 ± 0.30	NA[a]
6a	H	0.45 ± 0.11	0.27 ± 0.06	2.24 ± 0.32
6b	Me	0.24 ± 0.03	0.15 ± 0.04	1.19 ± 0.25
7	H	0.21 ± 0.03	0.11 ± 0.02	1.10 ± 0.06

[a]No inhibitory activity observed at 20 μg/mL. [b]Values are expressed as the mean ± SD, and the test was performed in triplicate.

While compounds **5c**, **5e**, and **5f** showed moderate inhibition to HDAC1 and 3, compounds **5a** and **5d** displayed no inhibitory activity. These results were consistent with previous reports regarding the importance of the trans double bond linker in largazole analogues.[30]

Further structural simplification, featuring replacement of the chiral L-valine moiety with a chained carbon linker, was achieved by condensation of amine **21** with 2,4′-bisthiazole-4-carboxylic acids obtained by hydrolysis of **10a**,**10b** to give intermediates **22a**,**22b**. Upon treatment with hydroxylamine, hydoxamic acids **6a**,**6b** were afforded (route 3, Scheme 1). Negishi coupling of methyl 2-bromothiazole-4-carboxylates **24** with 2-thiazole-zinc bromide, prepared by treating 2-bromothiazole **23** with activated zinc dust, afforded 2,2′-bisthiazole **25**,[50] which was hydrolyzed to the corresponding acid (route 4, Scheme 1). Condensation of amine **21** with this acid and subsequent treatment with hydroxylamine afforded compound **7** directly. Analogues **6a**,**6b** and **7** displayed improved inhibitory activity compared with **5a**−**5f** (Table 2). Interestingly, compound **7**, which contains a 2,2′-bisthiazole moiety instead of a 2,4′-bisthiazole moiety, showed a 2-fold increase in activity compared to **6a**.

Encouraged by the comparable inhibitory activity of compound **7** and its facile synthetic accessibility, a series of 2,2′-bisthiazole-based hydroxamic acids **8a**−**8n** were synthesized (route 4, Scheme 1). The preparation of compounds **8o**−**8t** is additionally described in the Supporting Information. This approach allowed for rapid diversification of the substituent on the left thiazole ring (alkyl, aryl, and alkenyl, Table 3) and efficient synthesis of a small focused 2,2′-bisthiazole-based library.

Pleasingly, most of the R_1 substituents were well tolerated (Table 3). Among compounds **8a**−**8t**, **8s** was found to be the most potent HDAC1 inhibitor with IC_{50} as low as 0.01 μM. The isopropyl group in **8s** might contribute to key hydrophobic interactions with the protein surface. In contrast, compound **8p**, which lacks a hydrophobic substitute on the left thiazole, showed much reduced activity. Moreover, compound **8f** not only displayed potent activity against nine HDACs except HDAC7 and HDAC9 (Table S1, Supporting Information) but also showed excellent pharmacokinetic profiles characterized by high C_{max} (7.04 μg/mL) and $AUC_{0\text{-}t}$ (12.07 μg·h/mL) and high absolute bioavailability (F% = 118.7%) in mice (20 mg/kg, P.O., Table S2, Supporting Information). We thus went on to evaluate its efficacy in vivo.

Compound **8f** induced hyperdeacetylation of histone H3 and H4 and apoptosis of T cells in vitro. The cellular histone acetylation levels after treatment with **8f** were determined using mouse embryonic fibroblast (MEF) cells. The cells were cultured with **8f** (0.3, 1, or 3 μM) for 6 h, and cell lysates were subjected to Western blot analysis. As shown in Figure 2a,b, resting levels of acetylated histone H3 and H4 were low in MEF cells. When treated with **8f**, acetylation levels of histone H3 and H4 increased significantly in a dose-dependent manner.

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Table 3. Inhibitory Activity against Human HDACs

Compd	R_1	IC_{50} (μM)[a]		
		HDAC1	HDAC3	HDAC6
8a	*i*-Bu	0.05±0.00	0.10±0.01	1.23±0.13
8b	Me	0.61±0.07	0.62±0.03	1.11±0.09
8c	Et	0.03±0.00	0.26±0.06	0.93±0.08
8d	*i*-Pr	0.34±0.04	0.36±0.03	1.21±0.11
8e	*n*-Bu	0.03±0.00	0.19±0.04	0.63±0.05
8f	cyclopropyl	0.07±0.01	0.26±0.05	0.79±0.14
8g	cyclohexyl	0.35±0.05	0.33±0.02	0.75±0.10
8h	Ph	0.11±0.02	0.38±0.07	0.47±0.05
8i	*p*-F-C_6H_4	0.20±0.05	0.31±0.04	0.50±0.07
8j	*o*-Cl-*p*-F-C_6H_3	0.49±0.08	0.59±0.07	4.99±0.55
8k	o-Cl-C_6H_4	0.16±0.02	0.45±0.08	5.17±1.0
8l	*p*-NO_2-C_6H_4	0.11±0.03	0.20±0.02	1.07±0.04
8m	Bn	0.06±0.00	0.08±0.01	1.75±0.12
8n	CH_2CH_2OBn	0.05±0.01	0.16±0.03	0.95±0.04
8o		0.17±0.03	0.20±0.01	0.18±0.02
8p		1.57±0.20	2.59±0.15	9.54±1.4
8q		0.02±0.00	0.11±0.01	0.38±0.05
8r		0.12±0.01	0.38±0.08	2.30±0.17
8s		0.01±0.00	0.14±0.02	0.18±0.03
8t		0.63±0.13	0.28±0.04	0.19±0.03

[a]Values are expressed as the mean ± SD, and the test was performed in triplicate.

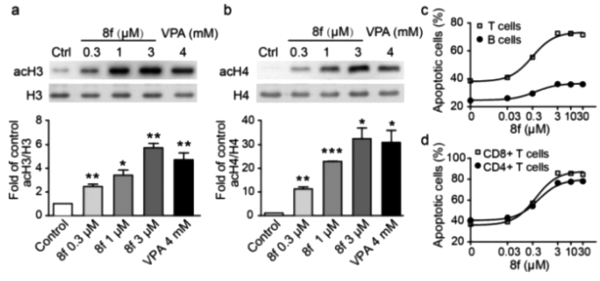

Figure 2. Compound **8f** induces histone acetylation in MEF cells and apoptosis in T cells. (a,b) MEF cells were treated with **8f** or VPA for 6 h and then lysed for Western blot analysis to detect the acetylation level of histone H3 and H4. Quantification of histone acetylation was analyzed with Quantity One software (Bio-Rad). (c,d) Mouse lymphocytes were stimulated in vitro and treated with **8f** for 36 h. Apoptosis was detected by Annexin V and PI staining, and the percentages of apoptotic cells (Annexin V single positive) were analyzed by flow cytometry.

Notably, at a concentration of 3 μM, **8f** displayed better inhibitory effects than VPA at 4 mM.

We next examined the effect of **8f** on apoptosis induction in lymphocytes; HDAC enzymes play important roles in cell survival and proliferation, and HDACis have been shown to induce apoptosis in several cell types.[51−53] Naive lymphocytes were isolated from mouse spleen and activated with anti-CD3 antibody or LPS, respectively, and cultured with various concentrations of **8f** for 36 h. Activated splenocytes displayed a relatively high apoptosis level (~40% for T cells) when cultured in vitro. Compound **8f** treatment further enhanced apoptosis in a dose-dependent way in T cells (~75%), but not in B cells (Figure 2c). Subpopulation analysis with cell surface markers revealed that **8f** enhanced apoptosis in both CD4+ and CD8+ T cells after activation in vitro (Figure 2d).

Compound 8f Ameliorates Clinical Symptoms of EAE Mice. HDACis have been shown to ameliorate inflammation in several autoimmune animal models,[54,55] including EAE.[23] To examine the in vivo effect of **8f**, EAE was induced in C57BL/6 mice by immunization with MOG_{35-55} peptide. Compound **8f** (10 mg/kg) was given b.i.d. via oral administration from day 3 or day 15 following immunization until the end of the experiment. PBS was used as a vehicle control. Compound **8f** treatment led to decreased incidence, reduced peak severity, and decreased cumulative clinical score of EAE when given from day 3 (Figure 3a and Table S2, Supporting Information).

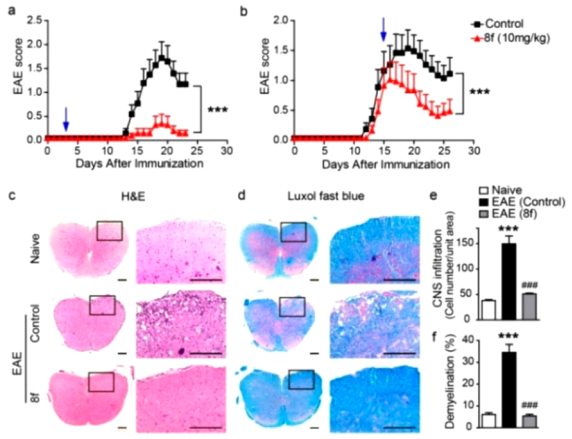

Figure 3. Compound **8f** alleviates clinical symptoms of EAE. (a,b) Clinical score of EAE mice treated with **8f** (n = 10) or PBS (n = 10) b.i.d. by oral administration on the day indicated by the arrow. Data are mean ± SEM, ***p < 0.001 (two-way ANOVA test). (c,d) Representative H&E or Luxol fast blue stained spinal cord sections isolated from naive, **8f**, or vehicle-treated EAE mice on day 22 postimmunization. Boxed areas in left columns are presented enlarged on the right. (e,f) Quantitative analysis of the number of spinal cord infiltrates in the sections presented in panel c and the amount of demyelination presented in panel d. Data are mean ± SEM; ***p < 0.001 versus naive group; ###p < 0.001 versus vehicle control (Student's t-test).

More interestingly, when given after the onset of the disease (day 15), **8f** (10 mg/kg) was still able to reduce the severity of EAE (Figure 3b and Table S2, Supporting Information), indicating both a therapeutic benefit and preventative effect of this drug.

Compound 8f Ameliorates CNS Pathology and Leukocytes Infiltration in EAE Mice. Histological examination of spinal cords was performed at day 22 postimmunization. A dramatic decrease in leukocyte infiltration in spinal cord was observed with **8f** treatment (Figures 3c,e). In vehicle-treated EAE mice, Luxol fast blue staining showed multiple widespread areas of myelin damage in white matter regions; demyelination was greatly ameliorated in **8f**-treated EAE mice (Figures 3d,f). Leukocyte infiltration was further assessed by immunofluorescent staining of the spinal cord sections with anti-CD45 antibody; **8f** treatment significantly reduced the number of CD45+ cells in the spinal cords (Figure S1a, Supporting Information).

Leukocyte infiltration into the CNS was isolated and quantified by flow cytometry at day 18 postimmunization. Results confirmed that the total CNS infiltrates (Figure S1b,

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Supporting Information) and CD4+ T cells accumulated in CNS (Figure S1c, Supporting Information) both decreased with **8f** treatment. IL-17-producing Th17 and IFN-γ-producing Th1 are the main pathogenic CD4+ T effector cells in EAE. With surface staining of CD4 and intracellular staining of IL-17 and IFN-γ, the number of both Th17 and Th1 cells was observed to decrease significantly in the CNS of **8f**-treated EAE mice (Figures S1c,d, Supporting Information).

In conclusion, a series of bisthiazole-based hydroxamic acids evolved from natural product largazole were synthesized as potent HDAC inhibitors. Several compounds exhibited low micromolar to midnanomolar IC_{50} values in in vitro HDAC inhibition assay. Compound **8f** was evaluated in vivo in an EAE model and showed efficacy in ameliorating clinical symptoms of EAE mice when administered orally. These bisthiazole-based HDACis are under further investigation for treatment of autoimmune diseases and cancer, and the results will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic details and characterization data for all compounds reported in this letter. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DME, ethylene glycol dimethyl ether; DMF, *N*, *N*-dimethylformamide; EAE, experimental autoimmune encephalomyelitis; EDCI, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride; IC_{50}, half maximal inhibitory concentration; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; HOBt, 1-hydroxybenztriazole; THF, tetrahydrofuran; Trt, triphenylmethyl

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