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Design and synthesis of novel hybrid benzamide–peptide histone deacetylase inhibitors

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ABSTRACT

We designed and synthesized a series of novel hybrid histone deacetylase inhibitors based on conjugation of benzamide-type inhibitors with either linear or cyclic peptides. Linear tetrapeptides (compounds **13** and **14**), cyclic tetrapeptides (compounds **1** and **11**), and heptanediamide–peptide conjugates (compounds **10**, **12**, **15** and **16**) were synthesized through on-resin solid-phase peptide synthesis (SPPS). All compounds were found to be moderate HDAC1 and HDAC3 inhibitors, with IC_{50} values ranging from 1.3 μM to 532 μM. Interestingly, compound **15** showed 19-fold selectivity for HDAC3 versus HDAC1.

Histone deacetylases (HDACs) play important roles in the regulation of gene expression, cell growth, and proliferation, by catalyzing the deacetylation of core histones, tubulin and other proteins.[1] HDAC inhibitors thus have the potential for use in cancer therapy.[2] Additionally, recent studies point to the potential therapeutic benefit of HDAC inhibitors in neurodegenerative diseases.[3] Eighteen HDACs have been identified in humans and are subdivided into four classes: Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8[4–7]), Class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10[8–11]), Class III HDACs (also known as Sirtuins, Sirt 1–7,[12] which are NAD^+-dependent enzymes) and the lone Class IV HDAC (HDAC11[13]). Class I, II and IV are Zn^{2+}-dependent enzymes.[14]

Recent studies[15,16] have demonstrated that HDAC inhibitors can induce growth arrest of tumor cells by inducing terminal differentiation and apoptosis. Numerous different HDAC inhibitors (HDACi) have been reported, including valproic acid (VPA),[17,18] suberoylanalide hydroxamic acid (SAHA),[19] **4b**,[20] and trapoxin[21] (Fig. 1). Five classes of HDAC inhibitors can be identified based on their structures: short chain aliphatic carboxylic acids (such as VPA), hydroxamic acids (such as SAHA), benzamides (such as **4b**), cyclic peptides (such as Trapoxin B), and the depsipeptides. Most HDAC inhibitors conform to a structural model, where (1) a cap region binds to the enzyme surface, (2) a Zn^{2+} coordinating group chelates this bound ion at the bottom of a tubular pocket, and (3) a five- to seven-atom spacer links the cap region to metal binding group.

We recently described a series of benzamide-type, pimelic diphenylamide HDAC inhibitors that show promise as therapeutics for the neurodegenerative diseases Friedreich's ataxia[20] and Huntington's disease.[22] In the course of these studies we identified both the enzyme specificities of these compounds (with a preference for HDAC3[23]) and specific amino acids in the core histones that are acetylated in cells after treatment with HDACi **4b**.[20]

In contrast to the pimelic diphenylamides, which exhibit high nM IC_{50} values for recombinant HDAC enzymes, cyclic tetrapeptides, such as trapoxin and apicidin, give low nanomolar IC_{50} values against HDACs. We reasoned that fusion of the benzamide-alkyl chain portion of **4b** with a cyclic peptide might yield a more

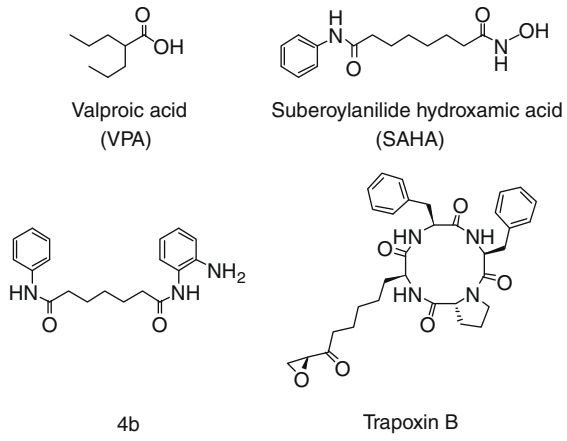

Figure 1. Chemical structures of HDAC inhibitors.

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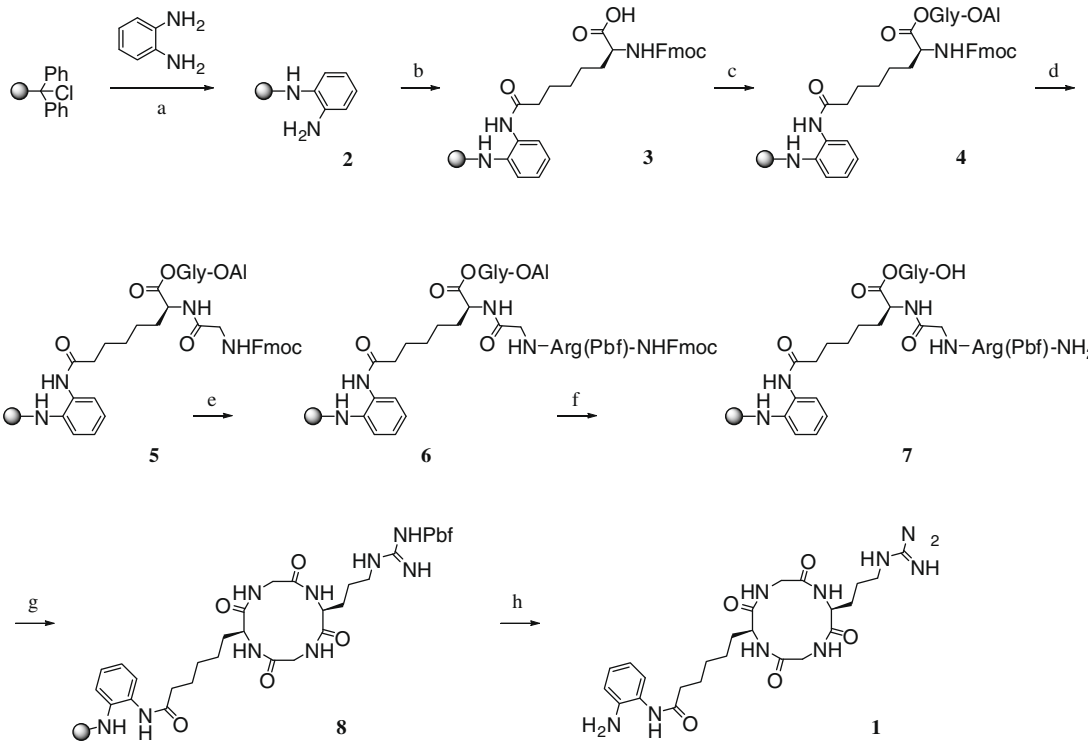


Scheme 1. Reagents and conditions: (a) 1,2-phenylenediamine (10 equiv), DIPEA (10 equiv), DMF, rt, overnight; (b) Fmoc-L-Asu, HBTU, HOBt, DIPEA, DMF, rt, 3 h, three times; (c) HBTU, HOBt, DIPEA, DMF, rt, 1 h then NH_2-Gly-OAl; (d) 20% piperidine in DMF (20 min × 2); Fmoc-Gly-OH, HBTU, HOBt, DIPEA, DMF; (e) 20% piperidine in DMF (20 min × 2); Fmoc-Arg(Pbf)-OH, HBTU, HOBt, DIPEA, DMF; (f) $Pd(PPh_3)_4$, Ph_3SiH, CH_2Cl_2 (anhy.) (2 h × 2) then 20% piperidine in DMF (20 min × 2); (g) TBTU, DIPEA, DMF, rt, 4 h; (h) TFA/H_2O (95: 5), 3 h, rt.

potent inhibitor than the parent compound **4b**. Cyclic tetrapeptide **1,** mimicking a four amino acid residue-sequence of histone H4 (RGKG, that is affected by **4b** in cells[20]), was designed, replacing the *K* residue with a benzamide-protected aminosuberic acid (Asu) residue, and making it a cyclic tetrapeptide. This compound has the same five carbon-linker length as in Trapoxin and Apicidin.

Cyclization is the key step in the synthesis of a cyclic peptide,[24,25] which generally involves preparation of the partially protected linear precursor by solution or solid phase approaches, followed by cyclization in solution under high dilution conditions.[26] Recently, Papini and co-workers[27] reported on-resin head-to-tail cyclization of cyclotetrapeptides, thus taking advantage of the pseudodilution phenomenon,[28] which favors intrachain resin-bound reactions, minimizing inter-chain interactions. In this study, Fmoc/tBu/OAl SPPS strategy[29] was applied as the orthogonal three-dimensional protection scheme in the synthesis of the linear tetrapeptide. The procedure is described in Scheme 1.

Compound **1** was prepared according to Scheme 1, starting from the trityl chloride resin, which was chosen for the trifunctional amino acids contained in the sequence and such that the final product would have a free amino group. This resin was found to react readily with an excess of 1,2-phenylenediamine[30] to give a high yield of the derivatized resin **2**, which after washing and drying was ready for the assembly of the desired ortho-diamine product.

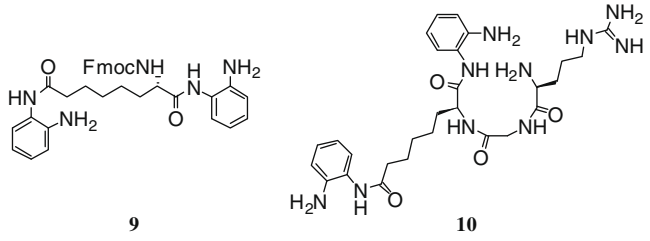


Figure 2. Structures of by-products **9** and **10**.

Linkage of Fmoc-L-Asu-OH to resin **2** was not as straight forward, since Fmoc-L-Asu-OH was a difunctional amino acid, in which both the functional groups were carboxylic groups. Several conditions for coupling were investigated, as shown in Table 1. When 2 equiv (based on Fmoc-L-Asu-OH) of coupling reagent (HATU) was used, by-product **9** (Fig. 2) was formed exclusively.

Table 1
Conditions for the linkage of Fmoc-L-Asu-OH to resin **2**

Condition	Equiv[b] of Fmoc-L-Asu-OH	Coupling reagents	Equiv[b] of coupling reagent	Ratio between **3** and **9**[a]
1	5	HATU, HOAt	10 equiv	Exclusive 9
2	5	HATU, HOAt	5 equiv	40: 60
3	5	PyBOP	5 equiv	50:50
4	5	EDCI, HOBt	5 equiv	60:40
5	5	HBTU, HOBt	5 equiv	70:30

[a] Ratio between **3** and **9** was determined by LC/MS.
[b] Equiv was compared to resin **2**.

When 1 equiv (based on Fmoc-L-Asu-OH) of coupling reagent was used, the ratio between **3** and **9** was decreased when coupling reagent varied from HATU (condition 2), PyBOP (condition 3), EDCI (condition 4), and HBTU (condition 5). The best result was obtained when HBTU, HOBt (1 equiv according to Fmoc-L-Asu-OH, 5 equiv according to resin **2**) was used and the ratio between **3** and **9** was 2.3 (condition 5). We use condition 5 for further synthesis.

With resin **3** in hand, we next installed NH_2-Gly-OAl directly onto resin **3** using HBTU/HOBt/DIPEA to afford resin **4**. Deprotection with 20% piperidine in DMF, followed by direct coupling with Fmoc-Gly-OH/HBTU/HOBt/DIPEA, affords resin **5**. Again treatment with 20% piperidine in DMF, followed by direct coupling with Fmoc-Arg(Pbf)-OH/HBTU/HOBt/DIPEA afforded resin **6**. Thus, the tetrapeptide skeleton was formed.

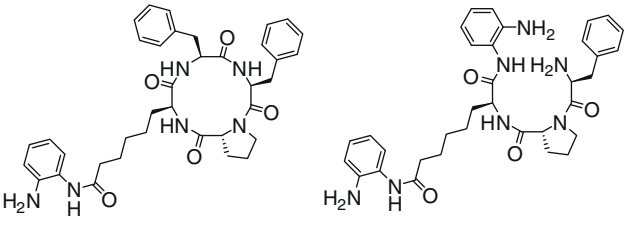
To avoid a nucleophilic attack of the free amino function leading to by-products, deprotection of the C-terminal carboxyl function must be carried out before the last Fmoc removal.[31] Allyl deprotection was performed using $Pd(PPh_3)_4/Ph_3SiH$ in CH_2Cl_2. Deprotection with 20% piperidine in DMF afforded the unprotected tetrapeptide **7** on resin. As to the cylization reaction, the following coupling conditions were tried: PyBOP/DIPEA, HATU/HOAt/DIPEA, and TBTU/DIPEA. The desired cyclic tetrapeptide was not obtained using the first two coupling conditions. TBTU/DIPEA gave the desired cyclic tetrapeptide on resin. Cleavage from the resin with TFA/H_2O (95:5) provided tetrapeptide **1** with 6% yield (over-all). By-product **10** (Fig. 2) was obtained with 4% yield (over-all) as product of compound **9**.

Cyclic tetrapeptide **11** (Fig. 3) was designed, mimicking trapoxin by replacing the side chain with benzamide protected aminosuberic acid. We synthesized compound **11** with 5% yield (overall) following the same procedure as described above (Scheme 1). Compound **12** was formed as the by-product with 3% yield (overall).

We also prepared compounds **13** and **14** (Fig. 4), the linear tetrapeptide versions of compounds **1** and **11**, respectively.

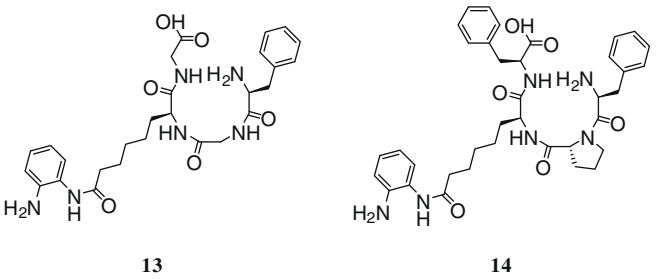
A new type of HDAC inhibitor, exemplified by **15** and **16,** was synthesized where amino acid units were incorporated into the aliphatic linker of **4b**. Compounds **15** and **16** were synthesized as shown in Scheme 2 using SPPS. Starting from resin **2**, Fmoc-β-Ala-OH was coupled to resin using HBTU/HOBt/DIPEA conditions. Treatment with 20% piperidine in DMF, followed by amino acid coupling (glycine for product **15**, arginine for product **16**) gave **18a** and **18b**. Deprotection of Fmoc and reaction with *p*-toluoyl chloride, followed by cleavage with TFA/H_2O (95:5), gave the desired products **15** and **16**.

The synthesized inhibitors **1**, **10**, **11**, **12**, **13**, **14**, **15**, and **16** were screened against recombinant human HDAC1 and HDAC3, and IC_{50}s for inhibition of these enzymes are summarized in Table 2. Some interesting trends were observed in these measurements. As far as activity is concerned, cyclo tetrapeptide **1** is stronger than linear tetrapeptide **13,** but weaker than N^1,N^7-bis(2-aminophenyl) heptanediamide-like compound **10**. The same trend is also observed with cyclo tetrapeptide **11**, linear tetrapeptide **14** and hep-



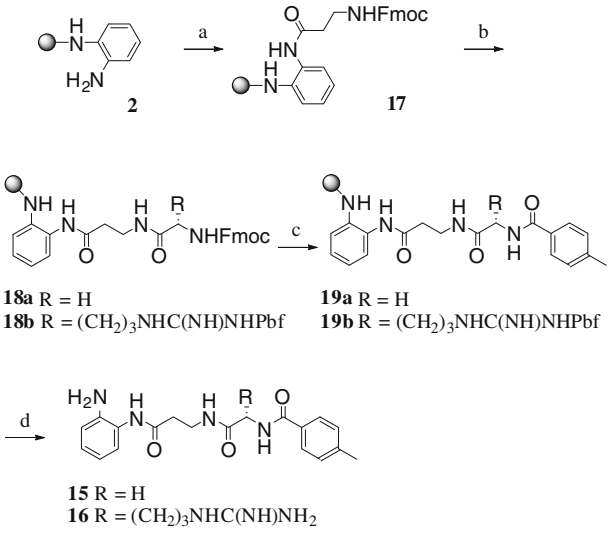
11 (Trapoxin mimic) **12**

Figure 3. Structures of **11** and by-product **12**.



13 **14**

Figure 4. Structures of **13** and **14**.



2 **17**

18a R = H
18b R = $(CH_2)_3NHC(NH)NHPbf$

19a R = H
19b R = $(CH_2)_3NHC(NH)NHPbf$

15 R = H
16 R = $(CH_2)_3NHC(NH)NH_2$

Scheme 2. Reagents and conditions: (a) Fmoc-β-Ala-OH, HBTU, HOBt, DIPEA, DMF; (b) 20% piperidine in DMF (20 min × 2); Fmoc-Gly-OH for **18a**/Fmoc-Arg(Pbf)-OH for **18b**, HBTU, HOBt, DIPEA, DMF; (c) 20% piperidine in DMF (20 min × 2); *p*-toluoyl chloride, DIPEA, CH_2Cl_2; (d) TFA/H_2O (95: 5), 3 h, rt.

Table 2
Inhibitory activity on recombinant HDAC1 and HDAC3

Compds	HDAC1 IC_{50}[a] (μM)	HDAC3 IC_{50}[a] (μM)
1	191(±27.8)	46.9 (±17.1)
10	4.7 (±0.2)	1.3 (±0.7)
13	532 (±37.7)	69.3 (±6.5)
15	83.9 (±21.4)	4.3 (±1.2)
16	9.2 (±0.5)	2.2 (±0.02)
11	20.5 (±3.0)	5.6 (±0.5)
12	7.3 (±1.4)	3.4 (±0.3)
14	84.2 (±15.9)	27.2 (±8.5)

[a] Values are means of two experiments with standard error given in parentheses.

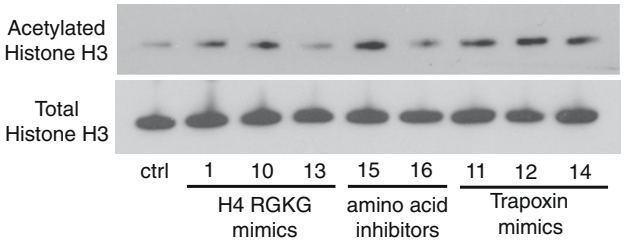


Figure 5. Effects of compounds **1**, **10**, **13**, **15**, **16**, **11**, **12**, and **14** on histone H3 in GM15850 cells.

tanediamide-like compound **12**. In conclusion, with the same amino acid sequence, the linear tetrapeptide is the weakest, cyclo tetrapeptide is somewhat stronger and the heptanediamide-like compound is the strongest inhibitor, respectively. When compared to the known cyclotetrapeptide Apicidin (IC_{50} = 4 nM)[32] and Trapoxin B (IC_{50} = 0.11 nM for HDAC1),[33] the activities of our compounds have decreased considerably. Optimal activity was observed with compound **10** (IC_{50} = 4.7 μM for HDAC1 and 1.3 μM for HDAC3). Surprisingly, compound **15** shows a 19-fold selectivity between HDAC1 and HDAC3, with a clear preference for HDAC3.

The effects of **1**, **10**, **11**, **12**, **13**, **14**, **15**, and **16** (at 20 μM concentration) on the acetylation levels of endogenous histone H3 in a human lymphoblastoid cell line (GM15850, Coriell Institute) were determined by western blot analysis, after 48 h incubations. The result of this experiment is shown in Fig. 5. For the H4 RGKG mimics, **10** showed relatively stronger effects on acetylated H3, **13** showed no effect and **1** showed moderate effects on acetylation. These results are consistent with the IC_{50} values for recombinant HDACs (Table 2). As for the trapoxin mimics, the same trend was observed; that is, the lower the IC_{50} value for each compound (Table 2), the stronger the effect on histone H3 acetylation in cells. As for the amino acid inhibitors, compound **15** showed a relatively strong effect on cellular histone H3 acetylation, while compound **16** showed no such effect. We believe that the positively charged Arg group in compound **16** may hinder the compound to penetrate cells, while the relative neutral compound **15** may allow cell penetration. This difference in charge may explain why compounds **15** and **16** have remarkable differences in their ability to cause histone H3 acetylation in cells but nearly equal IC_{50} values for inhibition of recombinant HDAC3, and compound **15** has a higher IC_{50} for HDAC1 than compound **16**.

In conclusion, we have synthesized two different types of benzamide compounds. Most show moderate inhibitory activity on HDAC1 and HDAC3. Compound **15** shows 19-fold selectivity between HDAC3 and HDAC1. Further studies will be carried out on compound **15**.

Acknowledgments

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