ORGANIC LETTERS

2003
Vol. 5, No. 26
5079−5082

Cyclic Tetrapeptides Bearing a Sulfhydryl Group Potently Inhibit Histone Deacetylases

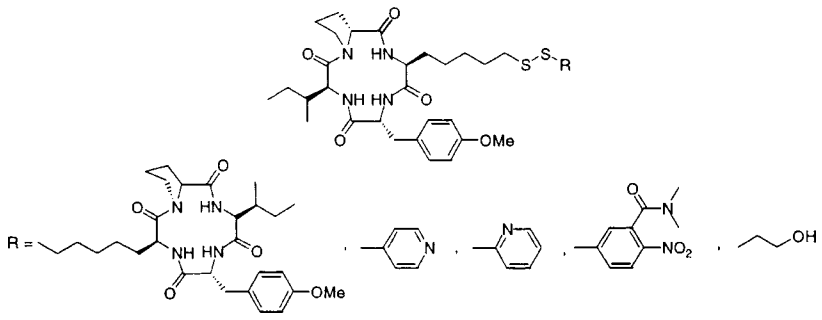
Norikazu Nishino,*,†,‡ Binoy Jose,‡ Shinji Okamura,† Shutoku Ebisusaki,† Tamaki Kato,† Yuko Sumida,‡ and Minoru Yoshida‡,§

Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan, RIKEN, Saitama 351-0198, Japan, and CREST Research Project, Japan Science and Technology Agency, Saitama 332-001, Japan

nishino@life.kyutech.ac.jp

Received October 28, 2003

ABSTRACT



New inhibitors of histone deacetylase (HDAC) containing a sulfhydryl group were designed on the basis of the corresponding hydroxamic acid (CHAP31) and FK228. Their disulfide dimers and hybrids exhibited potent HDAC inhibitory activity in vivo with potential as anticancer prodrugs.

The reversible acetylation of ϵ-amino groups of lysine residues clustered near the amino terminus of nucleosomal histones by histone deacetylases (HDACs) and histone acetyl transferases (HATs) has a significant influence on the chromatin superstructure and on the interactions of DNA with transcriptional regulators.[1] Modification of the level of histone acetylation and its consequences have received enormous interest in recent years, and increasing evidence supports their importance for basic cellular functions such as DNA replication, transcription, differentiation, and apoptosis. Aberrant histone acetylation emerging from HAT mutations or abnormal recruitment of HDACs has been correlated with carcinogenesis. Inappropriate recruitment of HDACs provides a common molecular mechanism by which genes necessary for proper differentiation or growth suppression can be silenced, leading to excessive proliferation. It is therefore proposed that HDACs are a potential target for the development of a small-molecule anticancer agent.

A number of natural and synthetic HDAC inhibitors have been reported, and in recent years importance of HDAC inhibitors has increased due to their efficacy against many malignant diseases. Several of these HDAC inhibitors inhibit tumor growth, and many of these are under clinical trials. Trichostatin A (TSA),[2] a cyclic tetrapeptide family, including trapoxin (TPX),[3] chlamydocin,[4] HC toxin,[5] Cyl-1, Cyl-2,[6]

† Kyushu Institute of Technology.
‡ CREST Research Project.
§ RIKEN.

(1) (a) Grozinger, C. M.; Schreiber. S. L. *Chem. Biol.* **2002**, *9*, 3−16. (b) Kouzarides, T. *Curr. Opin. Genev. Dev.* **1999**, *9*, 40−48. (c) Hassig, C. A.; Schreiber, S. L. *Curr. Opin. Chem. Biol.* **1997**, *1*, 300−308.

(2) Yoshida, M.; Kijima, M.; Akita, M.; Beppu, T. *J. Biol. Chem.* **1990**, *265*, 17174−17179.

(3) Kijima, M.; Yoshida M.; Suguta, K.; Horinouchi, S.; Beppu, T. *J. Biol. Chem.* **1993**, *268*, 22249−22435.

10.1021/ol036098e CCC: $25.00
Published on Web 12/04/2003

WF-3161,[7] apicidin,[8] and the recent depsipeptide FK228 (formerly FR901228)[9] are naturally occurring HDAC inhibitors (Figure 1). Inhibitors such as butyrate,[10] valproate,[11] suberoyl anilide hydroxamic acid,[12] analogues of TSA,[13] and benzamide derivatives of MS-275[14] were prepared through synthetic methods.

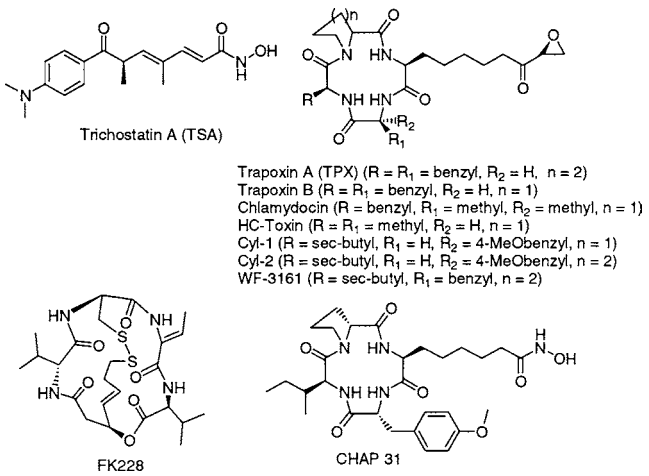


Figure 1. Structures of naturally occurring HDAC inhibitors and CHAP31.

We have reported a group of synthetic HDAC inhibitors (cyclic hydroxamic acid containing peptides; CHAPs) having a cyclic tetrapeptide scaffold and hydroxamic acid as the zinc-binding functional group.[15] The SAR study on CHAPs revealed that the biological activity of these inhibitors can be changed by the number of amino acids constituting the ring structure, the pattern of the combination of amino acid chirality, and the side chain structure of each amino acid. Recently, we demonstrated that FK228 serves as a stable natural prodrug that inhibits class I HDAC enzymes. FK228 is activated by the glutathione-mediated reduction of the disulfide bond to sulfhydryl, and this sulfhydryl group is coordinated to the zinc metal ion present in the HDAC binding pocket.[16] In this work, we report a new series of HDAC inhibitors, sulfur-containing cyclic peptides (SCOPs), by replacing the L-Aoe [(2*S*,9*S*)-2-amino-8-oxo-9,10-epoxydecanoic acid] moiety of natural cyclic tetrapeptides with L-2-amino-7-mercaptoheptanoic acid (L-Am7). On the basis of glutathione-mediated cellular reduction of disulfide bonds, we synthesized SCOPs as homodimers (disulfide dimers) and disulfide hybrids with several mercaptans.

Crystal structure studies on histone deacetylase-like protein (HDLP) revealed the presence of a zinc metal ion at the bottom of a narrow binding pocket, and the deacetylation proceeds through an oxyanion intermediate (Figure 1a).[17] Preliminary requirement for an effective inhibitor of HDAC is an efficient zinc-coordinating ligand connected to a hydrophobic scaffold by means of a spacer. The HDAC activity is varied with the binding nature of the zinc-chelating functionality, the length of the spacer, and the interactions of the groups present in the scaffold to the rim of the active site pocket. In our previous work, we found that the cyclic tetrapeptide is effective as a scaffold for potent HDAC inhibitors. CHAP 31, one of the several cyclic tetrapeptide inhibitors we synthesized having the same scaffold as that in natural cyclic tetrapeptide Cyl-1 and hydroxamic acid as a functional group, is an excellent inhibitor of HDAC and showed good selectivity during animal tests. Hydroxamic acid can chelate with zinc ion in HDAC effectively (Figure 1b). Because of the high HDAC inhibitory activity of FK228 and the increased activity in the presence of dithiotritol (DTT), which is known to reduce disulfide bonds, we speculate that a synthetic compound with a tetrapeptide scaffold and sulfhydryl zinc binding moiety can give better results ascribed to the strong ligation of zinc ion to sulfide ion (Figure 1c). Further, sulfhydryl-containing compounds such as thermolysin and carboxypeptidases are known as inhibitors of metalloproteases.[18] For these reasons, we proposed to synthesize new inhibitors for HDAC containing a natural cyclic tetrapeptide scaffold and a sulfhydryl group

(4) Closse, A.; Hugenin, R. *Helv. Chim. Acta* **1974**, *57*, 533−545.

(5) Shute, R. E.; Dunlap. B.; Rich, D. H. *J. Med. Chem.* **1987**, *30*, 71−78.

(6) Hirota, A.; Suzuki, A.; Aizawa, K.; Tamura, S. *Biomed. Mass. Specrom.* **2974**, *1*, 15−19.

(7) Umehara, K.; Nahahara, K.; Kiyota, S.; Iwami, M.; Okamoto, M.; Tanaka, H.; Kohsaka, M.; Aoki, H.; Imanaka, H. *J. Antibiot.* **1983**, *36*, 478−483.

(8) (a) Darkin-Rattray, S. J.; Gurnett, A. M.; Myers, R. W.; Dulski, P. M.; Crumley, T. M.; Allocco, J. J.; Cannova, C.; Meinke, P. T.; Colletti, S. L.; Bednarek, M. A.; Singh, S. B.; Goetz, M. A.; Dombrowski, A. W.; Polishook, J. D.; Schmatz, D. M. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 13143−13147. (b) Meinke, P. T.; Liberator, P. *Curr. Med. Chem.* **2001**, *8*, 211−235. (c) Han, J. W.; Ahn, S. H.; Park. S. H.; Wang, S. Y.; Bae, G. U.; Seo, D. W.; Known, H. K.; Hong, S.; Lee, Y. W.; Lee, H. W. *Cancer Res.* **2000**, *60*, 6068−6074.

(9) (a) Ueda, H.; Nakajima, H.; Hori, Y.; Fujita, T.; Nishimura, M.; Goto, T.; Okuhara, M. *J. Antibiot.* **1994**, *47*, 301−310. (b) Ueda, H.; Manda, T.; Matsumoto, S.; Mukumoto, S.; Nishigaki, F.; Kawamura, I.; Shimomura, K. *J. Antibiot.* **1994**, *47*, 315−323.

(10) Gore, S. D.; Carducci, M. A. *Exp. Opin. Invest. Drugs* **2000**, *9*, 2923−2934.

(11) Phiel, C. J.; Zhang, F.; Huang, E. Y.; Guenther, M. G.; Lazar, M. A.; Klein, P. S. *J. Biol. Chem.* **2001**, *76*, 36734−36741.

(12) Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 3003−3007.

(13) (a) Jung, M.; Brosch, G.; Kölle, D.; Scherf, H.; Gerhäuser, C.; Loidi P. *J. Med. Chem.* **1999**, *42*, 4669−4679. (b) Remiszewski, S. W.; Sambucetti, L. C.; Atadja, P.; Bair, K. W.; Cornell, W. D.; Green, M. A.; Howell, K. L.; Jung, M.; Known, P.; Trogani, N.; Walker, H. *J. Med. Chem.* **2002**, *45*, 753−757. (c) Woo, S. H.; Frechette, S.; Khalil, E. A.; Bouchain, G.; Vaisburg, A.; Bernstein, N.; Moradei, O.; Leit, S.; Allan, M.; Fournel, M.; Trachy-Bourget, M. C.; Li, Z.; Bestman, J. M.; Delorme, D. *J. Med. Chem.* **2002**, *45*, 2877−2885.

(14) (a) Suzuki, T.; Ando, T.; Tsuchiya, K.; Fukazawa, N.; Saito, A.; Mariko, Y.; Nakanishi, O. *J. Med. Chem.* **1999**, *42*, 3001−3003. (b) Saito, A.; Yamashita, T.; Mariko, Y.; Nosaka, Y.; Tsuchiya, K.; Ando, T.; Suzuki, T.; Tsuruo, T.; Nakanishi, O. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 4592−4597.

(15) (a) Furumai, R.; Komatsu, Y.; Nishino, N.; Khochbin, S.; Yoshida, M.; Horinouchi, S. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 87−92. (b) Komatsu, Y.; Tomizaki, K.; Tsukamoto, M.; Kato, T.; Nishino, N.; Sato, S.; Yamori, T.; Tsuruo, T.; Furumai, R.; Yoshida, M.; Horinouchi S.; Hayashi, H. *Cancer Res.* **2001**, *61*, 4459.

(16) Furumai, R.; Matsuyama, A.; Kobashi, M.; Lee, K.-H.; Nishiyama, M.; Nakajima, H.; Tanaka, A.; Komatsu, Y.; Nishino, N.; Yoshida, M.; Horinouchi S. *Cancer Res.* **2002**, *62*, 4916−4921.

(17) Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Rifkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. *Nature* **1999**, *401*, 188−193.

as the zinc-binding functional group. Since the free sulfhydryl group is unstable to oxidation, disulfides were prepared as homodimers. In addition, disulfide hybrids were also synthesized to reduce the molecular size, which might affect permeation across cell membranes.

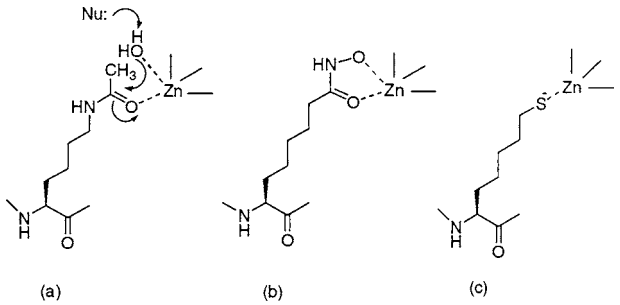
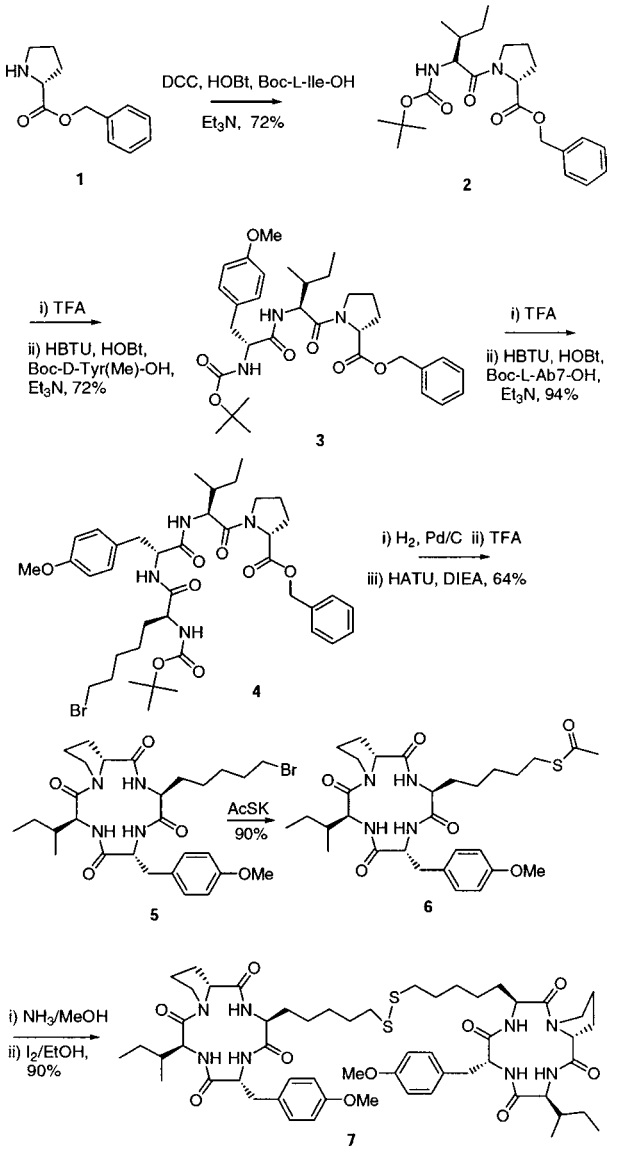


Figure 2. Binding of substrate and inhibitors to zinc ion with (a) substrate, (b) hydroxamic acid-containing inhibitor, and (c) sulfhydryl group-containing inhibitor.

First, we proposed to synthesize cyclic tetrapeptide **7** containing L-2-amino-7-bromoheptanoic acid (L-Ab7), which can be easily converted to sulfide. Synthesis of compound **7** is outlined in Scheme 1. Using a solution-phase peptide synthesis strategy, we started with benzyl-protected D-proline and coupled it with Boc-protected L-isoleucine to give protected dipeptide **2**. Boc protection was then removed using trifluoroacetic acid and again coupled with Boc-protected methyl ether of D-tyrosine to yield **3**. The *N*-terminal of the tripeptide **3** was deprotected and coupled with Boc-protected L-Ab7 to yield linear tetrapeptide **4**. After deprotection of both terminals by catalytic hydrogenation followed by trifluoroacetic acid treatment, the linear tetrapeptide was cyclized under high dilution conditions in DMF using HATU to give cyclic tetrapeptide **5** in 64% yield.[19] The side chain of the cyclic tetrapeptide **5** was modified from a bromo group to a thioacetyl group by treatment with potassium thioacetate to yield **6**. The acetyl group was then removed by the reaction with ammonia in methanol, and the sulfide derivative was converted to the corresponding dimer by the reaction with I_2 in ethanol. The cyclic tetrapeptide containing L-2-amino-7-mercaptoheptanoic acid as its disulfide dimer **7** was purified by filtration through a sephadex LH-20 column in 90% yield (Scheme 1).

Cyclic tetrapeptide disulfide dimers containing L-proline, D-pipecolic acid, and L-pipecolic acid in the position of D-proline of **7** were also synthesized to verify the effect of cyclic peptide configuration on the HDAC inhibitory activity. *cyclo*(L-Am7(−)-D-Tyr(Me)-L-Ile-L-Pro)dimer (**8**) was synthesized using the method described for **7**. *cyclo*(L-Am7(−)-D-Tyr(Me)-L-Ile-D-Pip)dimer (**9**) and *cyclo*(L-Am7(−)-D-Tyr(Me)-L-Ile-L-Pip)dimer (**10**) were synthesized using D,L-pipecolic acid, and the compounds were separated using column chromatography after cyclization reaction.

Scheme 1



To optimize the length of the side chain for effective zinc ligation, we synthesized cyclic tetrapeptides with varying chain lengths by increasing or decreasing the number of CH_2 groups. For this, we synthesized linear tetrapeptides by replacing Boc-L-Ab7 with Boc-L-Ab6, Boc-L-Ab8, and Boc-L-Ab9. After the deprotection of the terminals, the linear tetrapeptides were cyclized as before and converted to the corresponding disulfide dimers. Thus, the compounds with four methylene spacer *cyclo*(L-Am6(−)-D-Tyr(Me)-L-Ile-D-Pro)dimer (**11**), six methylene spacer *cyclo*(L-Am8(−)-D-Tyr(Me)-L-Ile-D-Pro)dimer (**12**), and seven methylene spacer *cyclo*(L-Am9(−)-D-Tyr(Me)-L-Ile-D-Pro)dimer (**13**) were synthesized.

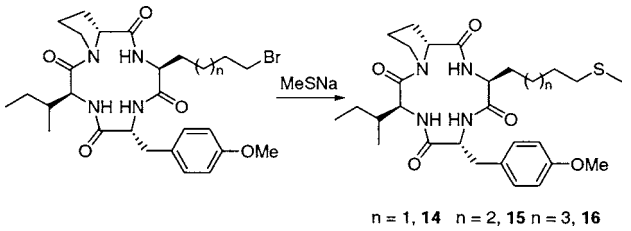
Methyl thioether derivatives of the cyclic tetrapeptide with varying chain lengths were synthesized to verify the effect

(18) (a) Nishino, N.; Powers, J. C. *Biochemistry* **1979**, *18*, 4340−4347. (b) Nishino, N.; Powers, J. C. *J. Biol. Chem.* **1980**, *255*, 3482−3486. (c) Ondetty, M. A.; Condon, M. E.; Reis, J.; Sabo, E. F.; Cheung, H. S.; Cushman, D. W. *Biochemistry* **1979**, *18*, 1427−1430.

(19) Cyclic tetrapeptides were characterized by ^{1}H NMR, COSY, HOHAHA, FAB-mass, and HR-MS. Purity of the compounds was determined by HPLC.

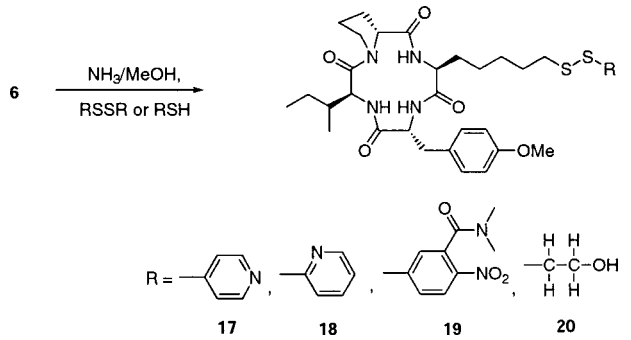
of the presence of a methyl group during the binding with zinc ions in HDAC. Compound **15** with a spacer of five CH_2 groups was synthesized from **5** by the reaction of sodium thiomethoxide in DMF. Compound **14** with a spacer of four CH_2 groups and compound **16** with a spacer of six CH_2 groups were also synthesized (Scheme 2).

Scheme 2



Disulfide hybrids were synthesized by treating the thioacetyl derivative **6** with ammonia/methanol in the presence of disulfide compounds such as 4,4′-dithiodipyridine, 2,2′-dithiodipyridine, and 2,2′-dithiobis-2-nitro-*N*,*N*-dimethylbenzamide to get the sulfur-exchanged products **17**, **18**, and **19**, respectively, in moderate yields (Scheme 3). Compound **20** was synthesized from **6** using ammonia/methanol and 2-mercaptoethanol.

Scheme 3



Results of initial biological evaluation of few SCOPs are given in Table 1. For comparison, the IC_{50} values for TSA and FK228 are also given in Table 1. The inhibitory activities of compounds **7** and **17** are comparable with that of FK228. The HDAC inhibitory activity varies with the methylene

Table 1. In Vitro and in Vivo HDAC Inhibitory Activities of Selected Compounds

	IC_{50}, μM (in vitro)				p21 promoter assay (in vivo)
compound	HDAC1	HDAC4	HDAC6	HDAC8	EC_{1000} (μM)
TSA	0.022	0.02	0.028	0.04	0.062
FK228	0.0358	0.512	>500	nt	0.0031
FK228 + DTT	0.001	nt	0.624	nt	0.0157
7	0.142	0.145	>500	>500	0.0368
7 + DTT	0.0046	0.0021	1.4	1.69	0.0677
11 + DTT	0.932	7.34	28.5	>500	7.55
12 + DTT	0.0091	0.091	8.05	1.88	0.631
13 + DTT	0.038	0.099	2.47	1.87	3.284
17	0.007	0.068	1.61	3.14	0.140
17 + DTT	0.00055	0.0011	2.01	0.494	0.0597
15	0.119	0.405	0.191	0.284	not tested

spacer length as shown in Table 1. From the results, it is clear that the spacer length of five methylene units is the optimum. After reduction of the disulfide bond using DDT, the HDAC inhibitory activity increased and compound **17** showed excellent activity in the presence of DTT. On the other hand, as expected, compound **15**, which contains a methyl sulfide showed a very weak inhibitory activity. This is due to the lack of capacity for ligation between the methyl sulfide and zinc ion present in the active site pocket of HDAC. Another interesting property of SCOPs is the target enzyme specificity. Both HDAC1 and HDAC4 are potently inhibited by SCOP compounds, while HDAC6 and HDAC8 were relatively resistant.

The reduction of the disulfide dimer **7** by DTT to the corresponding monomer was monitored at 37 °C using HPLC; 50% of **7** was converted to the monomer in 4 h, and complete conversion was observed in 24 h.

In summary, we have synthesized several sulfur-containing cyclic tetrapeptides (SCOPs) based on the CHAP31 skeleton and the HDAC-binding functional group of FK228. Initial biological studies showed that SCOPs are potent inhibitors of HDAC and this may allow the development of new theraputic agents for cancer. Complete biological studies on SCOPs will be reported elsewhere in the future.

Supporting Information Available: Complete experimental procedure for all reported compounds, ^{1}H NMR of key compounds, and biological assay methods. This material is available free of charge via the Internet at http://pubs.acs.org.

OL036098E