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The discovery of a potent and selective lethal factor inhibitor for adjunct therapy of anthrax infection

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Abstract—A potent and selective anthrax LF inhibitor **40**, (2*R*)-2-[(4-fluoro-3-methylphenyl)sulfonylamino]-*N*-hydroxy-2-(tetrahydro-2*H*-pyran-4-yl)acetamide, was identified through SAR study of a high throughput screen lead. It has an IC_{50} of 54 nM in the enzyme assay and an IC_{50} of 210 nM in the macrophage cytotoxicity assay. Compound **40** is also effective in vivo in several animal model studies.

Anthrax, caused by *Bacillus anthracis* spores, poses a significant threat to the public as a bioterrorist weapon. Current antibiotic treatment is less than optimal because it must be given early when symptoms are nonspecific and diagnosis is difficult.[1] At later stages of infection when large amounts of toxin are present, antibiotics are of less value because they have no effect on the toxin itself. There is an urgent need for an anthrax antitoxin for use in combination with antibiotics to provide improved therapy for anthrax.

Anthrax toxin consists of three proteins[2] secreted during vegetative growth, protective antigen (PA), edema factor (EF), and lethal factor (LF). PA facilitates the entry of EF and LF into target cells.[3] EF is a calmodulin-dependent adenylate cyclase and it raises the cellular cAMP level that results in edema. LF is a zinc-dependent metalloprotease and it cleaves, among other possible targets, several MAP kinase kinases,[4] disrupting the signaling pathway mediated by MAPKKs. Exactly how this MAPKK cleavage contributes to anthrax pathology is not understood, but it is clear that LF plays a critical role in all stages of anthrax infection. In the early stages, LF helps the bacteria evade the host immune system, promoting its survival and release from macrophages where the spores germinate.[5] Later when the infection becomes systemic, LF targets the endothelial cells[5,6] and causes vascular barrier dysfunction. *B. anthracis* strains with LF deletion are much less virulent than EF deletion or wild type in mice.[7] The combination of LF/PA is lethal when administered to mice, rats, and rabbits.[8] Infusion of purified LF/PA in rats caused symptoms[9] such as circulatory shock, pleural effusions, and hemorrhages, some of which are seen in late stage inhalational anthrax in humans.[10] Given the critical roles of LF in anthrax pathogenesis, a small molecule inhibitor of LF would be invaluable in combating the lethal toxin. Such an inhibitor may increase the survival rate of patients by reducing or preventing the damage to vascular circulation, winning precious time for the antibiotics to clear the bacteria. An LF inhibitor given prophylactically may even prevent anthrax by helping the host innate immune system to clear the infection at an early stage.

LF is an enzyme of 776 amino acid residues, with a typical zinc metalloprotease catalytic HExxH motif.

Keywords: Anthrax; Lethal factor; Hydroxyamic acid.
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The crystal structure of LF, and its complex with a 16-residue peptide from MAPKK-2 N-terminus (in the absence of zinc metal) were reported.[11] A higher resolution crystal structure of LF complexes with short peptides and small molecule inhibitors was reported more recently.[12] Our work started in earnest in late 2001 with the development of a high throughput fluorescence resonance energy transfer assay[13] (FRET) and a macrophage cytotoxicity assay[14] (MACK) using a murine J774A.1 cell line. We identified a potent and selective LF inhibitor **40** with good pharmacokinetic properties. This compound also proved to be efficacious in several animal models against anthrax spores.[15] In this letter, we report our medicinal chemistry effort in the discovery of the LF inhibitor **40**.

Targeted screening of known Zn-metalloprotease inhibitors[16] from Merck sample collections produced compound **1** as a lead. This compound is a potent inhibitor of stromelysin (IC_{50} 43 nM), and was developed as a treatment for osteoarthritis.[17] Compound **1** has broad activities against matrix metalloproteases (IC_{50} values: MMP-1, 33 nM, MMP-2, 20 nM, and MMP-9 8 nM). Against LF, it shows low micromolar enzyme inhibition and 12 μM IC_{50} in the macrophage assay (see Table 1).

We began by systematically changing various parts of compound **1**. Figure 1 lists a few of the compounds representing the structural modifications. First, we found that the hydroxyamic acid group is essential for LF inhibition, and any minor changes in this part of the molecule were not tolerated. Corresponding carboxylic acids were not active (data not shown) and compound **5** in Figure 1, for example, is totally inactive. The arylsulfonyl group is also crucial for the LF inhibition. Replacement of the arylsulfonyl group with alkyl sulfonyl groups or acyl groups (compounds **3** and **6**, for example) completely eliminated LF binding activity. On the other hand, removal of the substitution at sulfonamide-N position, such as in compound **2**, leads to a slightly higher affinity for LF. Other neutral substitutions at the N position (**7**, **8**, and **9** in Table 2 and other benzyl, alkyl substituted compounds, data not shown) do not increase the potency compared with the unsubstituted **2**. The absence of substitution at N^{α} also renders the molecule more water soluble, which is important for intravenous formulation of this emergency rescue medicine.

Our effort was then focused on optimizing the sulfonamide **1** and especially **2** for its better physical properties. A solid-phase synthesis of α-sulfonamido

Table 1. Structural modification of compound **1**

Compound	FRET assay	MACK assay
1	1.2	12
2	0.9	7.3
3	>40	>20
4	—	17
5	>40	>40
6	>40	>40

LF inhibition activities, IC_{50} in μM.

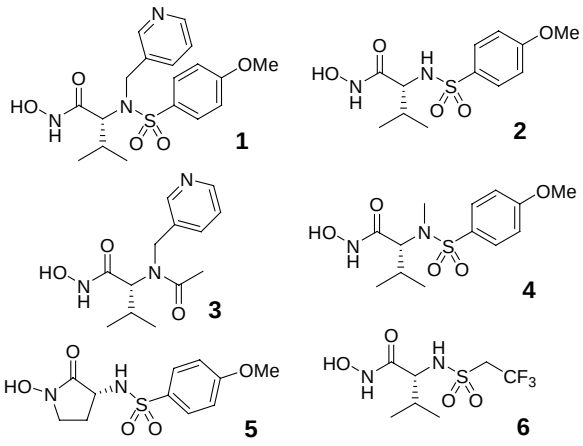


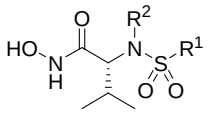
Figure 1.

N-hydroxy-carboxamide was developed based on modified published procedures,[18] Scheme 1. All compounds made from solid-phase synthesis were purified with semi-automated reverse-phase HPLC. We found that selective N^{α} alkylation (vs the hydroxyamic acid nitrogen) was only successful by using a bulky 2-chlorotrityl resin[19] as solid support. This solid-phase route allowed rapid syntheses of analogs of compounds **1** and **2**, where both the arylsulfonyl groups and amino acid side chain were varied. Compounds listed in Table 2 demonstrate a clear SAR surrounding the aryl sulfonamide group.

Anthrax LF cleaves MKK-1 and -2 at the Pro site near the N-termini. Substrate specificity study[12] using mixed short peptides revealed a strong preference at the S1′ site for hydrophobic amino acids like Ile and Tyr. The α-sulfonamido hydroxamic acid compounds, such as **1**, inhibit zinc metalloproteases by tying up of the zinc atom at the catalytic center with the hydroxamic acid group.[20] The aryl group interacts with the S1′ pocket of the enzyme. This interaction confers the selectivity of the inhibitors against various enzymes. We found that LF strongly favors small aromatic groups at the S1′ site. Small *para* and *meta* substitution groups (methyl, halogens) at the benzene ring enhance binding, while ortho substitution disrupts enzyme binding. Unlike the matrix metalloproteases,[17] the binding pocket at the S1′ site is small and tight. Compounds with larger substitutions at the benzene ring, such as **21**–**24**, are inactive against LF. Inserting flexible methylene groups between the benzene and sulfur atom, such as in compounds **19** and **20**, or removing the benzene ring, like in compound **6**, also completely abrogates the LF inhibition. The tight interaction of the aromatic group with the enzyme is clearly reflected in the progressive increase in activity from compound **13** to **15**, and to **16**, with the optimum group being the 4-fluoro-3-methylbenzene. The dramatic decrease in activity on exchanging positions of the fluorine and methyl groups (compounds **16** and **18**) further illustrates the tight fit of 4-fluoro-3-methylbenzene in LF S1′ pocket.

Next we began the optimization of the amino acid side-chain group (R^3 in Table 3) of the lead structure. The favorable interaction of 4-fluoro-3-methylbenzene with

Table 2. LF inhibition: varying R^1 and R^2



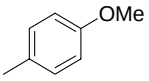
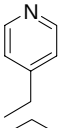
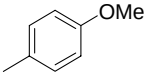
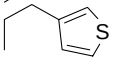
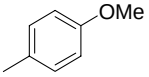
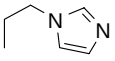
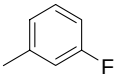
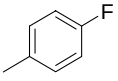
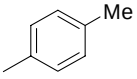
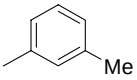
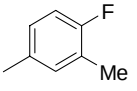
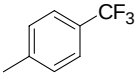
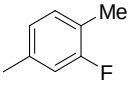
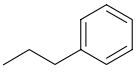
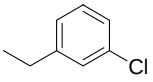
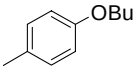
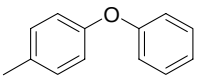
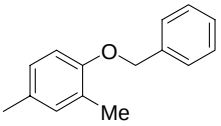
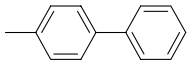
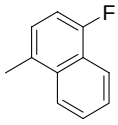
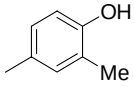
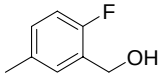
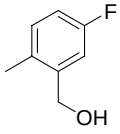
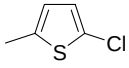
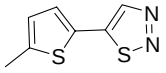
Compound	R^1	R^2	IC_{50} (μM)	
			FRET	MACK
7			3.10	9.50
8			2.10	—
9			19.1	—
10		H	1.10	7.80
11		H	6.60	>20
12		H	2.80	12.40
13		H	0.29	3.00
14		H	3.00	15.00
15		H	0.40	1.29
16		H	0.13	0.12
17		H	>40	>20
18		H	5.18	>20
19		H	11.88	>20
20		H	16.60	>20
21		H	>40	>20
22		H	>40	>20
23		H	>40	>20

Table 2 (*continued*)

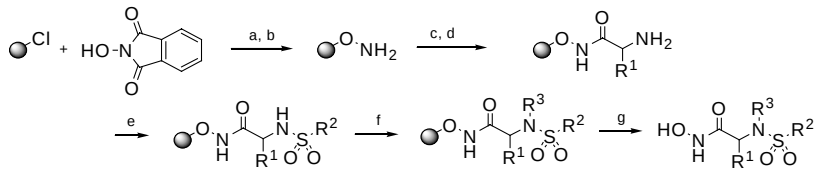
Compound	R^1	R^2	IC_{50} (μM)	
			FRET	MACK
24		H	>40	>20
25		H	0.46	2.76
26		H	1.64	>20
27		H	0.60	3.74
28		H	1.31	>20
29		H	1.31	6.45
30		H	0.81	10.20

—, Compounds not assayed in MACK.

LF is maintained with selected few groups as R^3 (data not shown), so we are comfortable with holding 4-fluoro-3-methylbenzene constant in probing SAR around the R^3 position. Table 3 lists the compounds selected to present the SAR around the R^3 site.

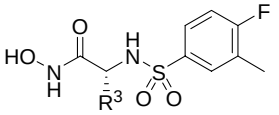
There is a strict preference for (R) configuration at the α-carbon and di-substitution at this α-carbon is not tolerated. Small hydrophobic groups are preferred at R^3, but the role of the R^3 group seems to pre-organize the inhibitor to the favored configuration, rather than to make a strong attractive interaction with the enzyme. Within certain limit, many of the small hydrophobic side chains confer the same level of activity to the inhibitor. Compounds with cyclic groups showed higher activities due to the favorable entropic effect. Cyclic ethers fared almost the same as their alkane analogs (compounds **38** and **40**; and **37** and **41**). The binding pocket around the R^3 group is very flexible, capable of accommodating various different groups. The pairs of diastereomeric compounds, **42** and **43**, and close analogs **44**, and **45**, all had similar activities.

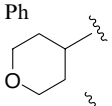
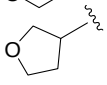
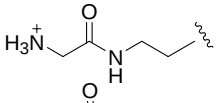
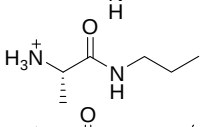
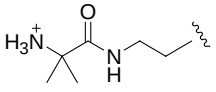
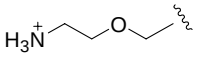
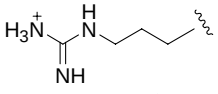
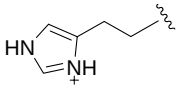
The two known substrates of LF, MKK-1 and MKK-2, all have several basic amino acids (Lys and Arg) two to three residues to the left of the scissile bond. The available X-ray structure also indicated a strongly acidic region of the protein surface making up the substrate binding area.[10] The side-chain group (R^3), as in many metalloprotease inhibitors, interacts with the unprimed side of the site of cleavage. Thus, a side



Scheme 1. Reagents and conditions: (a) DIEA/DCM:DMF (2:1), 15 h; (b) N_2H_4/THF, 6 h; (c) Fmoc-AA-OH (3 equiv), DIC (1.5 equiv)/DMF, 4 h; (d) 25% piperidine/DMF, 30 min; (e) R^2SO_2Cl (3 equiv), DIEA (6 equiv), DMAP (cat.) DCM-THF (1:1), 4 h; (f) R^3OH (6 equiv), TMAD (5 equiv), Bu_3P (5 equiv), DCM-THF, 15 h; (g) 5% TFA/DCM, 30 min.

Table 3. LF inhibition: varying R^3



Compound	R^3	IC_{50} (μM)	
		FRET	MACK
31	Me	0.130	2.10
32	Et	0.100	0.49
16	*i*-Pr	0.130	0.12
33	*n*-Bu	0.070	0.41
34	*t*-Bu	0.300	2.30
35	*c*-Pr	0.120	0.73
36	*c*-Bu	0.130	0.67
37	*c*-Pent	0.064	0.17
38	*c*-Hex	0.042	0.19
39	Ph	0.050	0.37
40		0.054	0.21
41[a]		0.120	0.45
42		0.067	0.23
43		0.067	0.13
44		0.051	0.13
45		0.065	0.46
46		0.037	7.40
47		0.059	7.47
48		0.057	8.70
40		0.048	3.50
50		0.040	1.70
51[b]		0.075	2.80

[a] Diastereomeric mixture.
[b] Racemic.

chain carrying a positive charge properly disposed is likely to interact with the acid region of LF binding area.

We synthesized a series of compounds incorporating basic groups in the side chain R^3 to pick up additional interaction with some acidic element around the S1 site. Indeed, these compounds (**46**–**51**) were very potent in the enzyme assay. We found that the optimum distance between the hydroxyamic acid carbonyl and the basic nitrogen is about 6–7 bonds length. Compound **46** is the most active inhibitor in our enzyme assay. But these compounds all showed much reduced activities in the cell based assay, perhaps due to their poor cell penetration. Addition of one or two methyl groups adjacent to the basic nitrogen (as in compounds **47** and **48**), a very effective approach to attenuate the basicity and hydrophilicity in the design of enalapril,[21] did not make much difference here. Even the compound with a weakly basic imidazole group, **51**, is only weakly active in the macrophage assay.

The flexible nature of the binding pocket around R^3 affords us certain freedom to choose a group that confers balanced overall properties to the LF inhibitor. Compound **40** is potent, has good water solubility (2.5 mg/ml), and shows an excellent selectivity profile against other MMPs and a wider array of enzyme and receptor targets. Table 4 shows the counterscreen results against a panel of relevant zinc metalloproteases. The highest inhibition besides LF is an IC_{50} of 1.4 μM for stromelysin (MMP-3), which is the target enzyme for the lead compound **1**. No other off-target activities were observed in a wider screening.

Table 4. Selectivity of **40** against other metalloproteases

Enzymes	IC_{50} (μM) or % inhibition
LF	0.054 μM
ACE	<10%@10 μM
ECE	<10%@10 μM
NEP	9.4 μM
TACE	4.3 μM
MMP-1	2.2 μM
MMP-2	2.0 μM
MMP-3	1.4 μM
MMP-9	2.0 μM
MMP-14	6.5 μM

ACE, angiotensin converting enzyme; ECE, endothelin converting enzyme; NEP, neutrophil endopeptidase; TACE, TNF-α converting enzyme; MMP: matrix metalloproteinase.

Table 5. Pharmacokinetic parameters of **40** in preclinical species

Parameter[a]	Mouse	Rat	Guinea pig	Rabbit	Dog	Monkey
Cl_p (mL/min/kg)	72	80	24	14(35)	15	23
V_d (L/kg)	1.0	3.9	1.1	1.5(3.8)	2.4	3.3
$T_{1/2}$ (h)	0.4	1.4	1.2	2.0(2.5)	4.7	2.4
C_{max} (μM/mpk)	0.12	0.24	0.25	0.012	0.73	0.12
T_{max} (h)	0.3	0.3	0.8	0.3	0.8	1.3
F_{oral} (%)[b]	20	51	22	ND	57	ND
F_{oral} (%)[c]	ND	71	ND	0.5	ND	16

ND, not determined.

[a] The iv and oral doses were 10 mg/kg in female Balb/c mice, 1 and 2 mg/kg, respectively, in male Sprague–Dawley rats and male Guinea pigs, 1 mg/kg in male Beagle dogs and male rhesus monkeys, and 2 and 5 mg/kg, respectively, in male Dutch Belted or New Zealand White (data in parentheses) rabbits.

[b] The formulation was 1 mg/ml in ethanol/PEG400/water (20/25/55, v/v/v).

[c] The formulation was 2 mg/ml in 0.5% methylcellulose.

Our objective was to find an LF inhibitor for emergency room use in IV formulation, but the pharmacokinetic properties of compound **40**, Table 5, indicate that it might be orally available as well. Compound **40** has oral bioavailability (F%) of 57% in the dog and about 16% in the rhesus monkey. Its half-life ($T_{1/2}$) is less than half hour in mouse and about 5 h in dog. Compound **40** is cleared mostly as its glucuronidation conjugate at the hydroxyamic acid site, accompanied by a minor degree of hydrolysis of the hydroxamic acid group.

Due to its excellent overall properties, compound **40** has been extensively tested in pharmacological and animal model studies.[22] When compared to antibiotic monotherapy, **40** in combination with ciprofloxacin significantly increases the survival rate of mice and rabbits treated with anthrax.[15b] It is even effective prophylactically in preventing anthrax infection in spore challenges without antibiotics. Accordingly compound **40** has been selected as a candidate for clinical studies and drug development.

References and notes

1. Dixon, T. C.; Meselson, M.; Guillemin, J.; Hanna, P. C. *New Eng. J. Med.* **1999**, *341*, 815.
2. Collier, R. J.; Young, J. A. *Annu. Rev. Cell Dev. Biol.* **2003**, *19*, 45.
3. (a) Pemental, R. L.; Christensen, K. A.; Krantz, B. A.; Collier, R. J. *Biochem. Biophys. Res. Commun.* **2004**, *322*, 258; (b) Zhang, S.; Finkelstein, A.; Collier, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16756.
4. (a) Duesbery, N. S.; Webb, C. P.; Leppla, S. H.; Gordon, V. M.; Kimpel, K. R.; Copeland, T. D.; Ahn, N. G.; Oskarsson, M. K.; Fukasawa, K.; Paull, K. D.; Woude, G. F. *Science* **1998**, *280*, 734; (b) Vitale, G.; Bernardi, L.; Napolitani, G.; Mock, M.; Montecucco, C. *Biochem. J.* **2000**, *352*, 739.
5. Moayeri, M.; Leppla, S. H. *Curr. Opin. Microbiol.* **2004**, *7*, 19.
6. (a) Warfel, J. M.; Steele, A. D.; D'Agnillo, F. *Am. J. Pathol.* **2005**, *166*, 1872; (b) Pandey, J.; Warburton, D. *Microbes Infect.* **2004**, *6*, 835.
7. Pezard, C.; Berche, P.; Mock, M. *Infect. Immun.* **1991**, *59*, 3472.
8. Moayeri, M.; Hanies, D.; Young, H. A.; Leppla, S. H. *J. Clin. Invest.* **2003**, *112*, 670.
9. (a) Cui, X.; Moayeri, M.; Li, Y.; Li, X.; Haley, M.; Fitz, Y.; Corren-Araujo, R.; Banks, S. M.; Leppla, S. H.; Eichacker, P. Q. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *286*, R699; (b) Moayeri, M.; Hanies, D.; Young, H. A.; Leppla, S. H. *J. Clin. Invest.* **2003**, *112*, 670.
10. Jernigan, J. A.; Stephens, D. S.; Ashford, D. A.; Omenaca, C.; Topiel, M. S.; Galbraith, M.; Tapper, M.; Fisk, T. L.; Zaki, S.; Popovic, T.; Meyer, R. F.; Quinn, C. P.; Harper, S. A.; Fridkin, S. K.; Sejva, J. J.; Shepard, C. W.; McConnell, M.; Guarner, J.; Shieh, W.-J.; Malecki, J. M.; Berberding, J. L.; Hughes, J. M.; Perkins, B. A. *EID* **2001**, *7*, 933.
11. Pennifer, A. D.; Wong, T. Y.; Schwarzenbacher, R.; Renatus, M.; Petosa, C.; Blenkowska, J.; Lacy, D. B.; Collier, R. J.; Park, S.; Leppla, S. H.; Hanna, P.; Liddington, R. C. *Nature* **2001**, *414*, 229.
12. Turk, B. E.; Wong, T. Y.; Schwarzenbacher, R.; Jarrell, E. T.; Leppla, S. H.; Collier, R. J.; Liddington, R. C.; Cantley, L. C. *Nat. Struct. Mol. Biol.* **2004**, *11*, 60.
13. Cummings, R. T.; Salowe, S. P.; Cunningham, B. R.; Wiltsie, J.; Park, Y. W.; Sonatore, L. M.; Wisneiwski, D.; Douglas, C. M.; Hermes, J. D.; Scolnick, E. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *10*, 6603.
14. Tang, G.; Leppla, S. *Infect. Immun.* **1999**, *67*, 3055.
15. (a) Xiong, Y.; Shoop, W. L.; Wiltsie, J.; Woods, A.; Guo, J.; Pivnichny, J. V.; Felcetto, T.; Michael, B. F.; Bansal, A.; Cummings, R. T.; Cunningham, B. R.; Friedlander, A. M.; Douglas, C. M.; Patel, S. B.; Scapin, G.; Salowe, S. P.; Zaller, D. M.; Chapman, K. T.; Scolnick, E. M.; Schmatz, D. M.; Bartizal, K.; Hermes, J. D.; MacCoss, M. *Abstracts of Papers*, 228th National Meeting of the American Chemical Society, Philadelphia, PA, Aug 22–26, 2004; (b). *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7958.
16. Compounds of hydroxamic acid, thiol, carboxylic acid, and aminomethylcaboxylic acid groups are selected for this screen.
17. MacPherson, L. J.; Bayburt, E. K.; Capparelli, M. P.; Carroll, B. J.; Goldstein, R.; Justice, M. R.; Zhu, L.; Hu, S.; Melton, R. A.; Fryer, L.; Goldberg, R. L.; Doughty, J. R.; Spirito, S.; Blancuzzi, V.; Wilson, D.; O'Byrne, E. M.; Canu, V.; Parker, D. T. *J. Med. Chem.* **1997**, *40*, 2525.
18. (a) Floyd, C. D.; Lewis, C. N.; Patel, S. R.; Whittaker, M. *Tetrahedron Lett.* **1996**, *37*, 8045; (b) Richter, L. S.; Desai, M. C. *Tetrahedron Lett.* **1997**, *38*, 321; (c) Mellor, S. L.; McGuire, C.; Chan, W. C. *Tetrahedron Lett.* **1997**, *38*, 3311.
19. (a) Bauer, U.; Ho, W. B.; Koskinen, A. M. P. *Tetrahedron Lett.* **1997**, *38*, 7233; (b) Khan, S. I.; Grinstaff, M. W. *Tetrahedron Lett.* **1998**, *39*, 8031.
20. (a) Babine, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359; (b) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735.
21. Wyvratt, M. J.; Patchett, A. *Med. Res. Rev.* **1985**, *5*, 483.
22. A process route for synthesis of 40 was developed, see Shultz, C. S. et al. *Org. Lett.* **2005**, *7*, 3405.