Drug Discovery

DOI: 10.1002/anie.201402428

Total Synthesis of Syringolin A and Improvement of Its Biological Activity

*Takuya Chiba, Hidetaka Hosono, Koji Nakagawa, Masahiro Asaka, Hiroshi Takeda, Akira Matsuda, and Satoshi Ichikawa**

Abstract: *The development process for syringolin A analogues having improved proteasome inhibitory and antitumor activity is described. The strategy was to first establish a convergent synthesis of syringolin A using a rare intramolecular Ugi three-component reaction in the last stage of the synthesis, so as to gain access toa set of structure-based analogues. The inhibitory activity of chymotrypsin-like activity of 20S proteasome was largely improved by targeting the S3 subsite of the β5 subunit. Cytotoxic activity was also improved by installing the membrane-permeable substituent. These biological properties are comparable to those of bortezomib, a clinically used first-line proteasome inhibitor.*

Around 50% of new drug entries between 1981 and 2010 were natural-product-based compounds,[1] and it is no doubt that natural products are a rich source for drug development. Improvement of biological activity is a key issue associated with natural-product-based drug development because it is difficult to use the natural product itself without modifying its structure, as found in many cases, and much effort has to be devoted to adding desired properties to make them into drug candidates. Therefore it is necessary to develop a synthetic strategy that allows an efficient route and also provides a range of analogues to investigate structure–activity relationships so as to improve potency. Syringolins[2] and glidobactins[3] constitute members of the syrbactins, which are 12-membered macrolactams containing an *E*-configured α,β-unsaturated carboxamide moiety with an appended side chain (Figure 1).[4] In 2008, they were revealed to be irreversible inhibitors of the 20S proteasome, which is a multicatalytic proteolysis machinery.[5] Proteasome inhibitors are promising candidates as anticancer agents,[6] and syrbactins are expected to be a next-generation proteasome inhibitor in cancer chemotherapy[7] because of its mode of inhibition is different from those of the currently used proteasome inhibitors bortezomib (Velcade) and carfilzomib (Kyprolis), which have several associated problems.[8] Syringolin A (**1**), however, exhibits a moderate proteasome inhibitory activity and weak cytotoxic activity against cancer cells in vitro. As in an effort to develop more potent syringolin A analogues by Kaiser et al., who synthesized hybrid-type analogues of **1** and **2**,[9] it is necessary to improve the biological activity in order to set an initial stage of anticancer drug development. Herein we describe the process to develop syringolin A analogues with improved biological activity. Our strategy was to first establish a synthetic strategy applicable to accessing a set of structure-based analogues. Upon improvement of the protea-

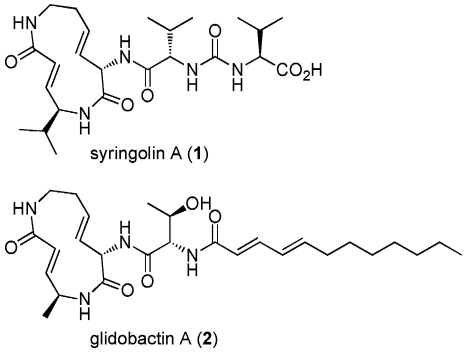


Figure 1. Structures of syrbactins.

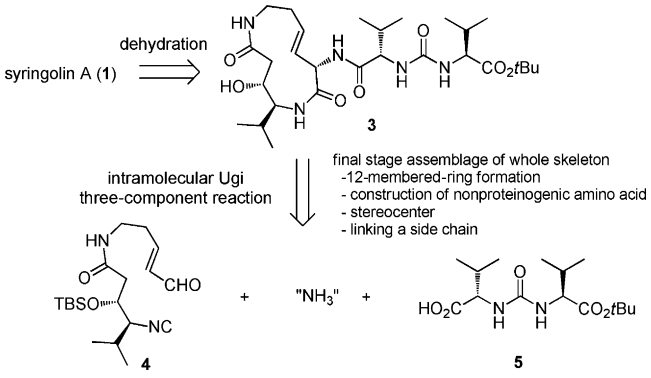
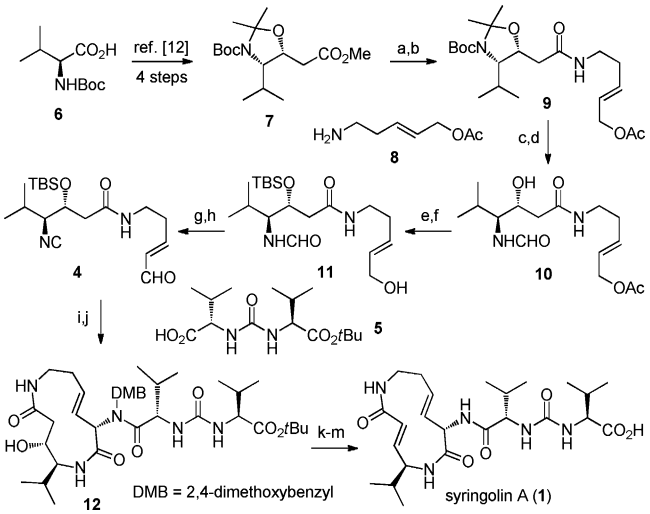


Figure 2. Retrosynthetic analysis of syringolin A.

[*] T. Chiba, K. Nakagawa, H. Takeda, A. Matsuda, S. Ichikawa
Faculty of Pharmaceutical Sciences, Hokkaido University
Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812 (Japan)
E-mail: ichikawa@pharm.hokudai.ac.jp
H. Hosono, M. Asaka
Graduate School of Medicine, Department of Cancer Preventive Medicine, Hokkaido University
Kita-12, Nishi-7, Kita-ku, Sapporo, 060-0812 (Japan)
S. Ichikawa
Center for Research and Education on Drug Discovery
Hokkaido University
Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812 (Japan)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201402428. This research was supported by JSPS Grant-in-Aid for Challenging Exploratory Research (SI, grant number 22659020), Scientific Research on Innovative Areas "Chemical Biology of Natural Products" (SI, grant number 24102502), and Scientific Research (B) (SI, grant number 25293026). We thank S. Oka and A. Tokumitsu (Center for Instrumental Analysis, Hokkaido University) for measurement of the mass spectra.



Scheme 1. Total synthesis of syringolin A. Reagents and conditions. a) LiOH, H_2O/THF/MeOH (60:75:75), 0°C to RT, 15 h; b) **8**, EDCI, iPr_2NEt, DMAP, CH_2Cl_2, 0°C to RT, 19 h, 89% over 2 steps; c) 80% aq. TFA, 0°C, 2 h; d) HCO_2H, iPr_2NEt, EDCI, CH_2Cl_2, 0°C, 72 h, 50% over 2 steps; e) TBSCl, imidazole, DMF, 0°C to RT, 30 h, f) K_2CO_3, MeOH, RT, 1 h, 72% over 2 steps; g) IBX, MeCN, 80°C, 1 h; f) triphosgene, Et_3N, CH_2Cl_2, −78°C, 30 min, 86% over 2 steps; i) 2,4-dimethoxybenzylamine, **5**, CH_2Cl_2, reflux, 36 h; j) 3 HF·Et_3N, MeCN, RT, 24 h, 32% over 2 steps; k) MsCl, Et_3N, CH_2Cl_2, 0°C, 10 min; l) DBN, CH_2Cl_2, RT, 48 h, 64% over 2 steps; m) 80% aq. TFA, Et_3SiH, RT, 4 h, 75%. DBN = 1,5-diazabicyclo[4.3.0]non-5-ene, DMAP = *N*,*N*-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, IBX = 2-iodoxybenzoic acid, Ms = methanesulfonyl, TBS = *tert*-butyldimethylsilyl, TFA = trifluoroacetic acid, THF = tetrahydrofuran.

some inhibitory activity, further modification was pursued to improve the cytotoxic activity. We planned to assemble **4**, **5**, and an ammonia equivalent by a rare intramolecular Ugi three-component reaction[10] (intra-U3CR) in the last stage of the synthesis (Figure 2). This reaction constructs the highly strained 12-membered macrolactam and a nonproteinogenic amino acid, β,γ-dehydrolysine, within the maclolactam, and links the ureadipeptide side chain **5** simultaneously. This strategy was completely different from those previously reported.[11]

The total synthesis of **1** is summarized in Scheme 1. The known methyl ester **7**,[12] prepared from L-valine, was hydrolyzed and the resulting carboxylic acid was condensed with the amine **8** to give the carboxamide **9**. Protecting group manipulations of **9** by way of **10** provided the formamide **11**. Oxidation of the primary alcohol of **11** followed by dehydration of the formamide moiety by triphosgene cleanly afforded the isocyanoaldehyde **4**, a precursor for the intra-U3CR, in 86% yield over two steps. A mixture of **4**, the ureadipeptide carboxylic acid **5**, and 2,4-dimethoxybenzylamine was heated under reflux in CH_2Cl_2 for 36 hours and gave the desired **12** in 32% over two steps after removal of the TBS group.[13] Sequential dehydration of **12** by mesylation and elimination with DBN (64% over 2 steps) gave a α,β-unsaturated carboxamide, which was deprotected further by TFA and Et_3SiH (75% yield) to complete the total synthesis of **1**. Synthetic **1** was obtained as a single diastereomer, and

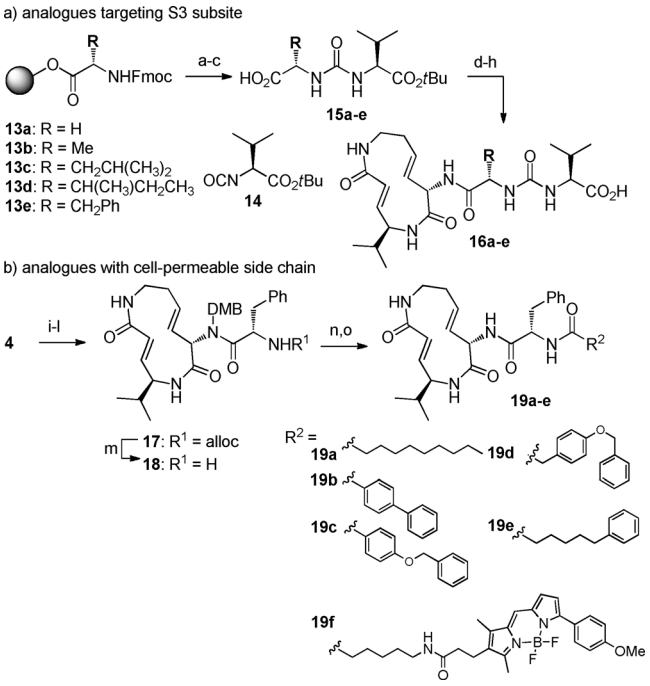
Table 1: Proteasome inhibitory[a] and cytotoxic activity[b] of **1** and its analogues.

	K_i [nM]		IC_{50} [nM]		
	isolated	cell-based	HCT116	A431	RPMI8226
1	680	–	2830	3240	1160
16e	44	–	2010	3370	947
19a	0.14	5.5	5.7	4.3	2.2
19b	1.1	6.0	16.5	20.9	4.7
19c	0.6	8.5	18.2	21.1	4.8
19d	0.2	11	9.1	12.6	5.7
19e	0.4	12	8.1	10.1	6.2
bortezomib	–	2.1	6.0	5.3	2.0

[a] Chymotrypsin-like activity of 20S proteasome. [b] HCT116: human colon cancer cells, A431: human epidermal cancer cells, RPMI8226: human myeloma cells.

the corresponding diastereomer at the dehydrolysine residue was not isolated in the intra-U3CR, dehydration, and deprotection steps. It was, therefore, presumed that the intra-U3CR proceeded stereoselectively.

Synthetic **1** moderately inhibited chymotrypsin-like (CT-L; β5 subunit) activity of a purified 20S proteasome with an apparent K_i value of 680 nM as reported. Weak cytotoxic



Scheme 2. Synthesis of syringolin A analogues. Reagents and conditions. a) piperidine, DMF, RT, 15 min; b) **14**, iPr_2NEt, CH_2Cl_2, RT, 1 h; c) 5% TFA/CH_2Cl_2, 10 min; d) **4**, 2,4-dimethoxybenzylamine, CH_2Cl_2, reflux, 41 h; e) 3 HF·Et_3N, MeCN, RT, 40 h, 17–29% over 2 steps; f) MsCl, Et_3N, CH_2Cl_2, 0°C, 10 min; g) DBU, CH_2Cl_2, RT, 17 h, 18–33% over 2 steps; h) 80% aq. TFA, Et_3SiH, RT, 5 h, 40–86%; i) *N*-Alloc phenylalanine, 2,4-dimethoxybenzylamine, CH_2Cl_2, reflux; j) 3 HF·Et_3N, MeCN, RT, 39 h, 57% over 2 steps; k) MsCl, Et_3N, CH_2Cl_2, 0°C, 10 min; l) DBN, CH_2Cl_2, RT, 6 h, 20% over 2 steps; m) [$Pd(PPh_3)_4$], morpholine, THF, RT, 30 min, 81%; n) carboxylic acid, EDCI, CH_2Cl_2, 0°C to RT, 12 h; o) 80% aq. TFA, Et_3SiH, RT, 30 min, 45–72% over 2 steps.

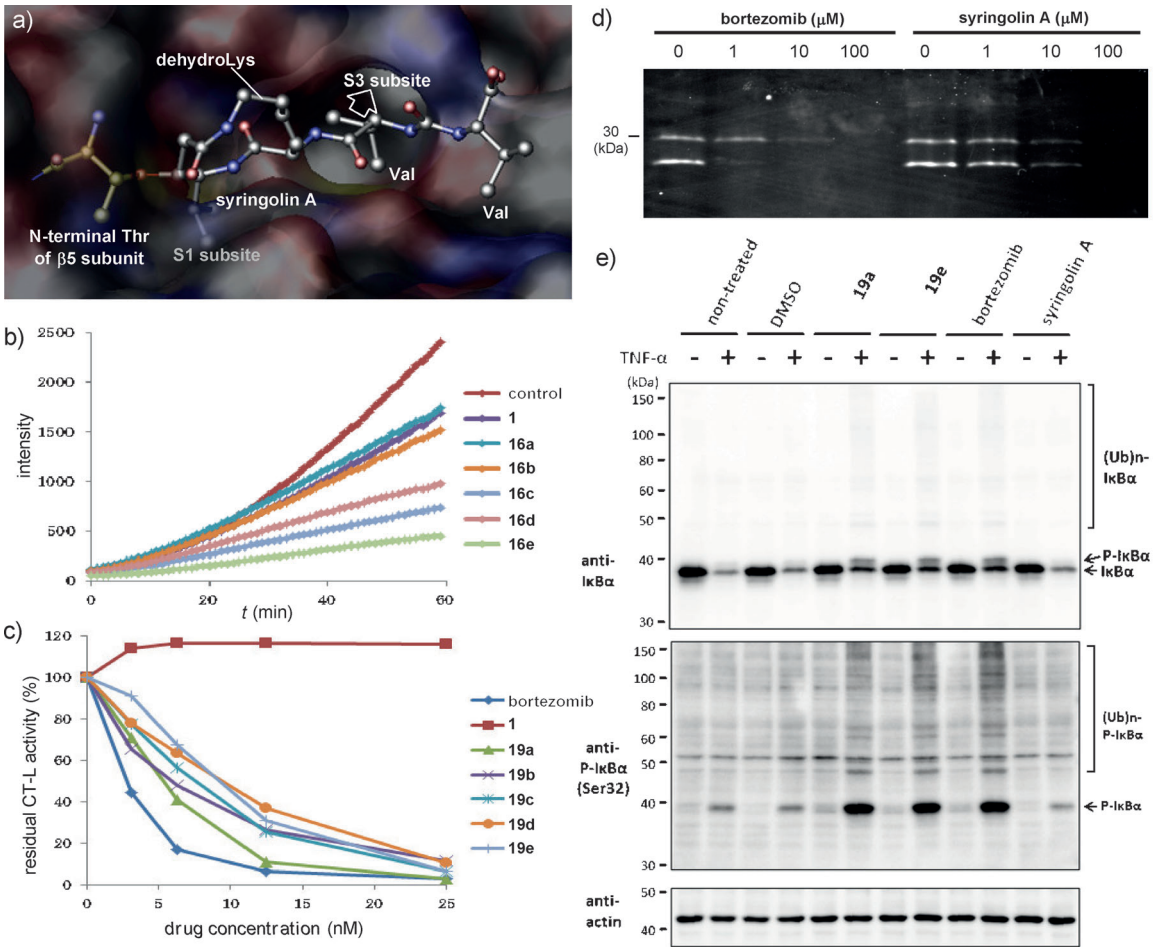


Figure 3. Biological properties of syringolin A analogues. a) X-ray crystal structure of β5-subunit of 20S proteasome bound to **1** (PDB code: 2ZCY). b) Comparison of CT-L inhibition of **1** and **16a–e** at 250 nM. c) Cell-based CT-L inhibitory activity of bortezomib, **1**, and **19a–e** with human embryonic kidney 293 (HEK293) cells. d) Profiling of HEK293 cell lysates with **19f**. e) Accumulation of poly-ubiquitinated IκBα in HEK293 cells treated with bortezomib, **1**, **19a**, and **19e** at 100 nM.

activity against a range of human cancer cells ($IC_{50} = 1160$–3240 nM) was observed for synthetic **1** (Table 1).

The α,β-unsaturated carboxamide moiety of **1** serves as a Michael acceptor to covalently bind to the secondary hydroxy group of the N-terminal threonine (Thr) residue of the β5 subunit of 20S proteasome. The X-ray crystal structure of the yeast 20S proteasome covalently bound to **1** (PDB code: 2ZCY)[5a] revealed that the isopropyl side chain of the internal valine (Val) residue was recognized by an S3 subsite, which constitutes a hydrophobic pocket (Figure 3a). Moreover, the hydrophobic pocket could accept a larger hydrophobic substituent than the isopropyl group. To improve the proteasome inhibitory activity by increasing the hydrophobic interaction, we designed the analogues **16a–e**, wherein the internal isopropyl group was replaced with a range of alkyl substituents, including a benzyl group (Scheme 2a). The ureadipeptide carboxylic acids **15a–e** were prepared using solid-phase synthesis to deliver a variety of analogues efficiently in a short time, and the analogues **16a–e** were synthesized in a manner similar to the synthesis of **1**. The CT-L inhibitory activity of **16a–e** at 250 nM was well correlated to the size of alkyl substituents (Figure 3b), and increasing the size of the hydrophobic substituents resulted in increased CT-L inhibitory activity. The benzyl-substituted analogue **16e** ($K_i = 44$ nM, Table 1) exhibited a 15-fold stronger CT-L inhibitory activity than **1**. The analogue **16e**, however, showed weak cytotoxic activity ($IC_{50} = 947$–3370 nM, Table 1) and this is because of the lack of cell membrane permeability of **16e**. It was reported that hybrid-type analogues of **1** and **2** exhibited an increased proteasome inhibition and cytotoxic activity against human cancer cells.[9,14] These results prompted us, likewise, to install a cell-permeable side chain on the core structure of **16e**. The analogues **19a–e**, which were prepared as shown in Scheme 2b, exhibited a strong isolated CT-L inhibitory activity with apparent K_i values in a range of 0.14–1.1 nM (Table 1). We also evaluated the ability of **19a–e** to inhibit the proteasome in living cells using a cell-based proteasome activity with human embryonic kidney 293 (HEK293) cells. The analogues **19a–e** showed a strong CT-L inhibitory activity (Figure 3c) as well as trypsin-like (T-L, β2 subunit) activity (see the Supporting Information). A strong cytotoxic activity was also observed and the analogue **19a**, possessing the decanoyl group, and it was the most potent with an IC_{50} value

of 2.2 nM for human myeloma RPMI8226 cells (Table 1). To confirm its target selectivity, the cell lysate, treated with the activity-based probe[15] **19f** (see the Supporting Information for its preparation), was analyzed by gel electrophoresis (Figure 3d). As a result, only two bands, corresponding to the β2 and β5 subunits, were fluorescently labeled with **19f**. Labeling of these bands was inhibited by bortezomib in a dose-dependent manner. This result clearly indicates that the syringolin A analogues **19** exhibit a high specificity to the 20S proteasome. The transcription factor NFκB, responsible for immune and inflammatory responses, is primarily regulated by interaction with inhibitory IκB proteins, which is further regulated by phosphorylation followed by proteasome degradation through the poly-ubiquitination. Treatment of HEK293 cells with **19a,e** led to the accumulation of phosphorylated and poly-ubiquitinated IκBα as a result of proteasome inhibition, and was similar in behavior to that of bortezomib (Figure 3e). These results confirm that **19a,e** are promising cell-permeable and selective proteasome inhibitors as anticancer agents.

In conclusion, the total synthesis of syringolin A was established by a rare intra-U3CR. The 20S proteasome inhibitory activity of **1** was improved and guided by the structure-based drug design targeting the S3 subsite of the β5 subunit. Cytotoxic activity was also greatly improved by installing the membrane-permeable substituent. Further studies to develop a next-generation proteasome inhibitor as an anticancer drug, including evaluation of in vivo anticancer activity, administration, distribution, metabolism, and toxicity are currently ongoing.

Received: February 14, 2014
Published online: March 25, 2014

Keywords: antitumor agents · drug discovery · lactams · medicinal chemistry · natural products

[1] a) D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2012**, *75*, 311 – 335; b) D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2007**, *70*, 461 – 477; c) R. O'Shea, H. E. Moser, *J. Med. Chem.* **2008**, *51*, 2871 – 2878.

[2] a) U. Wäspi, D. Blanc, T. Winkler, P. Ruedi, R. Dudler, *Mol. Plant-Microbe Interact.* **1998**, *11*, 727 – 733; b) U. Wäspi, P. Hassa, A. A. Staempfli, L. P. Molleyres, T. Winkler, R. Dudler, *Microbiol. Res.* **1999**, *154*, 89 – 93.

[3] a) M. Oka, K. Nishikawa, S. Ohta, H. Kamei, M. Konishi, T. Miyaki, T. Oki, H. Kawaguchi, *J. Antibiot.* **1988**, *41*, 1331 – 1337; b) M. Oka, K. Yaginuma, K. Numata, M. Konishi, T. Oki, H. Kawaguchi, *J. Antibiot.* **1988**, *41*, 1906 – 1909.

[4] D. Krahn, C. Ottmann, M. Kaiser, *Nat. Prod. Rep.* **2011**, *28*, 1854 – 1867.

[5] a) M. Groll, B. Schellenberg, A. S. Bachmann, *Nature* **2008**, *452*, 755 – 759; b) A. K. Nussbaum, T. P. Dick, W. Keilholz, M. Schirle, S. Stevanović, K. Dietz, W. Heinemeyer, M. Groll, D. H. Wolf, R. Huber, H.-G. Rammensee, H. Schild, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12504 – 12509.

[6] a) J. Adams, *Cancer Cell* **2004**, *5*, 412 – 417; b) P. Moreau, P. G. Richardson, M. Cavo, R. Z. Orlowski, J. F. San Miguel, A. Palumbo, J.-L. Harousseau, *Blood* **2012**, *120*, 947 – 959.

[7] See Ref. [4].

[8] C. Naujokat, D. Fuchs, C. Berges, *Biochim. Biophys. Acta Mol. Cell Res.* **2007**, *1773*, 1389 – 1397.

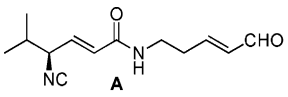
[9] a) J. Clerc, N. Li, D. Krahn, M. Groll, A. S. Bachmann, B. I. Florea, H. S. Overkleeft, M. Kaiser, *Chem. Commun.* **2011**, *47*, 385 – 387; b) C. R. Archer, M. Groll, M. L. Stein, B. Schellenberg, J. Clerc, M. Kaiser, T. P. Kondratyuk, J. M. Pezzuto, R. Dudler, A. S. Bachmann, *Biochemistry* **2012**, *51*, 6880 – 6888.

[10] Only one study has been reported in the literature. A. I. Khan, A. K. Saxene, *Tetrahedron* **2012**, *68*, 294 – 299.

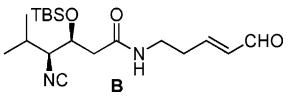
[11] For total synthesis, see: a) J. Clerc, M. Groll, D. J. Illich, A. S. Bachmann, R. Huber, B. Schellenberg, R. Dudler, M. Kaiser, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 6507 – 6512; b) J. Clerc, B. Schellenberg, M. Groll, A. S. Bachmann, R. Huber, R. Dudler, M. Kaiser, *Eur. J. Org. Chem.* **2010**, 3991 – 4003; c) C. Dai, C. R. Stephenson, *Org. Lett.* **2010**, *12*, 3453 – 3455; d) M. C. Pirrung, G. Biswas, T. R. Ibarra-Rivera, *Org. Lett.* **2010**, *12*, 2402 – 2405.

[12] K. M. Lassen, J. Lee, M. M. Joullie, *Tetrahedron Lett.* **2010**, *51*, 1635 – 1638.

[13] Initial attempt of the intra-U3CR with the α,β-unsaturated carboxamide **A** or the diastereomer at the silyloxy group (**B**) failed to give the corresponding macrocycle. Details will be described elsewhere.



A



B

[14] C. R. Archer, D. L. Koomoa, E. M. Mitsunaga, J. Clerc, M. Shimizu, M. Kaiser, B. Schellenberg, R. Dudler, A. S. Bachmann, *Biochem. Pharmacol.* **2010**, *80*, 170 – 178.

[15] J. Clerc, B. I. Florea, M. Kraus, M. Groll, R. Huber, A. S. Bachmann, R. Dudler, C. Driessen, H. S. Overkleeft, M. Kaiser, *ChemBioChem* **2009**, *10*, 2638 – 2643.

