

The Multiple Multicomponent Approach to Natural Product Mimics: Tubugis, N-Substituted Anticancer Peptides with Picomolar Activity

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 Supporting Information

ABSTRACT: The synthesis of a new generation of highly cytotoxic tubulysin analogues (i.e., tubugis) is described. In the key step, the rare, unstable, and synthetically difficult to introduce tertiary amide–*N,O*-acetal moiety required for high potency in natural tubulysins is replaced by a dipeptoid element formed in an Ugi four-component reaction. Two of the four components required are themselves produced by other multicomponent reactions (MCRs). Thus, the tubugis represent the first examples of the synthesis of natural-product-inspired compounds using three intertwined isonitrile MCRs.

Multicomponent reactions (MCRs) are among the most powerful synthetic tools available.¹ They allow rapid access to structural variation and complexity within single-step conversions. The potential of multiple bond formations among more than two building blocks in a one-step procedure has been widely exploited in combinatorial and medicinal chemistry, coming close to the concept of an ideal synthesis.^{1,2} Isonitrile-based MCRs are especially unrivalled in diversity-oriented synthesis (DOS) strategies.^{2a,b} The impact of MCRs on target-oriented synthesis (TOS) has been much less pronounced but is nevertheless remarkable because shorter and more elegant synthetic routes have emerged.³ For large molecules, the ideal of multiple MCRs, i.e., the use of two or more (different types of) MCRs within a target-oriented approach toward natural products or their derivatives, has remained unexplored. In this paper, we show the use of sequential multiple MCRs in the synthesis of a new generation of highly cytotoxic tubulysin⁴ analogues.

Tubulysins are among the most potent antimitotic agents known to date. These unusual tetrapeptides disrupt the microtubule spindle and were first isolated by Höfle's group from myxobacteria culture broths.^{4a} Average growth inhibition (GI₅₀) values range from nanomolar to picomolar concentrations and outperform those of taxoids and vinca alkaloids. The extraordinarily high cytotoxic activity, which also extends to multi-drug-resistant cell lines, makes the tubulysins a remarkable lead for the development of novel anticancer drugs.^{4b} They are especially suitable as "warheads" for use in conjugation strategies with targeting entities (i.e., antibodies, nanospheres, folates), as the cancer-cell-specific structures often are not abundant, meaning that a low concentration of the active moiety must suffice to kill the targeted tissue area.⁵ Not surprisingly, tremendous attention

has been given to the synthesis of natural tubulysins and simplified analogues.⁶

Tubulysins (Figure 1, box) are usually classified by the amino acid at the C-terminus, i.e., tubutyrosine (Tut, A series) or tubuphenylalanine (Tup, D series). Each series is produced by a specific strain of myxobacteria. The thiazole-containing amino acid tubuvaline (Tuv), L-isoleucine (Ile), and the hydrophobic D-*N*-methylpipecolic acid (Mep) are common to all tubulysins. The most potent tubulysins possess a rare tertiary amide that makes the middle part of the molecule extremely crowded and constitutes the major challenge in their synthesis.^{4b} Despite many efforts, only a few groups have successfully introduced this chemical motif during the total synthesis of tubulysins.^{6c,d,p} Previous studies have shown that the replacement of this functionality by simpler alkyl groups improves the synthetic accessibility and restores most of the activity.^{6e,o}

The *N*-alkyl amide function imparts to the peptide backbone greater stability toward enzymatic cleavage and, more importantly, reduces the energy barrier between the *s*-cis and *s*-trans configurations of the amide bond.⁷ The resulting conformational arrangements are essential for the biological activity. However, *N*-branched amides (peptoids in the wider sense) are still difficult to synthesize in a sterically challenged environment such as that in the tubulysins, with the resulting problems of low yields, low reproducibility, and sometimes instability of neighboring stereocenters during synthesis.

One of the best methods for generating *N*-substituted peptides and peptoids is the Ugi four-component reaction (Ugi-4CR), which has been used extensively by us for this purpose.⁸ The reaction normally is not sensitive to steric bulk. Thus, we envisioned that the tertiary amide functionality could be introduced by the suitable application of an Ugi-4CR as the key step (MCR-3; Figure 1, left). Hereby the acid- and base-labile *N,O*-acetal–ester moiety would be replaced by a stable retro-amide. We hoped that this approach would lead to the novel generation of tubulysin-type tertiary tetrapeptides (named "tubugis") with retention of the cytotoxic activity and improved hydrolytic stability.⁹ Further retroanalysis suggested that the two major building blocks required, i.e., the acid and amine components in the Ugi-4CR, would again be accessible by MCRs (MCR-1 and MCR-2; Figure 1), as detailed below.

The synthesis started with the multigram preparation of Boc-protected tubuvaline (Tuv) ethyl ester **4** using a Passerini-type reaction as the key step and of Boc-protected Tuv–Tup–OME

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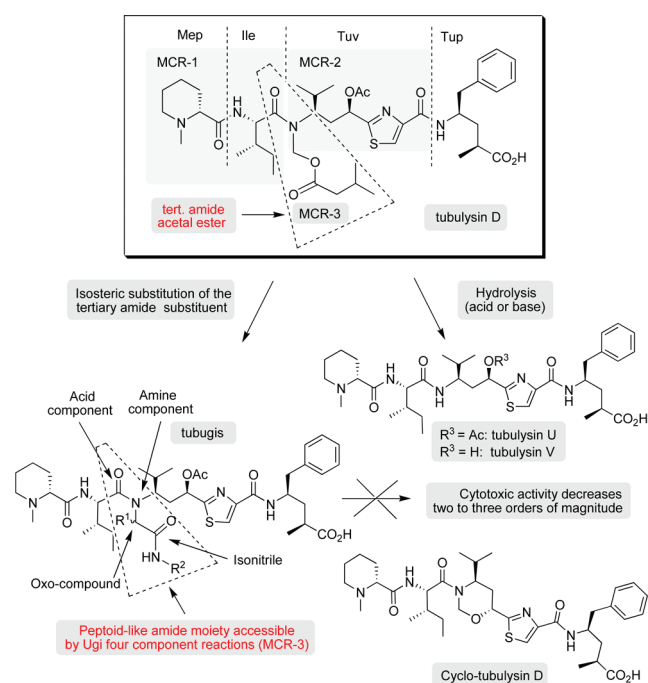
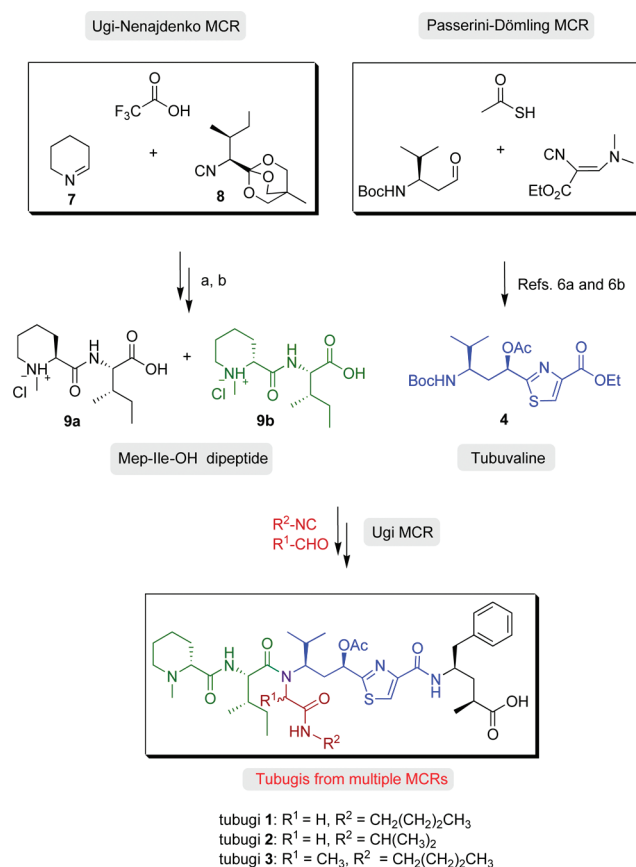


Figure 1. Natural tubulysin D and tubugis.

dipeptide by conventional peptide coupling, as described earlier by us^{6a,b} (Scheme 1). The Mep–Ile–OH fragment **9b** was synthesized through an Ugi-type MCR involving Δ^1 -piperidine (7),¹⁰ the 4-methyl-2,6,7-trioxabicyclo[2.2.2]octyl (OBO) ester (8) of isoleucine isocyanide,¹¹ and trifluoroacetic acid (TFA).¹² This strategy, combined with a basic hydrolysis/reductive amination protocol, allowed straightforward access to the hydrochloride salts of the Mep–Ileu–OH dipeptide fragment **9b** and its diastereomer **9a**, which were obtained in 67% overall yield in a ratio of ~1:1 and were easily separated by flash column chromatography (for the stereochemical assignments, see the Supporting Information). It was decided to use OBO ester **8** in order to decrease the acidity of the α -hydrogen atom and avoid the commonly observed epimerization at this center,¹¹ although isoleucine isocyanide methyl ester has been shown to be configurationally stable in some Ugi reactions under certain conditions.¹³ The wide range of readily available cyclic imines offers the potential for further exploration of derivatives that have been inaccessible in tubulysin syntheses to date, as the basic tertiary amine terminus is of remarkable importance for the cytotoxic activity of tubulysins and related compounds such as the dolastatins.^{6e,7,14}

The crucial Ugi-4CR-based coupling was initially attempted using as the amino component the Tuv–Tup–OMe dipeptide, which was generated in situ from its Boc-protected derivative; Mep–Ile–OH (**9b**) served as the carboxylic acid component, paraformaldehyde as the oxo component, and isopropyl isocyanide as the condensing agent. This small alkyl isocyanide was selected because it possesses the closest similarity to the natural counterpart and therefore was considered most likely to provide similar properties. Unfortunately, despite trials under many conditions (e.g., different solvents or proportions of the reactants, variation of the order and times of addition, catalysts), the coupling always gave the “double isocyanide addition product” **11** as the major compound in the reaction mixture (Scheme 2).

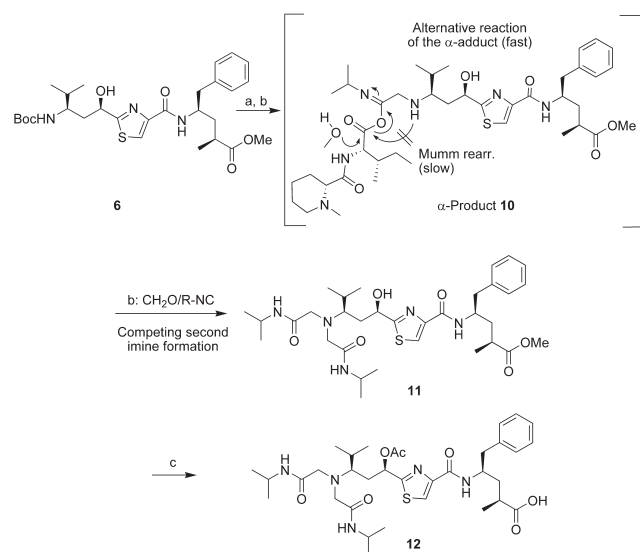
Scheme 1. Multiple Multicomponent Approach toward the Tubugis^a



^a Reagents and conditions: (a) (1) MeOH, stirring, 15 h; (2) CF₃COOH, stirring, 30 min; (3) NaOH, THF/H₂O; no purification. (b) (1) (CH₂O)_m, Pd(OH)₂/C, H₂, MeOH/H₂O (3:1 v/v), stirring, 16 h, separation of the C₂ diastereomers; (2) HCl, THF/H₂O (1:1 v/v); **9b** in 31% overall yield.

One possible explanation for this alternative product formation was the action of water as an acid substitute during the Ugi reaction.¹⁵ Such Ugi-3CR products are common byproducts that have been known since the first Ugi reaction was reported. In sterically dense reactions, bulky carboxylates are substituted by the usually less reactive water or, under very dry conditions, by small nucleophilic solvents such as methanol. However, in this case, a different escape route emerged: after reaction of the carboxylate in the usual manner, the intermediate α -product was unable to undergo a Mumm rearrangement, and the usually faster attack of the internal amine nucleophile was substituted by attack of the external nucleophile and solvent methanol. This was evidenced by formation of Mep–Ileu–OMe (the methyl ester of **9b**) during the coupling. The resulting α -product **10** could undergo a second Ugi-3CR of the same type via an iminium ion. The reaction outcome was identical when the experiment was performed under extremely dry conditions. Basic hydrolysis of methyl ester **11** under mild basic conditions followed by acetylation of the secondary alcohol led to branched amine **12**, which showed no cytotoxic activity against human cancer cell lines (see Table 1).

These findings prompted us to perform the key Ugi-4CR coupling at an earlier stage of the synthesis, using the tubuvaline

Scheme 2. Undesirable Alternative Ugi-3CR^a

^a Reagents and conditions: (a) (1) $\text{CH}_2\text{Cl}_2/\text{CF}_3\text{COOH}$ (4:1 v/v); (2) washing with aqueous NaHCO_3 solution. (b) $(\text{CH}_2\text{O})_m$, **9b**, isopropyl isocyanide, MeOH, stirring overnight, 60% overall yield. (c) (1) LiOH, THF/ H_2O (1:1 v/v); (2) $\text{Ac}_2\text{O}/\text{Py}$; 86% yield over two steps.

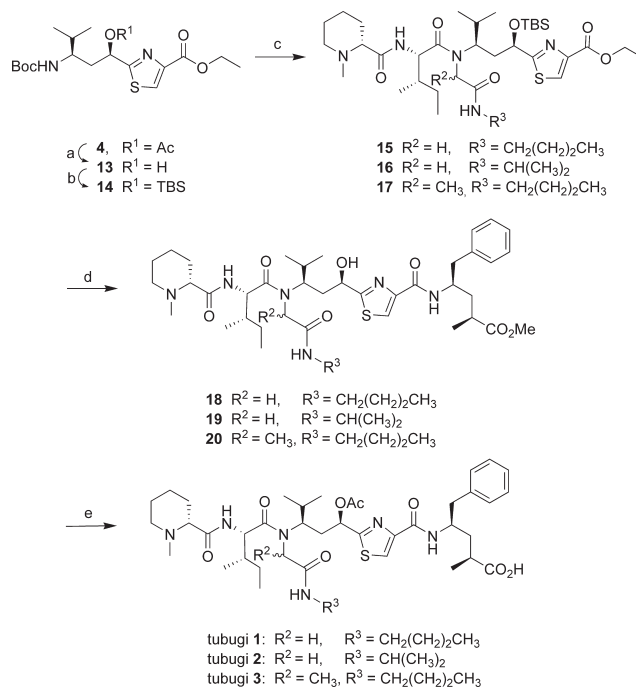
Table 1. Cytotoxic Activities

compound	GI_{50} (nM)	
	vs PC-3 ^a	vs HT-29 ^b
tubugi 1	0.23 ^c	0.14 \pm 0.02
tubugi 2	0.29 \pm 0.04	0.34 \pm 0.07
tubugi 3	0.22 \pm 0.01	0.56 \pm 0.04
branched amine 12	>1000 ^c	>1000 ^c
tubulysin A	0.21 \pm 0.05	0.32 \pm 0.06
taxol	7.2 \pm 4.3	5.3 \pm 1.2

^a Human prostate cancer cell line. ^b Human colon cancer cell line. ^c Single test or standard deviation > 0.2.

ethyl ester as the amino component (Scheme 3). To ensure optimum imine formation during the Ugi-4CR instead of reaction to give an undesired cyclic *N,O*-acetal species (cf. *cyclo*-tubulysin in Figure 1),^{6f} stable protection of the alcohol function was mandatory. The protected Tuv derivative **4** was converted from the reactive *O*-acetyl-protected to the sufficiently stable *O*-TBS-protected free amine by selective cleavage of the Boc group of **14** with $\text{CH}_2\text{Cl}_2/\text{TFA}$ (4:1 v/v) at 0 °C followed by removal of excess TFA with aqueous NaHCO_3 solution. This afforded the desired amino precursor for the Ugi-4CR. Once more, the Ugi-4CR was carried out using the same other components as before (paraformaldehyde, Mep-Ileu-OH, and isopropyl isocyanide).

The desired peptoid **16** was obtained as the major product only when the isopropyl isocyanide was added slowly over a period of 3 h using a syringe pump, thus allowing the Mumm rearrangement to take place before the concentration of isonitrile reached levels that would allow the formation of the double-addition product. This finding can be explained considering the fact that the competitive second imine formation is reversible and its capture would be favored only by fast addition, i.e., a high

Scheme 3. Tubugi Syntheses^a

^a Reagents and conditions: (a) NaOEt, EtOH, 92% yield. (b) TBSCl, imidazol, *N,N*-dimethylformamide (DMF); no purification. (c) (1) $\text{CH}_2\text{Cl}_2/\text{CF}_3\text{COOH}$ (4:1 v/v) at 0 °C; (2) NaHCO_3 ; (3) $(\text{CH}_2\text{O})_n$ for **15** and **16** and CH_3CHO for **17**, **9b**, isopropyl isocyanide for **16** and *n*-butyl isocyanide for **15** and **17**, MeOH; 35–52% overall yield. (d) (1) $\text{CF}_3\text{COOH}/\text{THF}/\text{H}_2\text{O}$ (2:2:1 v/v/v); (2) LiOH in THF/ H_2O (1:1 v/v); (3) DIC, PFP, CH_2Cl_2 ; (4) $\text{Tup}-\text{OMe}\cdot\text{HCl}$, diisopropylethylamine, DMF; 55–62% overall yield. (e) (1) LiOH, THF/ H_2O (v/v 1:1); (2) $\text{Ac}_2\text{O}/\text{Py}$ (in the case of tubugi 3, a catalytic amount of dimethylaminopyridine was added); 71–86% overall yield.

concentration of the isocyanide (see the Supporting Information for more details).

Completion of the synthesis was initiated by cleavage of silyl ether **16** followed by hydrolysis of the ethyl ester under basic conditions. Coupling with the hydrochloride salt of $\text{Tup}-\text{OMe}$ ^{6a,b} was performed using a standard *N,N'*-diisopropylcarbodiimide/pentafluorophenol (DIC/PFP) protocol, giving methyl ester **19** in 55% yield over three steps. Finally, hydrolysis of the methyl ester was completed under mild basic conditions to afford the desired tubugi **2** after acetylation of the secondary alcohol. The congeners, tubugis **1** and **3**, were similarly prepared by variation of the isocyanide and oxo component, respectively, in the crucial Ugi coupling. Interestingly, the highest yield for this Ugi coupling was obtained for compound **17**, which was formed when a higher aldehyde that introduced additional steric hindrance and a α -branched side chain was used. The reason might be that paraformaldehyde, which was used for **1** and **2**, is often one of the worst performers in Ugi reactions. As expected, isolation of **17** gave an almost 1:1 mixture of diastereomers,^{2b} which unfortunately could not be resolved at any stage of the synthesis of tubugi **3**.

The cytotoxic activity of tubugis **1**–**3** was evaluated against human cancer cell lines using tubulysin A and taxol as reference compounds (Table 1). Tubugis **1**, **2**, and **3** showed prominent biological activities, most likely acting as microtubule modifiers

like their lead natural products. They exhibited potential equal to that of tubulysin A within the error limits of the experiments.

In summary, we have reported the first synthesis of natural-product analogues by means of a combination of three different types of isonitrile-based multicomponent reactions in a concise and convergent manner. The new cytotoxic tubulysin analogues ("tubugis") show GI₅₀ values in the high picomolar range. The rare peptoid *N,O*-acetal-ester functionality has been replaced by a more stable *N*-branched peptide backbone with retention of the cytotoxic activity. Most importantly, the unreliable multistep generation of the sterically hindered tertiary amide function could be substituted with the more reliable one-pot multicomponent assembly. The simplicity of the synthesis highlights the extraordinary value and scope of multiple MCRs in the construction of complex target molecules.

The use of natural product leads and multiple MCRs together can be considered a valuable strategy for the generation of bioactive derivatives. To our knowledge, tubugis are among the most potent artificial anticancer agents ever discovered and represent the first example of a target-oriented synthesis approach using multiple MCRs. Other synthetic strategies based on multiple MCRs are currently under development in our group. We hope that the findings presented herein encourage further investigations of the application or development of new (combinations of) MCRs as a powerful means for the synthesis of complex natural products and related compounds.

■ ASSOCIATED CONTENT

S Supporting Information. Detailed experimental procedures; full characterization of new compounds; and selected copies of ¹H and ¹³C NMR spectra, HPLC chromatograms, and HRMS (ESI-FT-ICR) spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ REFERENCES

- (1) *Multicomponent Reactions*; Zhu, J., Bienyamé, H., Eds.; Wiley-VCH: Weinheim, Germany, 2005.
- (2) For reviews, see: (a) Dömling, A. *Chem. Rev.* **2006**, *106*, 17. (b) Dömling, A.; Ugi, I. *Angew. Chem., Int. Ed.* **2000**, *39*, 3168. (c) Lesanko-Ulaczyk, A.; Hall, D. G. *Curr. Opin. Chem. Biol.* **2005**, *9*, 266. (d) Nair, V.; Rajesh, C.; Vinod, A. U.; Bindu, S.; Sreekanth, A. R.; Mathen, J. S.; Balagopal, L. *Acc. Chem. Res.* **2003**, *36*, 899.
- (3) For recent reviews, see: (a) Toure, B. B.; Hall, D. G. *Chem. Rev.* **2009**, *109*, 4439. (b) Gulevich, A. V.; Zhdanko, A. G.; Orru, R. V. A.; Nenajdenko, V. G. *Chem. Rev.* **2010**, *110*, 5235.
- (4) (a) Sasse, F.; Steinmetz, H.; Heil, J.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **2000**, *53*, 879. (b) Steinmetz, H.; Glaser, N.; Herdtweck, E.; Sasse, F.; Reichenbach, H.; Höfle, G. *Angew. Chem., Int. Ed.* **2004**, *43*, 4888.
- (5) (a) Floyd, W. C., III; Datta, G. K.; Imamura, S.; Kieler-Ferguson, H. M.; Jerger, K.; Patterson, A. W.; Fox, M. E.; Szoka, F. C.; Fréchet, J. M. J.; Ellman, J. A. *ChemMedChem* **2011**, *6*, 49. (b) Kularatne, S. A.; Venkatesh, C.; Santhapuram, H. K. R.; Wang, K.; Vaitilingam, B.; Henne, W. A.; Low, P. S. *J. Med. Chem.* **2010**, *53*, 7767. (c) Schlupe, T.; Gunawan, P.; Ma, L.; Jensen, G. S.; Düringer, J.; Hinton, S.; Richter, W.; Hwang, J. *Clin. Cancer Res.* **2009**, *15*, 181. (d) Reddy, J. A.; Dorton, R.; Dawson, A.; Vetz, M.; Parker, N.; Nicoson, J. S.; Westrick, E.; Klein, P. J.; Wang, Y.; Vlahov, I. R.; Leamon, C. P. *Mol. Pharmaceutics* **2009**, *6*, 1518. (e) Leamon, C. P.; Reddy, J. A.; Vetz, M.; Dorton, R.; Westrick, E.; Parker, N.; Wang, Y.; Vlahov, I. *Cancer Res.* **2008**, *68*, 9839.
- (6) (a) Dömling, A.; Beck, B.; Eichelberger, U.; Sakamuri, S.; Menon, S.; Chen, Q.-Z.; Lu, Y.; Wessjohann, L. A. *Angew. Chem., Int. Ed.* **2006**, *45*, 7235. (b) Dömling, A.; Beck, B.; Eichelberger, U.; Sakamuri, S.; Menon, S.; Chen, Q.-Z.; Lu, Y.; Wessjohann, L. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 2347. (c) Pando, O.; Dörner, S.; Preusentanz, R.; Denkert, A.; Porzel, A.; Richter, W.; Wessjohann, L. *Org. Lett.* **2009**, *11*, 5567. (d) Peltier, H. M.; McMahon, J. P.; Patterson, A. W.; Ellman, J. A. *J. Am. Chem. Soc.* **2006**, *128*, 16018. (e) Patterson, A. W.; Peltier, H. M.; Sasse, F.; Ellman, J. A. *Chem.—Eur. J.* **2007**, *13*, 9534. (f) Patterson, A. W.; Peltier, H. M.; Ellman, J. A. *J. Org. Chem.* **2008**, *73*, 4362. (g) Sani, M.; Fossati, G.; Huguenot, F.; Zanda, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 3526. (h) Balasubramanian, R.; Raghavan, B.; Begaye, A.; Sackett, D. L.; Fecik, R. A. *J. Med. Chem.* **2009**, *52*, 238. (i) Raghavan, B.; Balasubramanian, R.; Steele, J. C.; Sackett, D. L.; Fecik, R. A. *J. Med. Chem.* **2008**, *51*, 1530. (j) Balasubramanian, R.; Raghavan, B.; Steele, J. C.; Sackett, D. L.; Fecik, R. A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2996. (k) Ulrich, A.; Chai, Y.; Pistorius, D.; Elnakady, Y. A.; Herrmann, J. E.; Weissman, K. J.; Kazmaier, U.; Müller, R. *Angew. Chem., Int. Ed.* **2009**, *48*, 4422. (l) Ulrich, A.; Herrmann, J.; Müller, R.; Kazmaier, U. *Eur. J. Org. Chem.* **2009**, 6367. (m) Burkhart, J. L.; Müller, R.; Kazmaier, U. *Eur. J. Org. Chem.* [Online early access]. DOI: 10.1002/ejoc.201100155. Published Online: April 18, 2011. (n) Wipf, P.; Takada, T.; Rishel, M. J. *Org. Lett.* **2004**, *6*, 4057. (o) Wipf, P.; Wang, Z. *Org. Lett.* **2007**, *9*, 1605. (p) Wang, Z.; McPherson, P. A.; Raccor, B. S.; Balachandran, R.; Zhu, G.; Day, B. W.; Vogt, A.; Wipf, P. *Chem. Biol. Drug Des.* **2007**, *70*, 75. (q) Shibue, T.; Hirai, T.; Okamoto, I.; Morita, N.; Masu, H.; Azumaya, I.; Tamura, O. *Chem.—Eur. J.* **2010**, *16*, 11678. (r) Shibue, T.; Okamoto, I.; Morita, N.; Morita, H.; Hirasawa, Y.; Hosoya, T.; Tamura, O. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 431. (s) Chandrasekhar, S.; Mahipal, B.; Kavitha, M. *J. Org. Chem.* **2009**, *74*, 9531.
- (7) Preusentanz, R.; Pando, O.; Wessjohann, L. *Nachr. Chem.* **2010**, *58*, 526.
- (8) (a) Wessjohann, L. A.; Rivera, D. G.; Vercillo, O. E. *Chem. Rev.* **2009**, *109*, 796. (b) Wessjohann, L. A.; Andrade, C. K. Z.; Vercillo, O. E.; Rivera, D. G. *Targets Heterocycl. Syst.* **2006**, *10*, 24. (c) Vercillo, O. E.; Andrade, C. K. Z.; Wessjohann, L. A. *Org. Lett.* **2008**, *10*, 205.
- (9) Patent application: Wessjohann, L. A.; Pando, O. *Tubugis*. EP 10 007 468.1, 2010.
- (10) Bender, D. R.; Bjeldanes, L. F.; Knapp, D. R.; Rapoport, H. *J. Org. Chem.* **1975**, *40*, 1264.
- (11) Zhdanko, A. G.; Nenajdenko, V. G. *J. Org. Chem.* **2009**, *74*, 884.
- (12) (a) Nenajdenko, V. G.; Gulevich, A. V.; Balenkova, E. S. *Tetrahedron* **2006**, *62*, 5922. (b) Gulevich, A. V.; Shevchenko, N. E.; Balenkova, E. S.; Rösenthaller, G. V.; Nenajdenko, V. G. *Synlett* **2009**, 403.
- (13) Bowers, M. M.; Caroll, P.; Joullie, M. M. *J. Chem. Soc., Perkin Trans. 1* **1989**, 857.
- (14) El Kaim, L.; Grimaud, L.; Oble, J.; Wagschal, S. *Tetrahedron Lett.* **2009**, *50*, 1741 and references cited therein.
- (15) Weber, L.; Wallbaum, S.; Broger, C.; Gubernator, K. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2280.