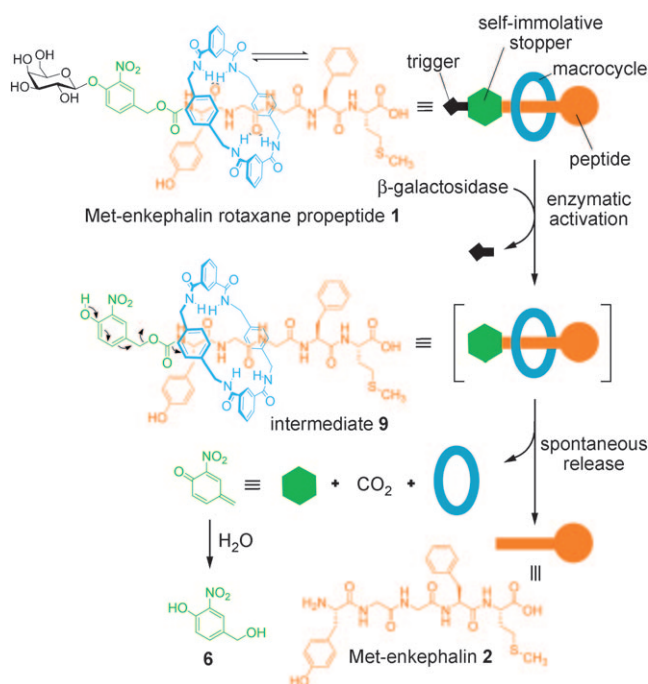


Rotaxane-Based Propeptides: Protection and Enzymatic Release of a Bioactive Pentapeptide**

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Rotaxane and pseudorotaxane architectures are starting to attract interest as a means of molecularly encapsulating substrates for potential biological applications.^[1–5] ‘Nano-valves’ that can store and release cargo molecules have been developed,^[1] and the encapsulation of squaraine dyes within rotaxanes has been shown to enhance dye fluorescence and stability as well as imparting different cell-localization propensities on the substrates.^[2] A stilbene-based rotaxane has been used to control the reactivity of a dipeptidic thread against protease hydrolysis,^[3] and functionalized rotaxanes have also been shown to serve as effective vehicles for the intracellular delivery^[4] of fluorescein-derivatized peptides. Polyrotaxanes have been studied for their potential as drug-delivery systems.^[5] Herein, we report the synthesis and properties of a rotaxane in which a macrocycle protects an extended pentapeptide thread from degradation by different types of peptidases and the cocktail of enzymes present in human plasma. The glycosidase-catalyzed cleavage of a carbohydrate unit in a ‘stopper’ of the rotaxane triggers the release of the bioactive parent peptide through a self-immolative mechanism (Scheme 1). The demonstration that a macrocycle is able to protect an oligopeptide of significant length from degradation by peptidases, whilst simultaneously allowing an orthogonal enzyme-activated release mechanism



Scheme 1. Enzyme-triggered release of Met-enkephalin **2** from [2]rotaxane propeptide **1**.

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to be built into the structure, is an important step toward the ultimate goal of a ‘molecular sheath’ peptide delivery system.

Benzylic amide macrocycles can be templated around dipeptides in which glycine is the N-terminal amino acid residue, to form [2]rotaxanes in good yields.^[6] Unfortunately, investigation of their efficacy in protecting peptide-based threads from enzymatic degradation (a common drawback to the use of oligopeptides in biological applications^[7]) has been hampered because the yield of the rotaxane-forming reaction decreases rapidly with increasing peptide length.^[6c,8] We envisaged that a solution might lie in employing a bulky nitrophenol ester^[9] as a stopper for an extendable,^[10] shorter, and more efficiently constructed, peptide rotaxane. We used this synthetic methodology to target a [2]rotaxane **1** based on Met-enkephalin **2** (H-Tyr-Gly-Gly-Phe-Met-OH), also known as opioid growth factor (OGF). Met-enkephalin **2** is a pentapeptide with a range of bioactivities that include regulation of nociception and also antitumor activity by control of cell growth.^[11] Release of the free pentapeptide was designed to occur through cleavage of an enzyme-accessible monosaccharide on one of the stoppers (Scheme 1) using *E. coli* β -galactosidase, an enzyme that has been successfully

targeted^[12] to specific cell types in antibody-directed enzyme prodrug therapy (ADEPT^[13]) strategies.

Met-enkephalin **2** possesses the glycine residue necessary for rotaxane assembly and a C-terminal amino acid sequence (Phe-Met) that is bulky enough to act as a stopper for a benzylic amide macrocycle. The carbohydrate trigger^[14] intended to release the bioactive peptide is covalently attached to the rest of the thread by a nitrobenzyloxycarbonyl linker. Following enzymatic activation with β -galactosidase, self-immolation of the deglycosylated stopper should result in the release of **2** along with the mechanical disassembly of the rotaxane architecture (Scheme 1).

Glycorotaxane^[15] **4** was prepared from galactosylated thread **3**, which bears a bulky 2,6-diphenyl-4-nitrophenyl stopper, in 26% yield (Scheme 2, step a). The rotaxane yield is not dissimilar to that found previously^[6c] using Phe-Gly threads (32%), despite the presence of both a nitrophenyl and tyrosyl ester in **3** that could potentially react with *p*-xylenediamine. We were delighted to find that although nucleophile access to the activated ester group was hindered by both the macrocycle (which was tightly locked on the short peptide portion of the thread) and the phenyl groups on the stopper, treatment of **4** with H-Phe-Met-OH in CHCl_3 at reflux afforded the elongated [2]rotaxane **5** in 40% yield (Scheme 2, step b), with no evidence of any dethreading of **4** or **5** (59% of **4** was recovered from the reaction). Deprotection of the hydroxy groups (Scheme 2, step c) furnished Met-enkephalin rotaxane propeptide **1**. The corresponding thread **7** was also prepared (see the Supporting Information).

The partial ^1H NMR spectra of [2]rotaxane **1** and thread **7** are shown in Figure 1. Apart from the signals that correspond to the carbohydrate protons (shown in black), most of the resonances in the thread are shifted upfield in the rotaxane, thus indicating that the macrocycle is able to move beyond the original template. Although

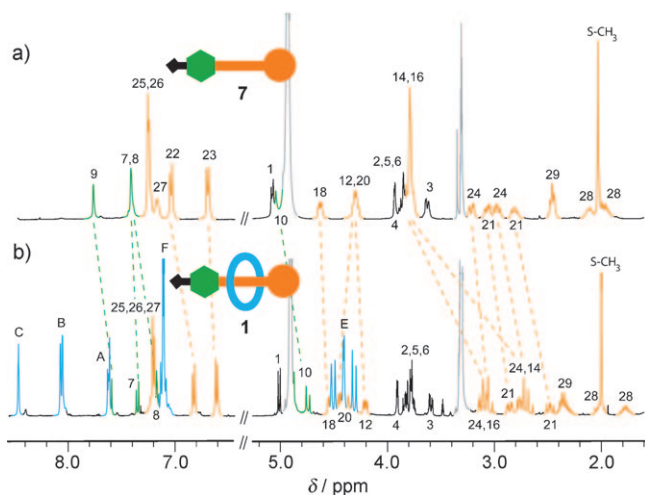
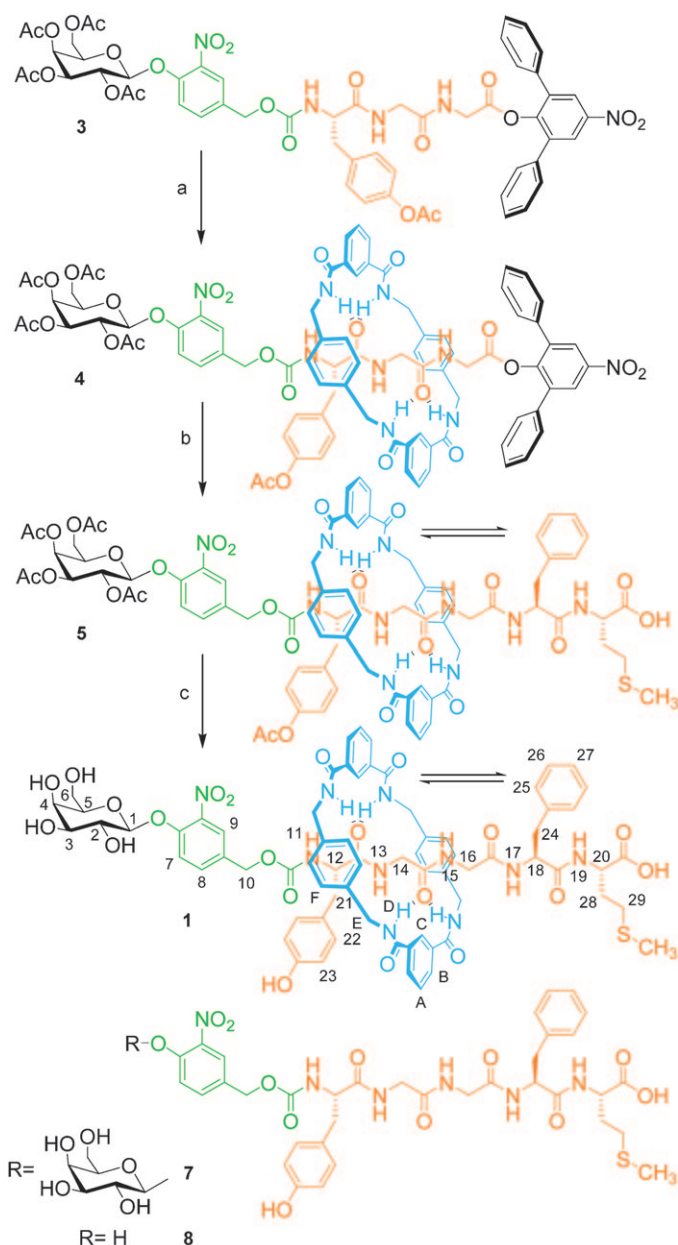


Figure 1. Partial ^1H NMR spectra (400 MHz, 298 K, CD_3OD) of a) thread **7** and b) rotaxane propeptide **1**. The assignments and coloring of signals correspond to that shown in Scheme 2. Residual solvent peaks are shown in gray.



Scheme 2. Synthesis of [2]rotaxane propeptide **1**. a) Isophthaloyl chloride, *p*-xylenediamine, Et_3N , CHCl_3 , 26%; b) H-Phe-Met-OH, *N,N*-diisopropylethylamine (DIPEA), CHCl_3 , reflux, 96 h, 40%; c) NaOMe, MeOH, 0°C , quantitative.

the rotaxane Gly-Gly methylene protons H_{14} and H_{16} are the most shielded, which indicates that the macrocycle spends most of its time in this region, various peptide protons (orange) near the C terminus (e.g., H_{24}) and protons in the nitrobenzyloxycarbonyl spacer (green) near the N terminus are shielded to some extent by the aromatic rings of the macrocycle.^[6]

The ability of the benzylic amide macrocycle to access almost the full length of the extended peptide augured well for its ability to protect the thread from degradation. We first confirmed that Met-enkephalin rotaxane **1** was stable in phosphate buffer at 37°C . After 48 hours, no decomposition

was observed by HPLC. The protease resistance of the rotaxane was then probed by comparing the behavior of Met-enkephalin **2**, rotaxane **1**, and the thread **7** under the action of different types of peptidases, the results of which are shown in Figures 2–4.

Enzymatic digestion of each of the three substrates with aminopeptidase M, an exopeptidase that hydrolyses Met-enkephalin sequentially from the N-terminal amino acid,^[16] was monitored by HPLC (Figure 2). While Met-enkephalin

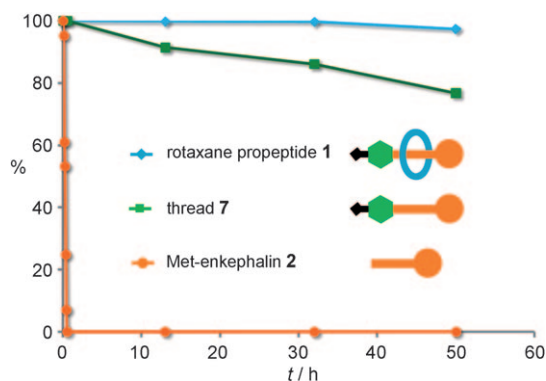


Figure 2. Enzymatic digestion of rotaxane propeptide **1** (blue diamonds), thread **7** (green squares), and Met-enkephalin **2** (orange circles) with porcine kidney aminopeptidase M. Reactions were carried out at 25 °C in 95 % phosphate buffer (0.05 M, pH 7.2), 5 % DMSO, using 0.005 μmol^{-1} of substrate. 0.0025 μmol^{-1} of enzyme was added every 12 h.

was rapidly degraded (< 30 minutes) using 0.005 units (U) of enzyme per μmol of substrate, thread **7** and rotaxane **1** proved stable for many hours under identical conditions and more than 70 % of **7** and more than 95 % of **1** were recovered after 2 days. In this case, the stability of both thread and rotaxane presumably arises from the fact that the part of Met-enkephalin that is recognized by the enzyme (the N terminus) is chemically derivatized in both **1** and **7**.

We next explored (Figure 3) the stability of **1**, **2**, and **7** towards angiotensin converting enzyme (ACE), a peptidase that hydrolyses Met-enkephalin between the Gly and Phe residues.^[17] HPLC showed that while both pristine Met-enkephalin **2** and the N-functionalized, but non-interlocked, derivative **7** were degraded within hours, rotaxane **1** exhibited exceptional resistance towards the action of ACE (> 90 % remaining after 2 days). The difference in stability between the rotaxane and thread can be attributed solely to the mechanical barrier to the enzyme provided by the macrocycle.

Finally, the stability of each of **1**, **2**, and **7** to the cocktail of peptidases and other enzymes present in human plasma was evaluated (Figure 4). Met-enkephalin **2** was completely consumed in less than 5 minutes in reconstituted human plasma at 37 °C. The non-interlocked thread **7** was degraded with a half-life of approximately 5 hours, whilst 90 % of rotaxane **1** was still present after 5 days incubation in the plasma.

Whilst the resistance to peptidases conferred by the macrocycle on the extended oligopeptide in the rotaxane is

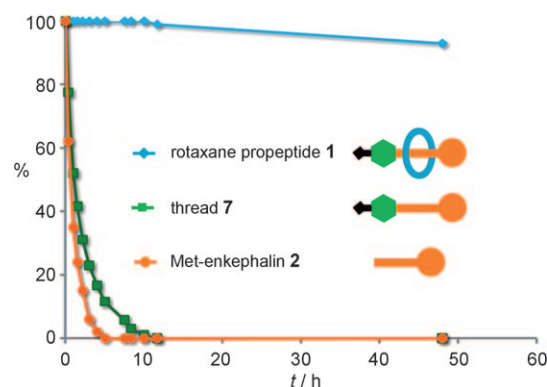


Figure 3. Enzymatic digestion of rotaxane propeptide **1** (blue diamonds), thread **7** (green squares), and Met-enkephalin **2** (orange circles) with rabbit lung angiotensin converting enzyme (ACE). Reactions were carried out at 25 °C in 95 % HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (0.05 M, pH 8.3), 5 % DMSO, 0.3 M NaCl, using 0.8 μmol^{-1} of substrate. 0.4 μmol^{-1} of enzyme was added every 12 h.

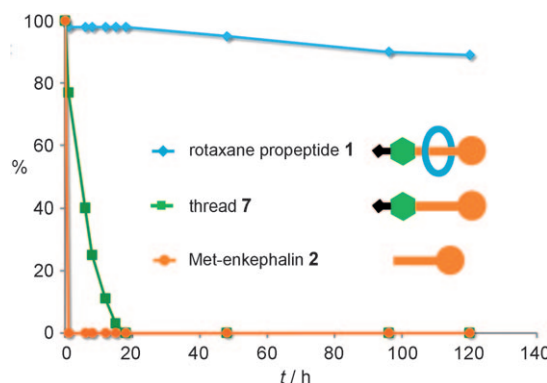


Figure 4. Stability of rotaxane propeptide **1** (blue diamonds), thread **7** (green squares), and Met-enkephalin **2** (orange circles) in human plasma reconstituted from lyophilized powder with 0.01 M Tris (tris(hydroxymethyl)aminomethane) buffer at pH 7.4, at 37 °C.

remarkable, if the parent peptide cannot ultimately be liberated from the molecular sheath then the structure can have no utility as a propeptide system. HPLC showed that the incubation of **1** with *E. coli* β -galactosidase in phosphate buffer (0.02 M, pH 7.0) at 37 °C resulted in the disappearance of **1** over 2–3 minutes (traces 2 and 3 in Figure 5b) and the emergence of a new peak with a retention time of 28 minutes and m/z 1318, which corresponds to the degalactosylated rotaxane **9**.^[18] This peak also quickly disappeared (traces 3 and 4 in Figure 5b) concomitantly with the appearance of benzylic alcohol **6** (the product formed by spontaneous reaction with water of the quinone methide intermediate released by self-immolation of the nitrohydroxybenzyl unit (Scheme 1)), the dethreaded macrocycle (m/z 532), and free Met-enkephalin **2**. Despite the presence of the macrocycle in **1**, and its efficacy in protecting the peptide portion of the rotaxane axle from enzymes, the remote siting of the β -galactose trigger results in no significant difference between

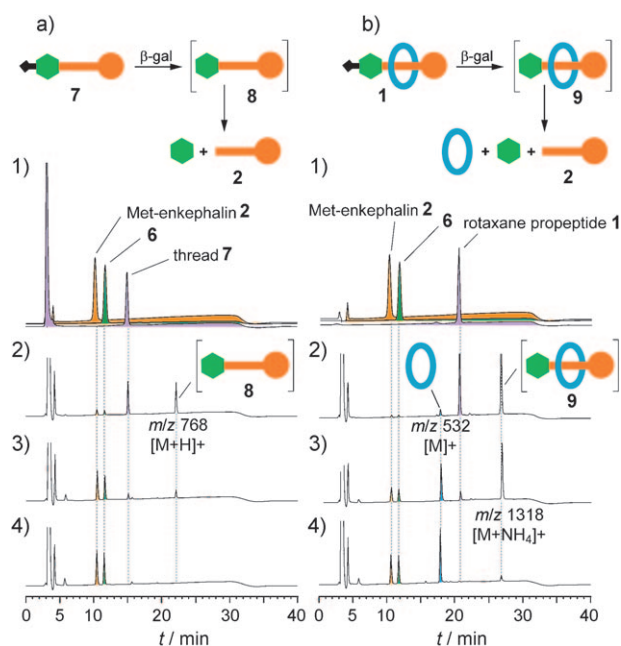


Figure 5. 1) Reference HPLC trace of individual components: a) **7**, **2** and **6**; b) **1**, **2** and **6**. 2–4) HPLC traces of the enzymatic hydrolysis of a) thread **7** and b) rotaxane propeptide **1** with *E. coli* β -galactosidase (β -gal) in 97.5% phosphate buffer (0.02 M, pH 7.0), 2.5% DMSO, at 37 °C using $10 \mu\text{mol}^{-1}$ of substrate: 2) after 1 min; 3) after 2 min; 4) after 10 min.

the rate of formation of free Met-enkephalin from rotaxane propeptide **1** and thread **7** (Figure 5).

In conclusion, we have demonstrated a rotaxane-based molecular device in which an oligopeptide can be protected from a broad range of peptidases for several days and then released in bioactive form in a matter of minutes through a specific glycosidase-mediated controlled-release mechanism. The concept may prove useful for the development of practical propeptide delivery methods, derivatizing the macrocycle to further improve the properties and characteristics of the system.

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- [1] a) T. D. Nguyen, H.-R. Tseng, P. C. Celestre, A. H. Flood, Y. Liu, J. F. Stoddart, J. I. Zink, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10029–10034; b) K. C.-F. Leung, T. D. Nguyen, J. F. Stoddart, J. I. Zink, *Chem. Mater.* **2006**, *18*, 5919–5928; c) T. D. Nguyen, K. C.-F. Leung, M. Liong, C. D. Pentecost, J. F. Stoddart, J. I. Zink, *Org. Lett.* **2006**, *8*, 3363–3366; d) T. D. Nguyen, Y. Liu, S. Saha, K. C.-F. Leung, J. F. Stoddart, J. I. Zink, *J. Am. Chem. Soc.* **2007**, *129*, 626–634; e) T. D. Nguyen, K. C.-F. Leung, M. Liong, Y. Liu, J. F. Stoddart, J. I. Zink, *Adv. Funct. Mater.* **2007**, *17*, 2101–2110; f) S. Angelos, Y.-W. Yang, K. Patel, J. F. Stoddart, J. I. Zink, *Angew. Chem.* **2008**, *120*, 2254–2258; *Angew. Chem. Int. Ed.* **2008**, *47*, 2222–2226; g) K. Patel, S. Angelos, W. R. Ditchel, A. Coskun, Y.-W. Yang, J. I. Zink, J. F. Stoddart, *J. Am.*

Chem. Soc. **2008**, *130*, 2382–2383; h) D. P. Ferris, Y.-L. Zhao, N. M. Khashab, H. A. Khatib, J. F. Stoddart, J. I. Zink, *J. Am. Chem. Soc.* **2009**, *131*, 1686–1688.

- [2] a) E. Arunkumar, C. C. Forbes, B. C. Noll, B. D. Smith, *J. Am. Chem. Soc.* **2005**, *127*, 3288–3289; b) E. Arunkumar, N. Fu, B. D. Smith, *Chem. Eur. J.* **2006**, *12*, 4684–4690; c) J. R. Johnson, N. Fu, E. Arunkumar, W. M. Leevy, S. T. Gammon, D. Piwnicka-Worms, B. D. Smith, *Angew. Chem.* **2007**, *119*, 5624–5627; *Angew. Chem. Int. Ed.* **2007**, *46*, 5528–5531; d) J. J. Gassensmith, E. Arunkumar, L. Barr, J. M. Baumes, K. M. DiVittorio, J. R. Johnson, B. C. Noll, B. D. Smith, *J. Am. Chem. Soc.* **2007**, *129*, 15054–15059.
- [3] A. G. Cheetham, M. G. Hutchings, T. D. W. Claridge, H. L. Anderson, *Angew. Chem.* **2006**, *118*, 1626–1629; *Angew. Chem. Int. Ed.* **2006**, *45*, 1596–1599.
- [4] a) V. Dvornikovs, B. E. House, M. Kaetzel, J. R. Dedman, D. B. Smithrud, *J. Am. Chem. Soc.* **2003**, *125*, 8290–8301; b) X. Bao, I. Isaacsohn, A. F. Drew, D. B. Smithrud, *J. Am. Chem. Soc.* **2006**, *128*, 12229–12238; c) J. Zhu, B. E. House, E. Fleck, I. Isaacsohn, A. F. Drew, D. B. Smithrud, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5058–5062; d) X. Wang, X. Bao, M. McFarland-Mancini, I. Isaacsohn, A. F. Drew, D. B. Smithrud, *J. Am. Chem. Soc.* **2007**, *129*, 7284–7293; e) J. Zhu, M. McFarland-Mancini, A. F. Drew, D. B. Smithrud, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 520–523.
- [5] a) T. Ooya, N. Yui, *Crit. Rev. Ther. Drug Carrier Syst.* **1999**, *16*, 289–330; b) T. Ooya, N. Yui, *J. Controlled Release* **1999**, *58*, 251–269; c) T. Ooya, K. Arizono, N. Yui, *Polym. Adv. Technol.* **2000**, *11*, 642–651; d) M. Eguchi, T. Ooya, N. Yui, *J. Controlled Release* **2004**, *96*, 301–307.
- [6] a) D. A. Leigh, A. Murphy, J. P. Smart, A. M. Z. Slawin, *Angew. Chem.* **1997**, *109*, 752–756; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 728–732; b) G. W. H. Wurpel, A. M. Brouwer, I. H. M. van Stokkum, A. Farran, D. A. Leigh, *J. Am. Chem. Soc.* **2001**, *123*, 11327–11328; c) G. Brancato, F. Coutrot, D. A. Leigh, A. Murphy, J. K. Y. Wong, F. Zerbetto, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4967–4971; d) M. Asakawa, G. Brancato, M. Fanti, D. A. Leigh, T. Shimizu, A. M. Z. Slawin, J. K. Y. Wong, F. Zerbetto, S. Zhang, *J. Am. Chem. Soc.* **2002**, *124*, 2939–2950; e) T. Da Ros, D. M. Guldi, A. Farran Morales, D. A. Leigh, M. Prato, R. Turco, *Org. Lett.* **2003**, *5*, 689–691; f) G. Bottari, D. A. Leigh, E. M. Pérez, *J. Am. Chem. Soc.* **2003**, *125*, 13360–13361; g) J. S. Hannam, S. M. Lacy, D. A. Leigh, C. G. Saiz, A. M. Z. Slawin, S. G. Stichele, *Angew. Chem.* **2004**, *116*, 3322–3326; *Angew. Chem. Int. Ed.* **2004**, *43*, 3260–3264; h) D. A. Leigh, M. A. F. Morales, E. M. Pérez, J. K. Y. Wong, C. G. Saiz, A. M. Z. Slawin, A. J. Carmichael, D. M. Haddleton, A. M. Brouwer, W. J. Buma, G. W. H. Wurpel, S. León, F. Zerbetto, *Angew. Chem.* **2005**, *117*, 3122–3127; *Angew. Chem. Int. Ed.* **2005**, *44*, 3062–3067.
- [7] *Peptide-Based Drug Design Methods in Molecular Biology*, Vol. 494 (Ed.: L. Otvos), Humana Press, New York, **2008**.
- [8] This is likely a result of the folding of the thread through strong intramolecular hydrogen bonding in the nonpolar solvents used to promote hydrogen bond-template rotaxane formation. Yields of rotaxanes from related pentapeptide threads attempted in our laboratory were typically less than 1%.
- [9] P. Garner, J. U. Yoo, *Tetrahedron Lett.* **1993**, *34*, 1275–1278.
- [10] a) S. J. Rowan, J. F. Stoddart, *J. Am. Chem. Soc.* **2000**, *122*, 164–165; b) D. W. Zehnder, D. B. Smithrud, *Org. Lett.* **2001**, *3*, 2485–2487; c) S.-H. Chiu, S. J. Rowan, S. J. Cantrill, J. F. Stoddart, A. J. P. White, D. J. Williams, *Chem. Eur. J.* **2002**, *8*, 5170–5183; d) T. J. Kidd, T. J. A. Loontjens, D. A. Leigh, J. K. Y. Wong, *Angew. Chem.* **2003**, *115*, 3501–3505; *Angew. Chem. Int. Ed.* **2003**, *42*, 3379–3383; e) O. Š. Miljanić, W. R. Dichtel, I. Aprahamian, R. D. Rohde, H. D. Agnew, J. R. Heath, J. F. Stoddart, *QSAR Comb. Sci.* **2007**, *26*, 1165–1174; f) J. M. Spruell, W. R.

- Dichtel, J. R. Heath, J. F. Stoddart, *Chem. Eur. J.* **2008**, *14*, 4168–4177.
- [11] a) I. S. Zagon, P. J. McLaughlin, *Neuropeptides* **2003**, *37*, 79–88; b) J. Fichna, A. Janecka, *Cancer Metastasis Rev.* **2004**, *23*, 351–366; c) I. S. Zagon, P. J. McLaughlin, *Neuropeptides* **2005**, *39*, 495–505; d) I. S. Zagon, K. A. Rahn, P. J. McLaughlin, *Neuropeptides* **2007**, *41*, 441–452; e) F. Cheng, I. S. Zagon, M. F. Verderame, P. J. McLaughlin, *Cancer Res.* **2007**, *67*, 10511–10518; f) F. Cheng, P. J. McLaughlin, M. F. Verderame, I. S. Zagon, *Mol. Biol. Cell* **2009**, *20*, 319–327.
- [12] a) H. Cheng, X. Cao, M. Xian, L. Fang, T. B. Cai, J. J. Ji, J. B. Tunac, D. Sun, P. G. Wang, *J. Med. Chem.* **2005**, *48*, 645–652; b) L. F. Tietze, F. Major, I. Schuberth, *Angew. Chem.* **2006**, *118*, 6724–6727; *Angew. Chem. Int. Ed.* **2006**, *45*, 6574–6577; c) A. Kamal, V. Tekumalla, A. Krishnan, M. Pal-Bhadra, U. Bhadra, *ChemMedChem* **2008**, *3*, 794–802.
- [13] a) P. D. Senter, C. J. Springer, *Adv. Drug Delivery Rev.* **2001**, *53*, 247–264; b) K. D. Bagshawe, *Expert Rev. Anticancer Ther.* **2006**, *6*, 1421–1431.
- [14] a) S. Papot, I. Tranoy, F. Tillequin, J.-C. Florent, J.-P. Gesson, *Curr. Med. Chem. Anti-Cancer Agents* **2002**, *2*, 155–185; b) I. Tranoy-Opalinski, A. Fernandes, M. Thomas, J.-P. Gesson, S. Papot, *Anti-Cancer Agents Med. Chem.* **2008**, *8*, 618–637.
- [15] a) F. Coutrot, E. Busseron, J.-L. Montero, *Org. Lett.* **2008**, *10*, 753–756; b) F. Coutrot, C. Romuald, E. Busseron, *Org. Lett.* **2008**, *10*, 3741–3744; c) F. Coutrot, E. Busseron, *Chem. Eur. J.* **2008**, *14*, 4784–4787; d) F. Coutrot, E. Busseron, *Chem. Eur. J.* **2009**, *15*, 5186–5190.
- [16] a) C. Gros, B. Giros, J. C. Schwartz, *Biochemistry* **1985**, *24*, 2179–2185; b) H. Terashima, N. Bunnett, *J. Gastroenterol.* **1995**, *30*, 696–704.
- [17] J. Lanzillo, J. Stevens, Y. Dasarathy, H. Yotsumoto, B. Fanburg, *J. Biol. Chem.* **1985**, *260*, 14938–14944.
- [18] Intermediates **8** and **9** are only stable for a few minutes at pH 7 at 37°C. However, they survive for hours at pH 2 at room temperature (the equilibrium between the phenol and phenolate forms is shifted toward the phenol and elimination occurs only slowly). In the β -gal-catalyzed reactions, aliquots were withdrawn from the reaction medium and diluted with a solution of trifluoroacetic acid (to pH 2) before being injected into the HPLC apparatus with the mobile phase buffered at pH 2 (see the Supporting Information).