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Open-chain half-bastadins mimic the effects of cyclic bastadins on calcium homeostasis in cultured neurons

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ABSTRACT

Constraining the catechol aryl ether moiety of bastadins by incorporation into a macrocyle is not necessary in order to mimic the effects of these marine natural products on neuronal calcium homeostasis. Simple, acyclic analogs that embody the 'western' or 'eastern' parts of bastadins were found to evoke comparable responses with bastadin 5.

Controlled fluctuations in intracellular calcium cation (Ca^{2+}) concentrations play an important role in many cellular processes such as muscle contraction, secretion, metabolism, and neuronal function.[1] Ryanodine receptors (RyR) are intracellular ion channels that mediate the release of calcium ions from internal stores and have been suggested as pharmacological targets for heart disease and neurodegenerative diseases.[2,3] Three isoforms (RyR1, RyR2, and RyR3) are expressed by mammalian tissues: RyR1 is present predominantly in skeletal muscle; RyR2 is found in cardiac muscle and is the major brain isoform; RyR3 has a wide tissue distribution and appears to be the major isoform in smooth muscle.[2a]

Bastadins (Fig. 1) are an ever-growing family of marine natural products isolated from sponges of the order Verongida.[4] Due to their ability to interact with RyR1 channels, they are considered useful chemical probes for related biological studies. Most of them are macrocyclic bis-diaryl ethers and, depending on the relative orientation of their diaryl ether segments, are further classified either as bastaranes or isobastaranes. They feature unique α-oximino amides while the degree of *ortho*-bromination of the diaryl ether moieties as well as the oxidation state of C5/C6 varies among family members.

Although bastadins share the same gross structural features, not all of them interact with RyR channels. Seemingly subtle differences in their substitution pattern suffice to alter the response observed.[5] Thus, bastarane bastadin 5 is the most active member (EC_{50} = 2.2 μM) and seems to stabilize both open and closed channel states but has little effect on the apparent sensitivity of the channel to Ca^{2+} activation. The isobastarane with the same bromination pattern, bastadin 19, although it competes for the same binding site, does not mobilize Ca^{2+} from the channel (EC_{50} > 100 μM). The unsaturated bastadin 7 (EC_{50} = 6.3 μM) follows closely bastadin 5 in terms of potency while its hydroxylated relative bastadin 10, in contrast to bastadin 5, stabilizes primarily the open-channel conformation and over sensitizes it to Ca^{2+} activation.

Until recently, further structure–activity relationship (SAR) studies were limited to the use of naturally occurring bastadins and were hampered by the fact that not all members are readily available. We have previously reported a general and flexible synthetic strategy towards bastadins, which allows for the synthesis of both bastaranes and isobastaranes with all possible bromination patterns.[6] Molinski et al. have prepared simplified cyclic analogues of the 'western' hemisphere of bastadin 5 by an alternative strategy and investigated their effect on RyR1 channels (Fig. 2).[7] This study reiterated the importance of the diarylether unit substitution pattern and illustrated that simpler analogs can retain the activity of more structurally complex natural bastadins.

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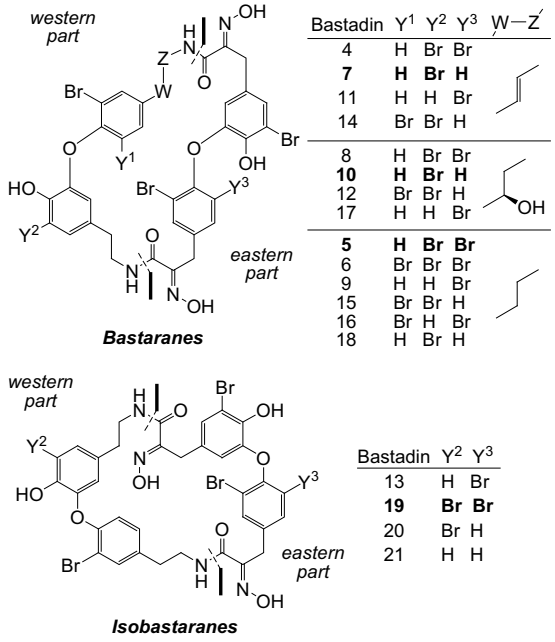


Bastadin	Y^1	Y^2	Y^3	W—Z
4	H	Br	Br	
7	**H**	**Br**	**H**	
11	H	H	Br	
14	Br	Br	H	
8	H	Br	Br	
10	**H**	**Br**	**H**	
12	Br	Br	H	
17	H	H	Br	
5	**H**	**Br**	**Br**	
6	Br	Br	Br	
9	H	H	Br	
15	Br	Br	H	
16	Br	H	Br	
18	H	Br	H	

Bastadin	Y^2	Y^3
13	H	Br
19	**Br**	**Br**
20	Br	H
21	H	H

Figure 1. Some naturally occurring bastadins.

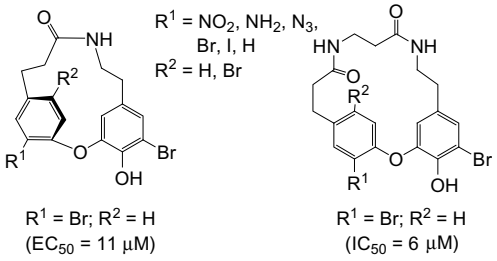
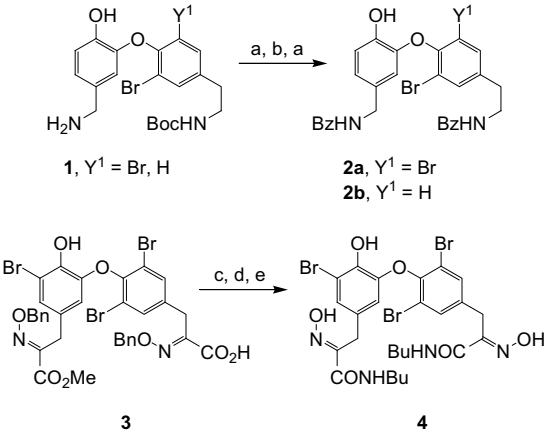


Figure 2. Simplified cyclic analogs of bastadin 5 prepared by Molinski et al. and their effect on RyR1 channels.[7]

Subsequently, we have investigated the effect of several synthetic bastadins, including bastadins 5 and 10, on the RyR2 receptors employing primary cultures of rat cerebellar granule neurons and observed that they elicit responses similar to the ones previously identified on skeletal RyR1 receptors.[8] Intrigued by the above mentioned structure–activity relationships, we examined and report herein the effect of simple acyclic analogues of 'eastern' and 'western' parts of bastadins (**2a**, **2b**, and **4**; Scheme 1) on calcium homeostasis in cultured neurons.

These compounds were readily prepared (Scheme 1) from key synthetic intermediates of our total synthesis effort.[6d] Thus, benzoylation of amine **1** (1:1 mixture of mono- and dibromo-derivative), followed by cleavage of the Boc protective group and a second benzoylation furnished a separable mixture of dibenzoylamides **2a** and **2b** in 76% overall yield.[9] On the other hand, hydrolysis of methyl ester **3** followed by coupling of the resulting diacid with *n*-butylamine and final benzyl ether cleavage, upon treatment with BBr_3 in the presence of thioanisole, yielded derivative **4** in 33% overall yield.[9]

The ability of bastadin 5 (positive control) and the acyclic analogues **2a**, **2b**, and **4** to increase the intracellular calcium concentration in cultured rat cerebellar granule cells[10] was evaluated using FLUO-3, a calcium-sensitive fluorescent probe. After loading the cells with the fluorescent probe, the compounds were applied to the incubation medium in equal concentration (20 μM) and the



Scheme 1. Reagents and conditions: (a) BzCl, Et_3N, benzene; (b) TFA, CH_2Cl_2; (c) LiOH, MeOH/THF/H_2O; (d) $BuNH_2$, EDC, HOBt, iPr_2EtN, DMF/CH_2Cl_2, 0 °C–rt; (e) BBr_3, thioanisole, 0 °C–rt.

initial level of fluorescence was measured using confocal microscopy (Fig. 3).[11] Synthetic bastadin 5, which in the control experiments demonstrated identical calcium mobilizing potential with the natural, commercially available product (results not shown), induced a substantial increase in the FLUO-3 fluorescence, indicating its ability to release calcium from the intracellular stores.[8] From the three analogs tested, **4** induced the most potent calcium transients comparable with the effects of bastadin 5 and even more dynamic. Analogue **2b** was less potent, whereas analog **2a** had practically no effect on the neuronal free calcium level.

Increases of intracellular Ca^{2+} concentration may arise from intracellular stores calcium release and/or from extracellular Ca^{2+} influx. Thus, in order to characterize bastadin-induced changes in calcium homeostasis, apart from measurements of the intracellular calcium level detected with FLUO-3 fluorescence, we monitored also the influx of extracellular calcium to neurons employing $^{45}Ca^{2+}$ isotope.[12] As presented in Fig. 4, application of bastadin 5 significantly increased uptake of $^{45}Ca^{2+}$ in the cultured neurons. Moreover the effect of the acyclic analogs on calcium accumulation mirrored the picture observed for their effect on the intracellular

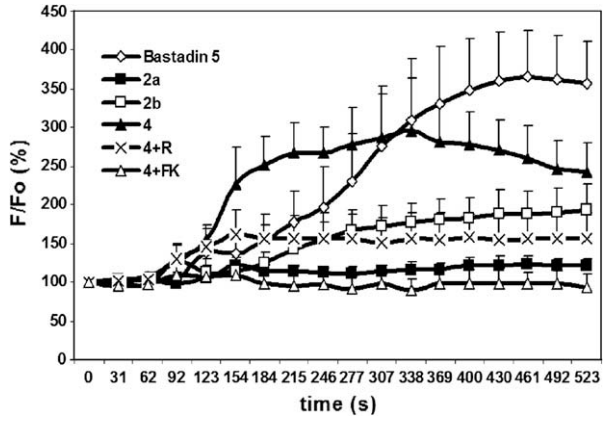


Figure 3. Effects of bastadin 5 and analogues on the intracellular calcium level in primary cultures of rat cerebellar granule cells. Synthetic bastadin 5 as well as analogues **2a**, 2**b**, and **4** (all compounds 20 μM) were applied after 60 s of incubation. Analog **4** was also applied in the presence of 200 μM ryanodine (**4 + R**) or 50 μM FK506 (**4 + FK**). Results, means ± SD ($n = 15$) are presented as percent changes in the intensity of fluorescence compared with the basal level (F/F_0). The presented data are from one of three independent experiments using different cultures that gave qualitatively identical results.

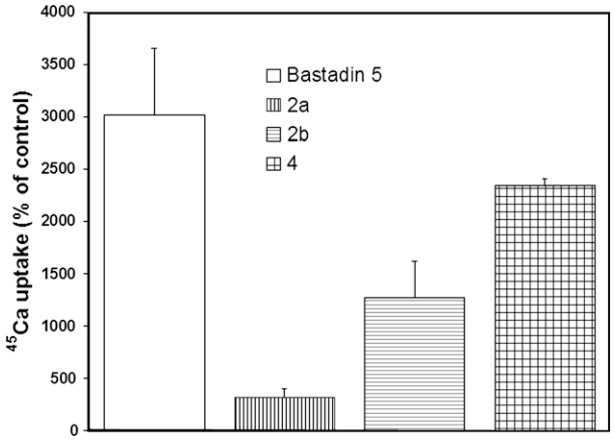


Figure 4. Effects of bastadin 5 and analogues **2a**, **2b**, and **4** (all compounds 20 μM) on $^{45}Ca^{2+}$ uptake in cultured cerebellar granule cells. Accumulation of $^{45}Ca^{2+}$ was measured after 10 min incubation with the compounds and results (means ± SD, $n = 6$) are presented as percent of control. All results were significantly different from the control ($P < 0.05$) as verified with ANOVA test.

calcium concentration, that is, the effect of **4** > **2b** > **2a**, the latter being very slight.

The fact that the influx of $^{45}Ca^{2+}$ to neurons evoked by application of bastadin 5 and some of the synthetic analogues correlates with increases in the intracellular calcium levels could be an indication that they directly activate the receptor-operated or voltage-sensitive calcium channels in neuronal plasma membranes. However, similarly to our previous studies on bastadins (including bastadin 5),[8] increases in the intracellular calcium levels induced by the most potent analog **4** were found to be sensitive to high concentrations of ryanodine and FK506 (Fig. 3).[13] This observation clearly indicates that ryanodine receptors are the primary targets for their pharmacological activity. Thus, the influx of extracellular calcium to bastadin-treated neurons appears to be a secondary effect resulting from the release of calcium from the ryanodine-sensitive pool. This release leads, in turn, to stimulation of glutamate release from the glutamatergic cerebellar granule neurons or otherwise leads to secondary activation of the NMDA receptors, as it has been recently described for PCB 95.[14] Therefore both bastadin-induced phenomena, an increase in FLUO-3 fluorescence and activation of $^{45}Ca^{2+}$ uptake, represent different aspects of the same primary biological phenomenon: the release of calcium via ryanodine receptors in neurons.

Our newly prepared acyclic analogues embody segments of different naturally occurring bastadins: compound **2a** embodies the 'western' part of bastadin 16 while compound **2b** embodies the corresponding segment of bastadins 9, 13 and 21; compound **4** embodies the 'eastern' part of bastadins 4, 5, 6, 8, 9, 11, 13,16, 17 and 19. It should be noted, however, that recent structure–activity relationship (SAR) studies by Molinski et al. indicated that the ketoximino groups present in the natural products are not required for activity and thus the difference between 'western' and 'eastern' parts becomes less profound.[7] Furthermore, the same studies strongly support the importance of the shape and size of the macrocyclic half-bastadins. Interestingly, our findings clearly indicate that constraining the C–O–C–C torsional angle by incorporating the diarylether in a macrocycle is not a prerequisite for activity. Thus, the catechol aryl ether substitution pattern potentially plays the decisive role regarding calcium mobilization activity. The available data would seem to indicate that a *para*-substituted catechol *ortho*-brominated-*para*-substituted aryl ether, like the one found in **2b**, is required. This pattern is maintained in compounds **2a** and **4**. Intriguingly, introduction of an extra bromine substituent *ortho*- to the ether linkage (**2a**) leads to diminished activity while introduction of an additional bromine *ortho*- to the catechol (**4**) has the opposite result. Clearly further studies are needed to decipher the effect of aryl substitution pattern.

In conclusion, our results confirm that the calcium-mobilizing potential of bastadins critically depends on the presence and location of aryl substituents and indicate that constraining of the diarylether moiety in a macrocycle is not a prerequisite for activity. Thus, a major simplification of the structure of bastadins, resulting in open-chain analogs that conserve the bromocatechol ether moiety, is possible without obligatory lose of their ability to release calcium via ryanodine receptors. Some of these compounds, in particular the one embodying the dibromocatechol ether moiety found in bastadin 5, retain this activity. This major structural simplification facilitates the development of novel chemical probes for the study of RyR channels and at the same time compels further investigations to illuminate the modulating effect that 'satellite' substituents (i.e., local and nature of further aromatic ring substitution, aliphatic chain length, and substitution pattern) may exert.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.080.

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9. *Compound* ***2a****:* 38% yield from **1**; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* 7.2 Hz, 2H), 7.59 (d, *J* 7.2 Hz, 2 H), 7.46–7.51 (m, 2H), 7.44–7.37 (m, 6H), 7.01 (d, *J* 8.2 Hz, 1H), 6.84 (d, *J* 7.9 Hz, 1H), 6.65–6.71 (br m, 1H), 6.24 (s, 1H), 5.97–6.04 (br m, 1H), 5.5–5.8 (br s, 1H), 3.65–3.69 (m, 2H), 3.57–3.61 (m, 2H), 2.89–2.92 (m, 2H), 2.76–2.79 (m, 2H); HRMS (ESI): *m*/*z*: 661.01161 [M+Na$^+$], $C_{30}H_{26}Br_2N_2O_4Na$ requires 661.01366.*Compound* ***2b****:* 38% yield from **1**; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 (d, *J* 7.4 Hz, 2H), 7.63 (d, *J* 7.4 Hz, 2H), 7.39–7.55 (m, 7H), 7.05 (d, *J* 8.1 Hz, 1H), 7.00 (d, *J* 8.2 Hz, 1H), 6.89–6.91 (m, 2H), 6.57 (s, 1H), 6.34–6.40 (br m, 1H), 6.03–6.10 (br m, 1H), 5.4–5.8 (br s, 1H), 3.66–3.70 (m, 2H), 3.59–3.63 (m, 2H), 2.88–2.91 (m, 2H), 2.78–2.81 (m, 2H); HRMS (ESI): *m*/*z*: 581.10407 [M+Na$^+$], $C_{30}H_{27}BrN_2O_4Na$ requires 581.10519.*Compound* ***4****:* ^{1}H NMR (500 MHz, acetone-d_6): δ 11.29 (br s, 1H), 10.89 (br s, 1H), 8,77 (br s, 1H), 7.67 (br s, 2H), 7.55 (br m, 1H), 7.31 (br m, 1H), 7.17 (br s, 1H), 6.40 (br s, 1H), 3.98 (br s, 2H), 3.69 (br s, 2H), 3.29 (br m, 2H), 3.21 (br m, 2H), 1.20–1.56 (m, 4H), 0.90 (br m, 6H); HRMS (ESI): *m*/*z*: 756.96752 [M+Na$^+$], $C_{26}H_{31}Br_3N_4O_6Na$ requires 756.96710.

10. Rat cerebellar granule neurons were prepared as described in Schousboe, A.; Drejer, J.; Hansen, G. H.; Meier, E. *Dev. Neurosci.* **1985**, *7*, 252 and cultivated in modified basal Eagle's medium containing 25 mM KCl, exactly as described previously in details.[8] The cells were used for experiments on the 7th day in vitro.
11. For loading with 16 μM FLUO-3 AM, the dye was added to the original growth medium for 30 min. After washing, the cells were incubated in magnesium-free low potassium Locke 5 buffer. In relevant trials, ryanodine (200 μM) or FK506 (50 μM) was added to the medium. The basal fluorescence reflecting the steady-state level of intracellular calcium concentration was monitored, using the confocal microscope Zeiss LSM 510 with excitation at 488 nm and emission measured at 530 nm. Then the tested compounds were added and the fluorescence was measured for 15 min using LSM 510 computer program (version 3.2), as described previously.[8]
12. Accumulation of ^{45}Ca in cultured neurons was determined in the magnesium-free low potassium Locke 5 buffer. After preincubation for 10 min at 37°C, $^{45}CaCl_2$ (1 μCi/well) was added together with tested compounds for 10 min. Then the medium was removed and the cells were washed, dissolved in 0.5 M NaOH, and radioactivity in neurons was measured with a Wallac 1409 liquid scintillation counter as described in Makarewicz, D.; Zieminska, E.; Lazarewicz, J. W. *Neurochem. Int.* **2003**, *43*, 273.
13. Ryanodine and FK506 had similar effects on the activity of compound **2b** (Fig. S1 in Supplementary data).
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