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SHORT COMMUNICATION

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Prevention of Mitochondrial Membrane Permeabilization and Pancreatic β-Cell Death by an Enantioenriched, Macrocyclic Small Molecule

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Mitochondria produce the majority of cellular energy through the process of oxidative phosphorylation and play a central role in regulating the functionality and survival of eukaryotic cells. Under physiological stress, mitochondrial membrane permeabilization results in the release of apoptogenic material such as cytochrome c in the cytoplasm, which thereby initiates caspase activation and the consequent cell death. In our present study, we screened a series of compounds for their ability to inhibit mitochondrial membrane permeabilization and to prevent cytochrome c release during the endoplasmic reticulum stress in cultured pancreatic β-cells. Three benzofuran-based macrocyclic small molecules, that is, **2.4c**, **c104**, and **c108**, were found to restore the depolarization of mitochondrial membrane potential and to prevent the release of cytochrome c from mitochondria. Interestingly, the acyclic precursor of **2.4c** (i.e., **2.3c**) did not show any effect, whereas the macrocyclic derivative obtained by utilizing ring-closing metathesis as the "stitching technology" led to this function. The macrocyclic architecture seems to play a crucial role in presenting various functional moieties in the right orientation to observe this effect.

Introduction

Mitochondria play an essential role in pancreatic β-cell homeostasis through their involvement in the modulation of stimulus-coupled insulin secretion[1] and in the regulation of cell survival.[2,3] The permeabilization of mitochondrial membranes under the influence of various cytokines results in the release of cytochrome c, which is known to activate the caspase cascade.[4] Most importantly, during chronic endoplasmic reticulum (ER) stress, the accumulation of unfolded proteins in the ER results in leakage of calcium from the ER, which leads to calcium overload in the mitochondria[5,6] and the consequent opening of the mitochondrial permeability transition pore. The latter process plays a decisive role in the depolarization of the mitochondrial membrane potential and programmed cell death.

In our present study, we induced chronic ER stress in cultured BRIN-BD11 pancreatic β-cells by treating them with the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pump inhibitor thapsigargin (note: structure not shown). Thapsigargin treatment causes the depolarization of the mitochondrial inner membrane potential and compromises cell survival.[7] To prevent this depolarization, we utilized a small-molecule toolbox having 18 enantioenriched, benzofuran-derived compounds with 12-membered macrocyclic rings that are rich in 3D architectures and that can be considered members of the broad family of natural-product-inspired compounds. Our data reveals that enantioenriched benzofuran-based macrocycles having a 12-membered ring, which we synthesized, prevent the depolarization of the mitochondrial membrane potential and inhibit apoptogenic cytochrome c release from mitochondria in cultured pancreatic β-cells.

Results and Discussion

There is growing interest in accessing small molecules that are inspired by bioactive natural products having 3D architectures to explore their biological functions.[8] They could have either multiple rings or macrocyclic architectures. The latter is quite interesting,[9] because there are numerous examples of complex macrocyclic natural products exhibiting a wide range of biological properties.[10] Owing to several advantages that are associated with macrocyclic rings, there is also growing interest[9,11] in developing modular synthesis methods that allow a diverse chemical toolbox having different types of macrocyclic architectures to be ob-

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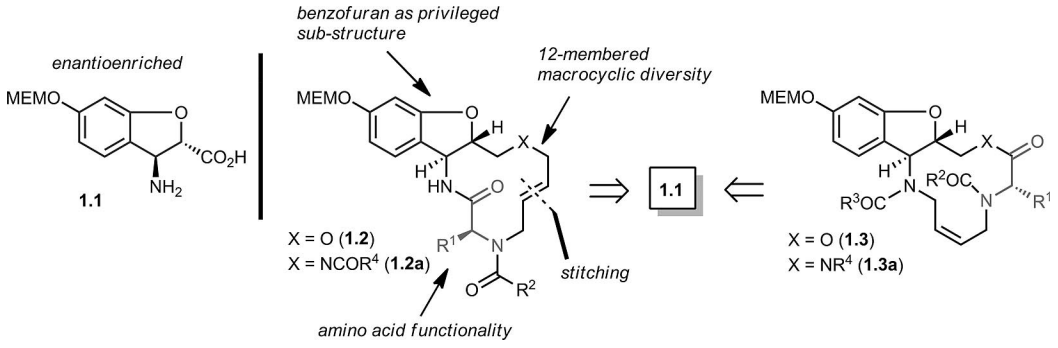
tained. Some of these advantages[10] include (1) preorganization, (2) enhanced cell permeation properties, and (3) the possibility of having numerous binding interactions, a property that could be highly relevant to search for small molecule modulators of protein–protein[12] and other types of biomacromolecular (e.g., DNA/RNA–protein)[13] interactions.

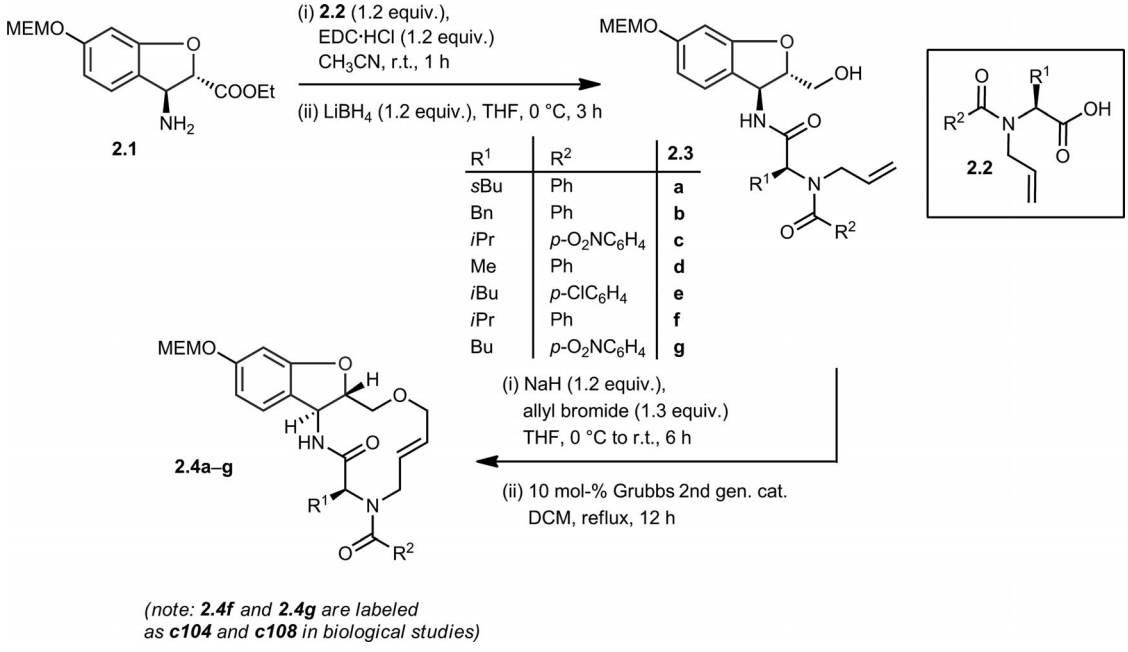
Earlier, we reported an enantioenriched synthesis of benzofuran-based, 1,2-*trans*-β-amino acid **1.1** (Scheme 1).[14] In this article, with the objective to explore further the additional large-ring chemical space, we report here a modular approach that allowed us to incorporate two different types of 12-membered macrocyclic rings onto the benzofuran scaffold (Scheme 1; see **1.2/1.2a** and **1.3/1.3a**). The presence of an amino acid moiety within the macrocyclic architecture is an attractive feature to introduce a diverse array of chiral side chains having a variety of polarities.

Shown in Scheme 2 is our plan to obtain a 12-membered macrocyclic ring that utilizes benzofuran-based *trans*-β-amino ester **2.1**. Compound **2.2**, a derivative of an *N*-protected amino acid, was prepared (see Supporting Information). In each case, following the derivatization of –OH as –OAllyl, the sample was submitted to ring-closing metathesis[15] (RCM, Grubbs 2nd generation catalyst) as the "stitching technology". All macrocyclic compounds that were synthesized are stable at room temperature.

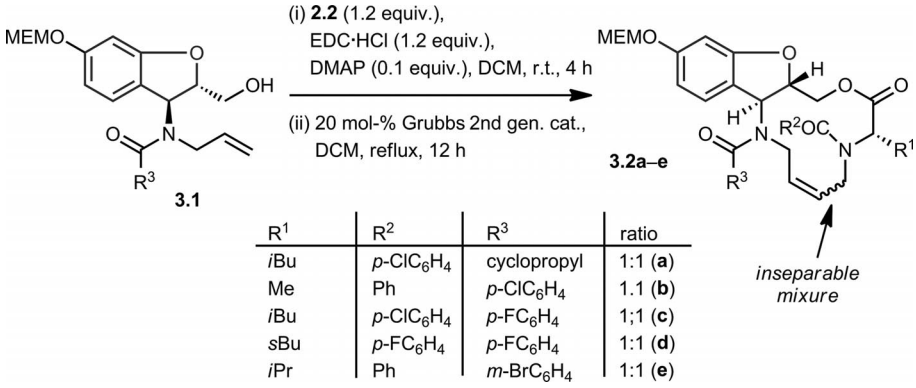
In our next approach, we were interested in accessing a different type of macrocyclic architecture, that is, **3.2** (Scheme 3), that would arise from coupling the amino acid moiety to the primary hydroxy side of the benzofuran scaffold. To achieve this, we synthesized **3.1** as the *N*-protected chiral amine-based benzofuran derivative. This was further coupled with the *N*-allyl derivative of the *N*-protected amino acid that produced the starting material required for the Grubbs RCM-based stitching technology. Our last ap-



Scheme 1. Proposed synthesis of four different types of benzofuran-derived macrocyclic compounds, that is, **1.2/1.2a** and **1.3/1.3a**, from enantioenriched **1.1**. MEM = (2-methoxyethoxy)methyl.



Scheme 2. Synthesis of 12-membered macrocyclic derivative **2.4**, which is derived from *N*-functionalized 1,2-*trans*-amino alcohol **2.3**. EDC = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide,.

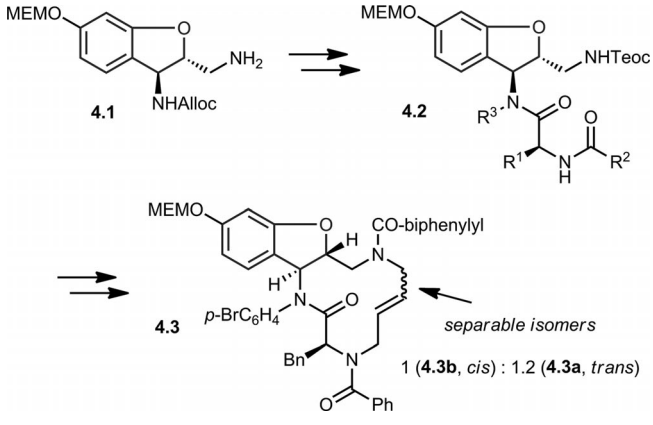


R^1	R^2	R^3	ratio
*i*Bu	*p*-ClC_6H_4	cyclopropyl	1:1 (**a**)
Me	Ph	*p*-ClC_6H_4	1.1 (**b**)
*i*Bu	*p*-ClC_6H_4	*p*-FC_6H_4	1;1 (**c**)
*s*Bu	*p*-FC_6H_4	*p*-FC_6H_4	1:1 (**d**)
*i*Pr	Ph	*m*-BrC_6H_4	1:1 (**e**)

Scheme 3. Macrocycle **3.2** from *N*-functionalized, enantioenriched, 1,2-*trans*-amino alcohol **3.1**.

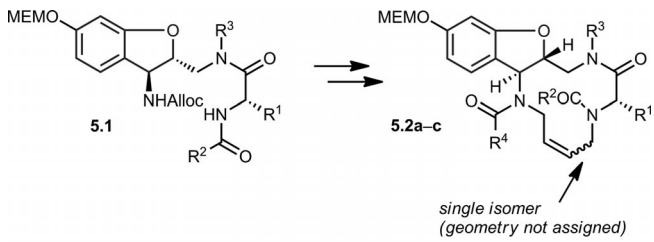
proach involved the synthesis of **3.2** (Scheme 3; see also Supporting Information) that would arise from coupling the amino acid moiety to the primary hydroxy side of the benzofuran scaffold. The detailed synthesis plan is shown in the Supporting Information.

In another plan (Scheme 4; see also Supporting Information), the goal was to replace the primary –OH group by an $–NH_2$ group to allow the incorporation of three diversity sites into the 12-membered ring. To achieve this synthesis, **4.1** as the starting material was utilized. This produced **4.2** in several easy steps that included (1) primary $–NH_2$ protection as an –NTeoc group {Teoc = [2-(trimethylsilyl)ethoxy]carbonyl}, (2) –NAlloc removal (Alloc = allyloxycarbonyl), (3) reductive alkylation (R^3 as the diversity point), (4) coupling with the protected amino acid (to introduce two diversity sites as R^1 and R^2). Finally, **4.2** was subjected to –NTeoc removal, amidation, which further upon bis(allylation) produced the key precursor for the RCM-based stitching technology. Once again, the RCM approach worked well, and **4.3** as the macrocyclic derivative was obtained as a mixture of two olefinic compounds. The details of the synthesis plan are provided in the Supporting Information.



Scheme 4. 12-Membered macrocycle **4.3** from 1,2-*trans* chiral diamine **4.1** (see Supporting Information).

As described earlier, a similar approach that utilized **5.1** (Scheme 5) as a starting material led to the synthesis of macrocyclic derivative **5.2**. Using the orthogonally protected chiral amine derivative of benzofuran, **5.1** was easily prepared. This compound has the coupled protected amino acid moiety on the primary amine and contains three diversity sites (R^1, R^2, and R^3). Once subjected to –NAlloc removal, it was then derivatized through amide coupling, followed by bis(allylation) needed for the RCM technology. This approach was attempted with three cases, and macrocyclic product **5.2** was easily obtained as a single isomer, although the olefin geometry remains to be assigned. The detailed synthesis methods are provided in the Supporting Information.



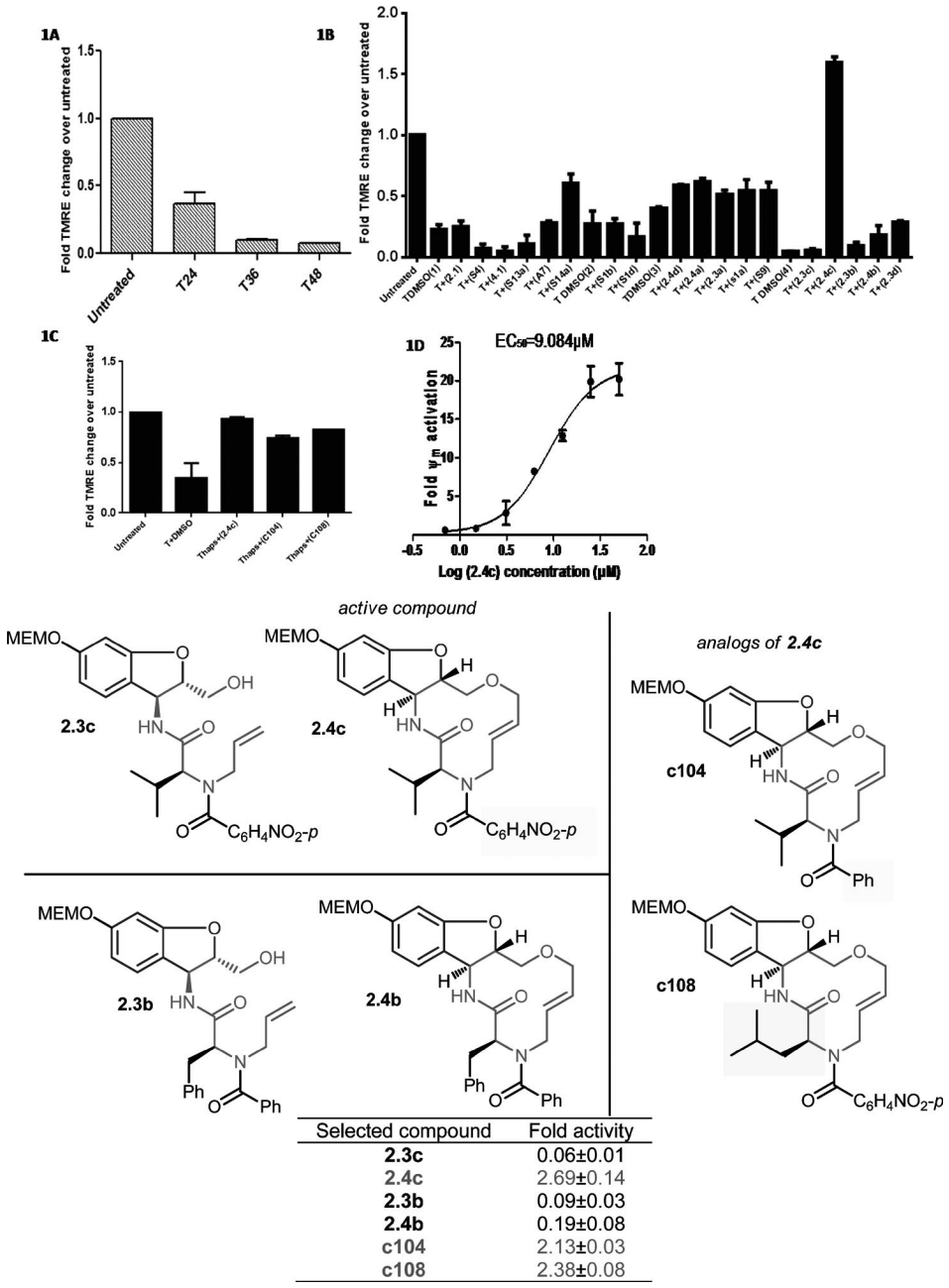
R^1	R^2	R^3	R^4	**5.2**
Bn	Ph	*p*-$BrC_6H_4C(=O)$	biphenylyl	**a**
*i*Pr	naphthyl	*p*-$BrC_6H_4C(=O)$	Ph	**b**
*i*Bu	*p*-ClC_6H_4	Bn	*m*-BrC_6H_4	**c**

Scheme 5. Synthesis of macrocycle **5.2** from *N*-functionalized, chiral 1,2-*trans* diamine **5.1** (see Supporting Information).

Prevention of Thapsigargin-Induced Mitochondrial Depolarization

Benzofuran-based macrocycles reported in this study showed remarkable activity to prevent the depolarization of the mitochondrial membrane potential (MMP) under thapsigargin-induced ER stress. Figure 1a shows the temporal effect of thapsigargin on the depolarization of the MMP. As the data reveals, a 36 h treatment of thapsigargin at a concentration of 5.0 μM caused a 10-fold reduction in the mitochondrial membrane potential. To prevent this depolarization of the MMP, we screened a library of benzofuran-based compounds to study their efficacy to rescue the phenotype (Figure 1b,c). Compound **2.4c** was found to prevent the depolarization of the MMP induced on thapsigar-

gin treatment in cultured pancreatic β-cells. Compound **2.4c** possessing a 12-membered macrocyclic ring and having an *N*-(4-nitrobenzoyl)valine amino acid moiety fused to the benzofuran scaffold showed the highest activity for the prevention of thapsigargin-induced depolarization of the mitochondrial membrane potential. To validate the structural features of this compound, we further synthesized two more related macrocyclic compounds, that is, **c104** and **c108**. In **c104**, the *N*-(4-nitrobenzoyl)valine is replaced by an *N*-benzoylvaline unit (i.e., no NO_2 group), whereas the amino acid moiety in **2.4c** is replaced by leucine to obtain **c108**. A comparative account of the efficacy of all these macrocycles to prevent thapsigargin-induced depolarization of the MMP is shown in Figure 1. Compounds **2.4c**, **c104**, and **c108** showed comparable efficacy in the prevention of thapsigargin-induced depolarization of the MMP at a concentration of 10.0 μM. Interestingly, the acyclic precursor of **2.4c** (i.e. **2.3c**) did not show any effect. In addition, replacement of the *N*-benzoylvaline amino acid moiety with an *N*-benzoyl-(phenylalanine) (i.e., **2.4b**) group dramatically reduced the



Selected compound	Fold activity
2.3c	0.06±0.01
2.4c	2.69±0.14
2.3b	0.09±0.03
2.4b	0.19±0.08
c104	2.13±0.03
c108	2.38±0.08

Figure 1. Attenuation of thapsigargin-induced mitochondrial depolarization: (a) Temporal depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$) in pancreatic β-cells. (b and c) Screening potential of compounds that prevent the depolarization of the MMP. (d) Dose–response curve for the rescue of thapsigargin-induced depolarization of $\Delta\Psi_m$ by **2.4c**. (Note: for detailed structural information of all compounds tested, see Supporting Information).

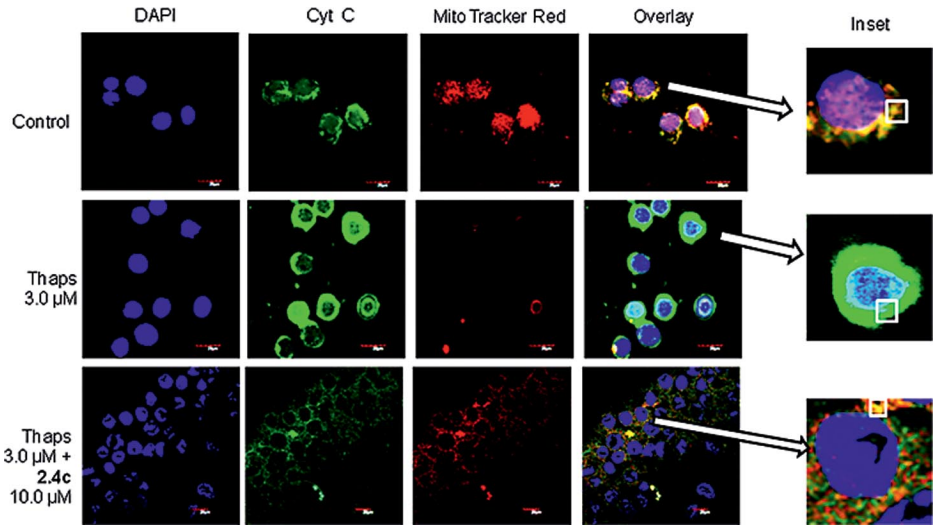


Figure 2. Prevention of cytochrome c release from thapsigargin-treated cells upon treatment with **2.4c**. Confocal microscopy images of BRIN-BD11 cells immune-labeled with primary monoclonal mouse anti-cytochrome c antibody (green) depicting release of cytochrome c from the mitochondria. Mitochondria are labeled by Mito-tracker Red (red), and the nucleus is visualized by DAPI (blue) staining. Yellow indicates co-localization of cytochrome c with Mito-tracker Red in control and **2.4c**-treated cells in mitochondria. Diffused staining in thapsigargin-treated cells shows the release of cytochrome c from the mitochondria.

activity. A dose–response curve for the prevention of the depolarization of the MMP by **2.4c** is shown in Figure 1d, and the EC_{50} of the response was found to be 9.04 μM (Figure 1c).

Prevention of Cytochrome C Release

In the next step, we evaluated the distribution of cytochrome c in untreated, thapsigargin-treated, and **2.4c**-treated cells. As Figure 2a reveals, in normal cells, there was a complete overlap of cytochrome c staining with Mito-Tracker Red; this indicated its presence in the mitochondria. Treatment with thapsigargin for 18 h caused a marked loss of Mito-Tracker Red and a concomitant release of cytochrome c into the cytoplasm (Figure 2b), which was totally prevented with the use of **2.4c** (Figure 2c). The data suggest the role of **2.4c** in preventing the release of cytochrome c from the mitochondria, which is known to activate cell death in pancreatic β-cells.

Conclusions

To the best of our knowledge, this is the first report of a macrocyclic small molecule that modulates the mitochondrial membrane potential ($\Delta\Psi_m$) and further prevents the release of cytochrome c from mitochondria from thapsigargin-induced ER stress in pancreatic β-cells. Given the role of small molecule **2.4c** in preventing mitochondria from high cytosolic calcium insult, this compound may also have interesting applications related to neurological disorders, such as, cortical spreading depression (CSD). Whether **2.4c** regulates protein misfolding and/or clustering in mitochondria or participates in chaperone-mediated regulation of mitochondrial membrane permeabilization is yet to be determined.

Supporting Information (see footnote on the first page of this article): Experimental details, characterization data, and copies of the ^1H and ^{13}C NMR spectra of all key intermediates and final products.

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