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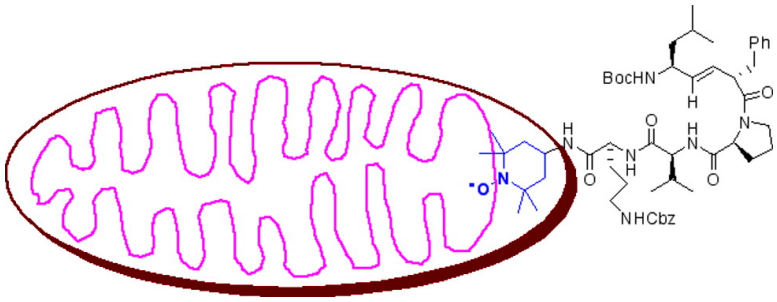

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Mitochondrial Targeting of Selective Electron Scavengers: Synthesis and Biological Analysis of Hemigramicidin−TEMPO Conjugates

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The chemistry and biology of mitochondria, in particular, the effects of intracellular reactive oxygen species (ROS, superoxide radicals and H_2O_2) that are byproducts of the oxidative phosphorylation cascade, is under intense study.[1] Cellular injury, aging, and death, as well as suspended animation, neuro-, and cardioprotection are influenced by events in the mitochondrial membrane that lead to an imbalance in ATP production and O_2 consumption.[2] Recently, dysregulated electron transport and generation of ROS were linked to a mitochondria-specific phospholipid, cardiolipin (CL), and involvement of CL oxidation products in apoptosis.[3] Nitroxide radicals prevent the formation of ROS, particularly superoxide, due to their reduction by the mitochondrial electron transport to hydroxylamine radical scavengers.[4] Nitroxides also exert superoxide dismutase and catalase activities,[5] thus offering additional protective benefits against oxidative stress. However, delivery of sufficient amounts of nitroxides into mitochondria has proven difficult.[6]

A selective delivery of TEMPO[7] to mitochondria could lead to a therapeutically beneficial reduction of ROS; therefore, we have investigated the use of conjugates[8] of 4-amino-TEMPO (4-AT)[9] and employed as targeting sequence fragments of the membrane-active antibiotic GS as well as the corresponding alkene isosteres (Figure 1).[10] We selected the Leu-^{D}Phe-Pro-Val-Orn fragment of GS as the targeting sequence, because it encompasses the β-turn motif that directs most of the polar functionality of the peptide strand into the core, and acylated the amino functions of Leu and Orn in order to reduce GS-related cytotoxicity.[11]

The preparation of (*E*)-alkene dipeptide isostere **3** was based on our Zr/Zn methodology (Scheme 1).[12] Hydrozirconation[13] of alkyne **1**[14] with Cp_2ZrHCl followed by transmetalation to Me_2Zn and addition of *N*-Boc-isovaleraldimine[15] afforded diastereomeric allylic amides, which were separated after desilylation and acetylation. A two-step oxidation of **2** provided peptide isostere **3**. The segment assembly of **3** and tripeptide H-Pro-Val-Orn(Cbz)-OMe was accomplished using EDC as a coupling agent. Saponification of **4a** followed by coupling with 4-AT afforded the desired conjugate **5a**, in which the Leu-^{D}Phe peptide bond had been replaced with an (*E*)-alkene. Conjugates **5b** and **5c** were prepared by coupling of pentapeptide **4b**[16] and isostere **3** to 4-AT.

We used EPR spectroscopy to monitor the cellular delivery and metabolic fate of **5a** and **5b**. Distinctive characteristic triplet signals of nitroxide radicals (with hyperfine splitting constants of 16.6 G) were detected in mouse embryonic cells (MECs) incubated with 10 μM **5a** as well as in mitochondria isolated from these cells (Figure 2). The cytosolic fraction did not elicit EPR signals of nitroxide radicals. Similar results were observed with conjugate **5b** (data not shown). In contrast, 4-AT did not effectively partition into either cells or mitochondria. Incubation of MECs in the

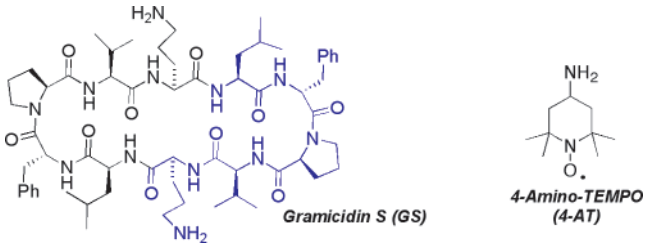
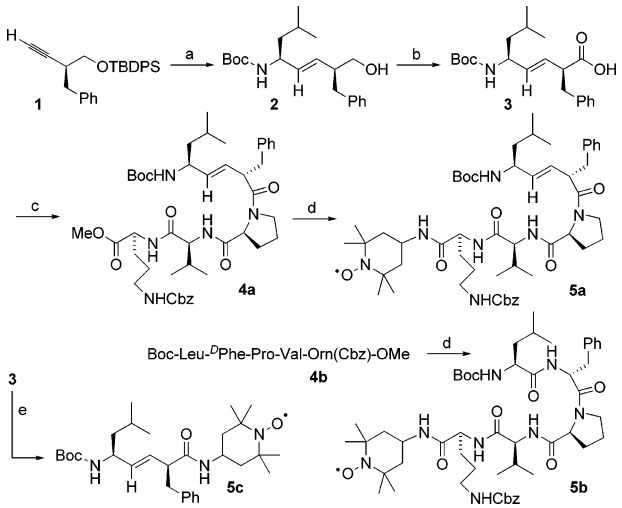


Figure 1. Gramicidin S (targeting sequence in blue) and 4-AT.

Scheme 1. Synthesis of Peptide Conjugates[a]



[a] Conditions: (a) (i) Cp_2ZrHCl, Me_2Zn, *N*-Boc-isovaleraldimine, then TBAF, 74%; (ii) Ac_2O, TEA, DMAP, 94%; (iii) K_2CO_3, MeOH, quant.; (b) (i) Dess−Martin periodinane; (ii) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene; (c) H-Pro-Val-Orn(Cbz)-OMe, EDC, HOBt, DMAP, 94% from **2**; (d) (i) 1 N NaOH; (ii) 4-AT, EDC, HOBt, DMAP; **5a**, 99%; **5b**, 99%; (e) 4-AT, EDC, DMAP, 91%.

presence of **5a** resulted not only in its integration but also in its one-electron reduction, as evidenced by a significant increase in the magnitude of the EPR signal intensity upon addition of a one-electron oxidant, ferricyanide (Figure 2B). Thus, not only delivery but also the reduction of **5a** and **5b** occurred in MEC mitochondria. We tested the ability of **5a** and **5b** to prevent intracellular superoxide generation (by flow cytometric monitoring of oxidation of dihydroethidium (DHE) to a fluorescent ethidium) and protect cells against apoptosis triggered by actinomycin D (ActD). Both **5a** and **5b** (but not 4-AT) completely inhibited ActD-induced (∼2-fold) increase of superoxide production in MECs (Figure 3A). Apoptotic cell responses were documented using three biomarkers: (1) Externalization of phosphatidylserine (PS) on the cell surface (by flow cytometry using an FITC-labeled PS-binding protein, annexin V); (2) Activation of caspase-3 (by cleavage of its specific substrate, Z-DEVD-AMC); and (3) DNA fragmentation (by flow cytometry of propidium iodide stained DNA). ActD effectively induced apoptosis, as revealed by an increased number of annexin V-positive

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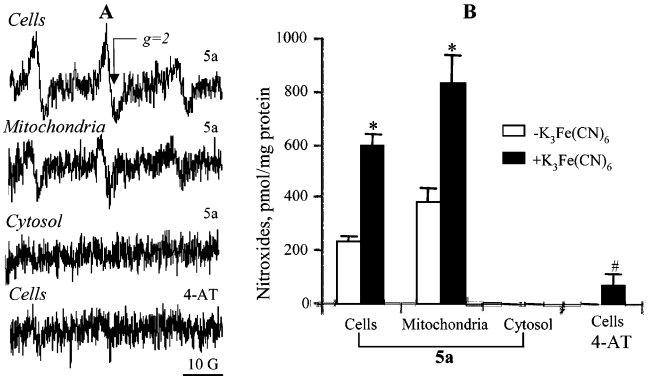

Figure 2. EPR-based analysis of integration and reduction of nitroxide GS−peptidyl conjugates in MECs. Cells (10 million/mL) were incubated with 10 μM of 4-AT or **5a** for 15 min. Recovered nitroxide radicals in whole cells, mitochondria, or cytosol fractions were resuspended in PBS in the presence or absence of 2 mM $K_3Fe(CN)_6$ (JEOL-RE1X EPR spectrometer under the following conditions: 3350 G center field; 25 G scan range; 0.79 G field modulation, 20 mW microwave power; 0.1 s time constant; 4 min scan time). (A) Representative EPR spectra of **5a** in different fractions of MECs in the presence of $K_3Fe(CN)_6$. (B) Assessment of integrated nitroxides ($n = 3$); *$p < 0.01$ vs $K_3Fe(CN)_6$; #$p < 0.01$ vs **5a** under the same conditions.

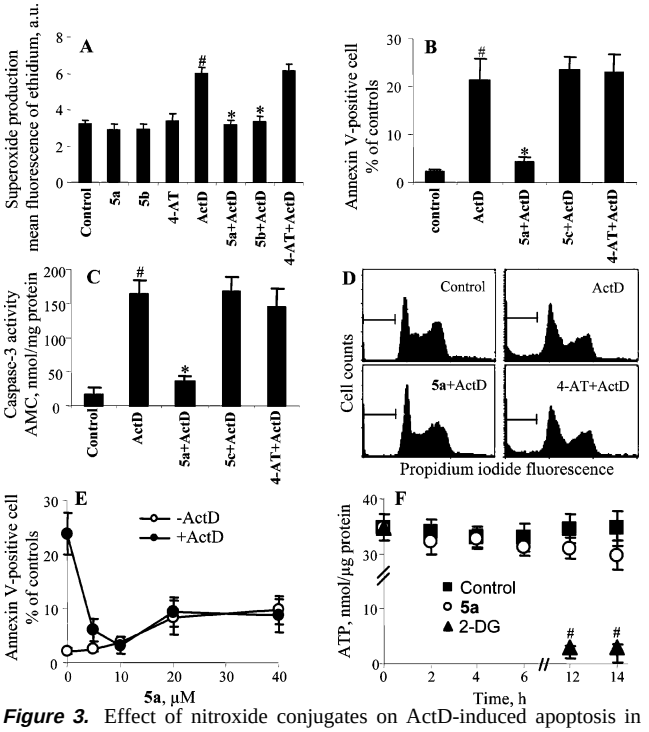

Figure 3. Effect of nitroxide conjugates on ActD-induced apoptosis in MECs. Cells were pretreated with 10 μM 4-AT, **5a**, **5b**, or **5c** for 1 h, then incubated with ActD (100 ng/mL). (A) Superoxide production: mean fluorescence intensity from 10 000 cells. (B) PS externalization. (C) Caspase-3 activation. (D) DNA fragmentation. (E) PS externalization at different concentrations of **5a**. (F) ATP levels in MECs in the presence or absence of **5a** or 2-deoxyglucose (2-DG), as a positive control. Data are means ±SD ($n = 3$), #$p < 0.01$ vs control, *$p < 0.01$ vs ActD-treated cells.

cells (Figure 3B), caspase activation (Figure 3C), and DNA fragmentation (Figure 3D). **5a** (Figure 3) and **5b** reduced the number of annexin V-positive cells and prevented caspase-3 activation and DNA fragmentation. In contrast, 4-AT afforded no protection.

Protective effects of **5a** and **5b** were achieved at relatively low 10 μM concentrations. At higher concentrations, both **5a** (Figure 3E) and **5b** were either less protective or exerted cytotoxicity. Both **5a** and **5b** are very hydrophobic compounds with a cLogP of 6.4 and 4.5, respectively. To determine whether their protective anti-apoptotic effects resulted from unspecific lipophilicity rather than from specific interactions with cellular and mitochondrial membranes, we tested nitroxide conjugate **5c**, which is similarly lipophilic (cLogP 5.5) but does not have a complete targeting moiety. We found that **5c** was ineffective in protecting MECs against ActD-induced apoptosis (Figure 3B,C). Thus, the GS−peptidyl targeting structure is required for anti-apoptotic activity of nitroxide conjugates. Since the reduction of **5a** and **5b** could also cause inhibition of mitochondrial oxidative phosphorylation, we tested whether ATP levels were changed in cells treated with these compounds. At concentrations at which anti-apoptotic effects were maximal (**5a**, 10 μM, Figure 3E), nitroxide conjugates did not cause significant changes in the cellular ATP level (Figure 3F). Thus, synthetic GS−peptidyl conjugates migrate into cells and mitochondria, where they are reduced (likely by electron-transporting proteins) and exert protection against apoptosis. Previously, spin trapping nitrones have demonstrated promise in aging research.[17] Our radical scavenger delivery approach is based on the use of specific GS-derived mitochondria targeting sequences[11] and offers similar potential for future anti-apoptotic interventions.[6b,c,18]

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Supporting Information Available: Experimental procedures, ^1H and ^{13}C spectra, and procedures for biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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