7264 J. Med. Chem. 2010, 53, 7264–7268
DOI: 10.1021/jm100637k

Journal of Medicinal Chemistry
Brief Article

# Polyamine Conjugation of Curcumin Analogues toward the Discovery of Mitochondria-Directed Neuroprotective Agents†

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Received May 26, 2010

Mitochondria-directed antioxidants **2**−**5** were designed by conjugating curcumin congeners with different polyamine motifs as vehicle tools. The conjugates emerged as efficient antioxidants in mitochondria and fibroblasts and also exerted a protecting role through heme oxygenase-1 activation. Notably, the insertion of a polyamine function into the curcumin-like moiety allowed an efficient intracellular uptake and mitochondria targeting. It also resulted in a significant decrease in the cytotoxicity effects. **2**−**5** are therefore promising molecules for neuroprotectant lead discovery.

## Introduction

Mitochondria undertake essential and diverse roles in the physiology of eukaryotic cells.[1] In addition to being the major source of ATP, they participate in numerous metabolic reactions and regulate a variety of cellular functions including proliferation, differentiation, and apoptosis. Mitochondria are both a major site of production and a primary target of reactive oxygen species (ROS[a]). ROS act as signaling molecules, but can cause damage if produced excessively. It is therefore not surprising that mitochondrial dysfunctions are associated with a wide variety of human pathologies ranging from neurodegenerative and neuromuscular diseases to diabetes and cancer.[1]

The unique structural and functional characteristics of mitochondria prompted researchers to design drugs able to selectively modulate the function of this organelle for therapeutic gain. This has led to the emergence of "mitochondria-directed therapeutics" as a new field of biomedical research.[2,3] In particular, in recent years, much attention has been devoted to identifying purposely designed mitochondria-directed antioxidants. This has led to innovative therapeutic molecules, some of which are now undergoing phase II clinical trials for neurodegenerative diseases.[3−5] In addition, because of their proapoptotic potential, mitochondria have recently emerged as intriguing targets for anticancer drugs.[6−8]

Curcumin (**1**) is a polyphenolic compound isolated from the rhizomes of the *Curcuma longa* plant. It is a pleiotropic agent with multiple molecular targets and biological activities. In addition to anti-inflammatory, antiviral, and neuroprotective actions,[9] it possesses direct primary[10] and indirect secondary antioxidant activity.[11] It also asserts antiproliferative and/or apoptotic effects in cancer cells targeting cell cycle regulatory molecules.[12,13] Despite its broad effects on the biological functions of cells, the potential use of **1** as a therapeutic agent is severely affected by its low water solubility, poor in vivo bioavailability, and rapid metabolism.[14] In recent years, its structure has been widely modified, focusing mainly on changes in the $\beta$-diketone structure and aryl substitution pattern of the molecule. Among the obtained compounds, the 3,5-dibenzylidenepiperidin-4-one (DBP) framework attracted our attention because of its higher antioxidant and antiproliferative actions with respect to **1**[15−17] and the possibility for the amino group to be easily functionalized. This moiety may also offer the chance of an improved pharmacokinetic profile.[18]

In this context, the aim of the present study is to selectively direct the above-mentioned moiety into the mitochondrion, which is the cellular compartment where ROS production and apoptosis modulation mainly occur. This should contribute to the development of effective drugs for therapeutic intervention in pathologies, such as cancer and neurodegenerative diseases, where these processes are strongly compromised.

So far, most research effort in developing mitochondria-directed molecules has been based on two distinct mitochondrial features: the organelle's protein import machinery and the high membrane potential across the inner membrane.[19] In particular, the latter offers a unique chemical opportunity for selectively targeting the mitochondrion, as verified for several lipophilic cations that efficiently accumulate into this subcellular compartment in a gradient-dependent manner.[3] On this basis and also because polyamine chains have already been used to improve the cellular import of drug molecules,[20,21] we selected a polyamine scaffold, protonated at physiological pH, because it could direct the bioactive functionality into

†Dedicated to Professor Goffredo Rosini on the occasion of his 70th birthday.

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[a]Abbreviations: ROS, reactive oxygen species; DBP, 3,5-dibenzylidenepiperidin-4-one; PPAA, propylphosphonic anhydride; SMP, submitochondrial particles; HO-1, heme oxygenase-1; BAEC, bovine aortic endothelial cells; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

 Published on Web 09/10/2010 

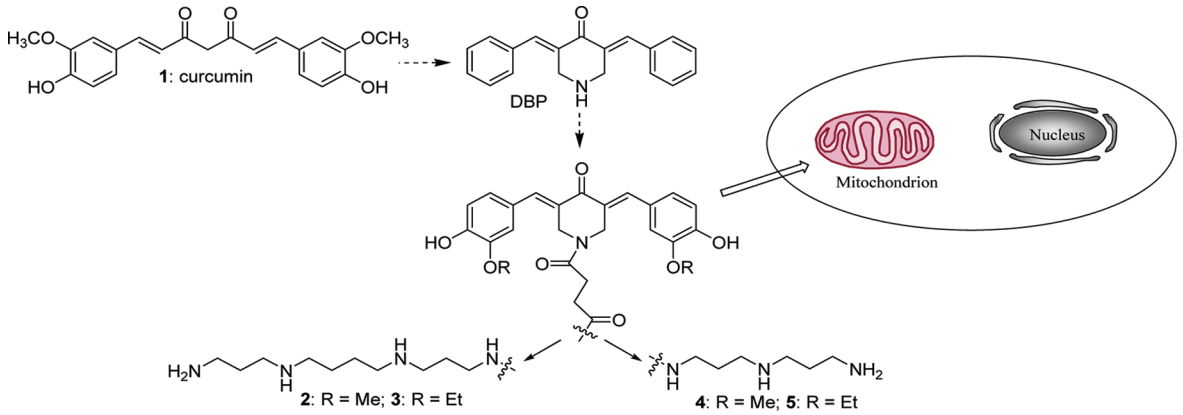



**Figure 1.** Design strategy for compounds **2**−**5**.

mitochondria passing through cell and mitochondria membranes. We synthesized a series of polyamine-based derivatives, conjugating differently substituted DBP scaffolds with spermine and norspermidine chains (**2**−**5**) (Figure 1), which were then biologically investigated to assess their antioxidant and antiproliferative effects. We synthesized the fluorescent probe **6** to validate whether or not the synthesized compounds were preferentially taken up by mitochondria.

## Results and Discussion

**2**−**5** were synthesized following the convergent synthetic approach depicted in Scheme 1. To incorporate a polyamine backbone into the polyphenolic moiety, partially protected spermine **7**,[22] norspermidine **8**,[23] and **9** having only one free amine function were first provided with an acidic functionality through reaction with succinic anhydride to afford **11**−**13**. Compound **9** was obtained by reductive amination of **7** with the commercially available 4-(benzothiazol-2-yl)benzaldehyde (**10**). Then (3*E*,5*E*)-3,5-bis-(-4-hydroxy-3-methoxybenzylidene)piperidin-4-one (**14**) and (3*E*,5*E*)-3,5-bis-(-4-hydroxy-3-ethoxybenzylidene)piperidin-4-one (**15**), synthesized by following reported procedures,[24] were condensed with **11**−**13** in the presence of propylphosphonic anhydride (PPAA) to give **16**−**20**. This protocol for amidation via mixed phosphoric anhydrides allowed an excellent selectivity for *N*- versus *O*-(phenolic) acylation, making hydroxyl protection unnecessary.[25] Removal of BOC groups with trifluoroacetic acid in $CH_2Cl_2$ gave the targets **2**−**6** as trifluoroacetate salts (Scheme 1).

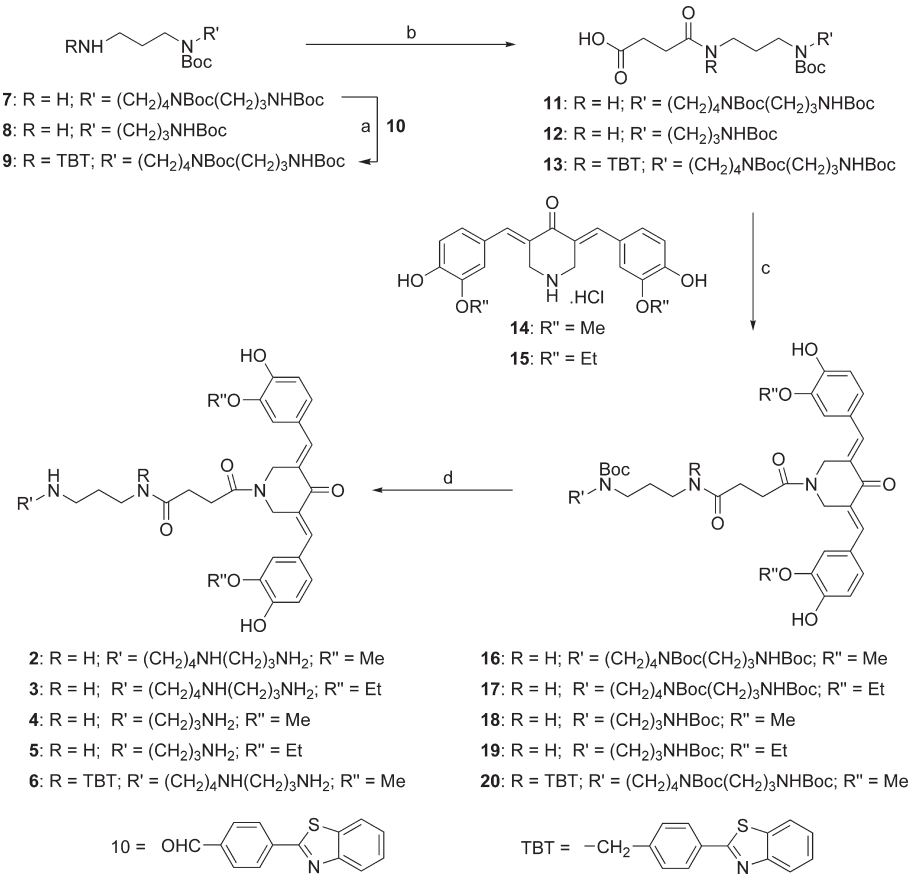
The antioxidant activity was first tested on submitochondrial particles (SMP) of bovine heart mitochondria. Addition of antimycin A to SMP treated with NADH induced a strong increase in ROS production, which was detected by the fluorogenic probe DCFDA. At 10 $\mu$M, **2**−**5** significantly decreased ROS production, similar to the reference compound **1**. Conversely, the unexpected very low fluorescence detected in the presence of **14** and antimycin A suggested that this compound could interfere with the electron transport chain. The ROS decrease could be explained by two different mechanisms: the compound may act directly on the sites of ROS production or it may have a strong scavenger activity (Figure 2).

To further explore the antioxidant potential of the synthesized compounds, their activity against oxidative insults was assayed in human fibroblasts, following treatment with *tert*-butyl hydroperoxide. Interestingly, **2**−**5** at 10 $\mu$M decreased ROS production by 38−45% (see Table 3SI in Supporting Information). Owing to its cytotoxicity, the antioxidant efficacy of the lonely polyphenolic moiety **14** could not be verified in the conditions used for **2**−**5**. The replacement of the methoxy function of **1** with an ethoxy function, meant to modulate the stability of the phenoxy free radical, did not effectively result in significant improvement of the antioxidant activity. Moreover, as expected, inserting different polyamine chains did not influence the antioxidant efficacy.

**1** not only exhibits free radical scavenging properties but also protects against oxidative stress by means of indirect antioxidant mechanisms such as heme oxygenase-1 (HO-1) induction.[11] HO-1 exerts its protective role by degrading the intracellular levels of pro-oxidant heme and by producing biliverdin, the precursor of bilirubin, this latter being an endogenous molecule with powerful antioxidant features.[26] For this reason, **14** and polyamines **2** and **4**, which carry the synthone **14**, were assayed as HO-1 modulators in bovine aortic endothelial cells (BAEC) using 10 $\mu$M of each compound incubated for 6 h. Interestingly, **14** turned out to be more efficacious than **1** in activating HO-1. Including a spermine tail in the constrained polyphenolic nucleus only slightly affected its efficacy (Figure 3). This promising result suggests that the synthesized compounds are, like **1**, multimodal antioxidants able to raise cell defenses by means of direct and indirect mechanisms. To verify how different substituents on the two aromatic rings can tune the profile of the DBP moiety in relation to its ability to increase HO-1 activity, a detailed structure−activity relationships study is in progress and will be published in due course.

The reported antitumoral efficacy of the DBP nucleus,[15−17,27] together with the high degree of cytotoxicity verified for **14** in the T67 cell line, prompted us to further explore the antiproliferative effect of this series of compounds. To verify their effect on tumor and normal cell proliferation, **2**−**5** and reference compounds **1** and **14** were assayed in human colonic carcinoma Caco-2, human neuroblastoma SH-SY5Y, rat glioma C6, and human prostate cancer LNCaP cell lines, mouse embryo BALB/c 3T3 fibroblasts line, and murine hippocampal neuronal HT22 cell lines. Antiproliferative activities were determined after 72 h of treatment with various concentrations (0.1−30 $\mu$M) of the tested compound. Cell proliferation was measured by MTT assay, the results being expressed as $IC_{50}$ (concentration of compound resulting in 50% inhibition of cell proliferation). As depicted in Table 1, **2**−**5** showed antiproliferative effects similar to that of **1**, while **14** was significantly more cytotoxic. In particular, the different efficacy of the polyamine derivatives and **14** is highlighted in Figure 4, where the cell proliferation of normal (a) and cancer

**Scheme 1**[a]




[a] Reaction conditions: (a) toluene, reflux, 2 h, and then ethanol, $NaBH_4$, room temp, overnight; (b) succinic anhydride, $Et_3N$, $CH_2Cl_2$, room temp, 24 h; (c) PPAA, $Et_3N$, dry DMF, room temp, 3−4 h; (d) TFA, $CH_2Cl_2$, room temp, 4 h.

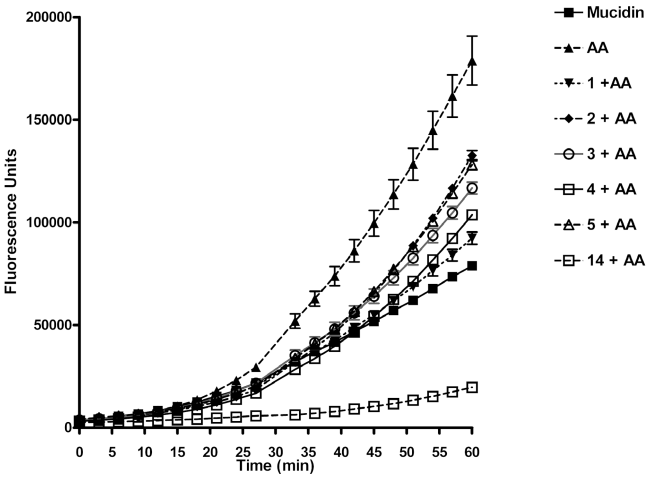



**Figure 2.** Effect of **1**−**5** and **14** on ROS production in submitochondrial particles induced by 2 $\mu$M antimycin A using NADH as substrate. 1.8 $\mu$M mucidin is used as a negative control.

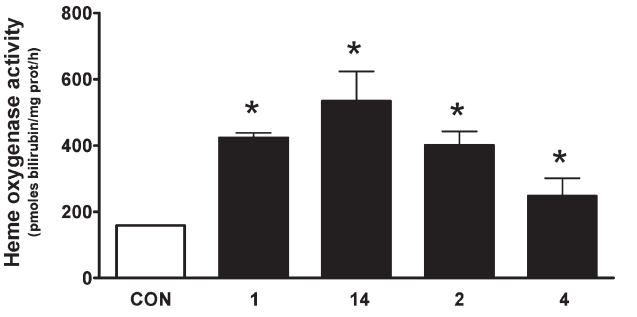



**Figure 3.** Effect of **1**, **2**, **4**, and **14** on HO-1 activity in endothelial cells. Bovine aortic endothelial cells were exposed to 10 $\mu$M compound, and HO-1 activity was measured after 6 h of incubation as described in the Experimental Section. Each bar represents the average of three independent experiments, and results are expressed as (pmol of bilirubin/mg of protein)/h. The control group (CON) is represented by cells exposed to medium alone: (*) $p < 0.05$ vs control (CON).

(b) cells is reported for **14** and **2**, as examples. In agreement with that observed in T67 glioma cells, 10 $\mu$M **14** exerted a strong inhibition of the cell proliferation at all the tested cell lines. Conversely, at the same concentration of **2**, the decrease of cell proliferation was about 40% in cancer cell lines and about 10% in normal ones. Compared to healthy cells, this suggests that cancer cells have a differential sensitivity to polyamine derivatives. This has already been reported for **1**.[13]

Therefore, inserting a polyamine into the DBP nucleus (with the intention of conveying the compound into mitochondria) significantly reduced the antiproliferative potency of the polyphenolic moiety, with the antioxidant efficacy being preserved. In particular, we were intrigued to find that 10 $\mu$M **2**−**5** significantly reduced ROS formation in fibroblasts without causing considerable cytotoxic effects at the studied healthy cell lines. This prompted us to regard these compounds as useful pharmacological tools for developing valid neuroprotective agents.

**Table 1.** Antiproliferative Effects of **1**−**5** and **14** on Tumor and Normal Cell Lines

| | $IC_{50}$ ($\mu$M)[a] | | | | | |
|---|---|---|---|---|---|---|
| | tumor cell line | | | normal cell line | | |
| compd | Caco-2 | SH-SY5Y | C6 | HT22 | HaCaT | 3T3 |
| **1** | 15.0 | 12.8 | 16.1 | 20.4 | 21.9 | 14.2 |
| **2** | 11.9 | 14.2 | 13.9 | 19.8 | 15.6 | 18.4 |
| **3** | 14.4 | 20.8 | 14.2 | 18.4 | 15.7 | 16.8 |
| **4** | 16.5 | 17.8 | 19.9 | 24.5 | 17.7 | 14.1 |
| **5** | 16.2 | 14.6 | 14.5 | 18.9 | 15.5 | 15.3 |
| **14** | 0.2 | 2.1 | 3.9 | 3.1 | 3.0 | 4.2 |

[a] Concentration of compound resulting in 50% inhibition of tumor and normal cell proliferation. The cell proliferation was determined by the MTT assay (as described in the Experimental Section) after 72 h of incubation with the tested compound (0.1−30 $\mu$M). The values are reported as the mean of two independent experiments.

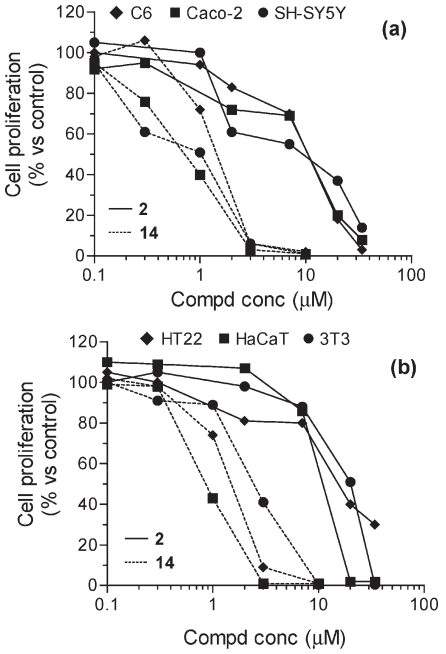



**Figure 4.** Antiproliferative effects of **2** and **14** on tumor (a) and normal (b) cell lines. The cell proliferation was determined by the MTT assay (as described in the Experimental Section) after 72 h of incubation with compounds (0.1−30 $\mu$M). The results were expressed as percentage of control cells. The values are reported as the mean of two independent experiments.

On the basis of these promising results, we wanted to verify if the synthesized compounds enabled efficient intracellular uptake and mitochondria targeting in a cellular context. To this end, mitochondria delivery was preliminarily assessed by inverted fluorescence microscopy using **6** as the fluorescent probe. To verify the direct involvement of mitochondrial membrane potential in polyamine localization, T67 glioma cells were pretreated with 10 $\mu$M **6** and 1 mg/mL oligomycin, an ATP-synthase inhibitor that enhances the mitochondrial potential favoring proton accumulation within the intermembrane space. Fluorescence images were obtained after 30 min of incubation in the presence (Figure 5a) and absence (Figure 5b) of the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The high fluorescence intensity measured in the former case is consistent with an active cellular uptake of **6**, while the loss of fluorescence observed in the presence of CCCP strongly suggests that the uptake is driven by the mitochondrial potential. To test this

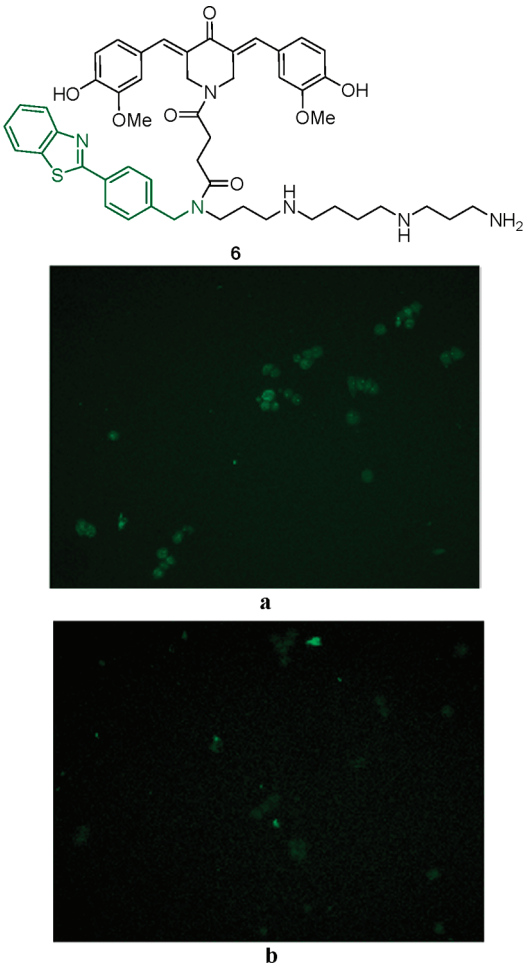



**Figure 5.** Fluorescence images of T67 cells treated with 10 $\mu$M **6** in the absence (a) and in the presence (b) of 20 $\mu$M CCCP.

hypothesis, we performed fluorimetric measurement in intact cells following **6**'s fluorescence in the presence of different amounts of CCCP, until complete mitochondrial depolarization. Our data show that the leaking of **6** follows the gradient dissipation induced by the uncoupler in a dose-dependent manner (see Figure 1SI in Supporting Information). The residual fluorescence observed after CCCP treatment (Figure 5b) is related to the partial distribution of **6** in the lipid phase as confirmed by the results obtained titrating **6**'s fluorescence in the presence of increasing amounts of liposomes (see Figure 2SI in Supporting Information). Moreover, CCCP pretreated cells were not able to accumulate **6** (Figure 3SI in Supporting Information).

In conclusion, this study suggests that **2**−**5** are efficient multimodal antioxidants able to penetrate cells and locate themselves inside the mitochondria. In particular, polyamines, already identified as excellent vectors for the polyamine transport system mediated cell import process, also emerged for their ability to specifically convey a bioactive functionality into mitochondria, taking advantage of electrostatic forces. Even more intriguing is the finding that, in addition to representing a promising tool in the considerable challenge of targeted drug delivery, polyamines might also be able to finely tune the bioactivity of a compound. In particular, in the present study, the well-retained antioxidant activity observed for DBP-polyamine conjugates with respect to **14** is accompanied by a significant loss of cytotoxicity, suggesting that

these compounds may represent promising molecules for neuroprotectant lead discovery. This thesis is further corroborated by the finding that naturally occurring polyamines have been covalently linked to CNS active therapeutic agents to significantly increase their permeability through the blood−brain barrier.[28]

## Experimental Section

**General Information.** Chemical reagents were purchased from Sigma Aldrich, Fluka and Lancaster (Italy). $CH_2Cl_2$ was distilled from calcium chloride. TLC experiments were performed on 0.20 mm silica gel 60 F254 plates (Merck, Germany). Nuclear magnetic resonance spectra (NMR) were recorded at 200 and 400 MHz on Varian VXR 200 and 400 spectrometers and reported in parts per million. Direct infusion ESI mass spectra were recorded on a Waters ZQ 4000 apparatus. The purity of compounds was determined as >95% by HPLC and elemental analyses.

**General Procedure for 11−13.** Succinic anhydride (1 equiv) and $Et_3N$ (2 equiv) were added to a solution of the appropriate Boc-protected polyamine (**7−9**) (1 equiv) in dry $CH_2Cl_2$. After being stirred at room temperature for 24 h, the mixture was worked up by washing with $NaHCO_3$. Then the aqueous layer was acidified using $KHSO_4$, extracted with $CH_2Cl_2$, and dried over $Na_2SO_4$. Removal of the solvent gave oily compounds that were used in the next step without further purification.

**General Procedure for 16−20.** The appropriate amine hydrochloride (**14**, **15**) (1 equiv) was added to a stirred solution of the corresponding acid (**11−13**) (1 equiv) in dry DMF, PPAA (2 equiv), and $Et_3N$ (3 equiv). After the mixture was stirred at room temperature for 3−4 h, the solvent was evaporated and the crude product purified by flash chromatography on silica gel.

**General Procedure for 2−6.** Trifluoroacetic acid (30% in DCM) was carefully added to a stirred solution of the appropriate Boc-compound (**16−20**) in $CH_2Cl_2$ at 0 °C and was allowed to stir at room temperature for 4 h. The solvent was evaporated under reduced pressure, adding heptane for the azeotropic removal of trifluoroacetic acid traces. The trifluoroacetate salts were washed with ether to obtain **2−6** in quantitative yield.

For the chemical characterization of **2−6**, **9**, **11−13**, and **16−20**, see Supporting Information.

**Acknowledgment.** This research was supported by grants from the University of Bologna, Italy, and MIUR (Grant PRIN 20073EWPF9_003).

**Supporting Information Available:** Synthesis of intermediate and final compounds, results from elemental and HPLC analyses, and biological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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