Potent Inhibitors of the Qi Site of the Mitochondrial Respiration Complex III

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A series of azole-fused salicylamides were prepared as analogues of antimycin and assayed for activity at complex III of the mitochondrial respiratory chain. The activity of these compounds approached that of antimycin in inhibitory potency and some showed growth reduction of *Septoria nodorum* in vitro. Compound **8a** was shown to bind at the Qi site of complex III by red-shift titration of the bc_1 complex.

Introduction

Antimycin A_1 (**1**, Figure 1, antimycin) is a dilactone salicylamide that was isolated from *Streptomyces sp.*[1] It is a potent inhibitor of the Qi site of complex III (bc_1 complex) in the mitochondrial respiratory chain, with a dissociation constant with bovine heart mitochondrial particles of 32 pM.[2] It and the related blastomycins and UK-2A have attracted interest as antifungal agents from the pharmaceutical arena[3] and have also served as a starting point for the synthesis of agricultural insecticides and fungicides.[4] However, unlike the Qo site of the bc_1 complex, which is the target of inhibitors such as myxothiazole and strobilurins[5] and commercial agricultural fungicides developed from these such as Azoxystrobin[6] and Kresoxim-methyl,[7] the Qi site has witnessed only limited commercial exploitation.[8] As such it represents an attractive target that might not suffer resistance issues that surround the Qo site inhibitors.[9]

Two recent crystal structure determinations of the bc_1 complex bound to antimycin illuminate the binding mode of antimycin but differ in their assessment of the conformation of the ligand.[10] In the structure with the higher resolution of 2.28 Å,[10b] it can be seen that antimycin forms an extensive hydrogen-bonded network to the enzyme as shown in Figure 1. The formamide oxygen receives a water-mediated hydrogen bond from Lys227 The formamide NH acts as a hydrogen-bond donor to Asp228, which also forms a hydrogen bond with the phenol. The formamide oxygen is bent back toward and coplanar with the benzene ring as in the crystal structure of the unbound ligand.[11] A hydrogen bond between the phenolic oxygen and the benzamide NH maintains a coplanar arrangement of these two groups, and the benzamide oxygen is then involved in a water-mediated hydrogen bond to His201. With respect to the native ligand in the solid state, the benzamide has been rotated 180° in binding to the bc_1 complex.

Tokutake et al.[12a] have prepared simpler analogues of the natural product that possess comparable in vitro activity by replacing the dilactone portion of the molecule with biphenyl and biphenyl ether groups such as compound **2** (Figure 2). These analogues also possess moderate to good levels of in vivo activity on a range of agricultural pathogens but fall short of commercial standards such as Azoxystrobin.[4]

The formamide group is an important element of the pharmacophore[13] but one which represents a potential metabolic liability that could result in lower in vivo efficacy than might be expected on the basis of the intrinsic activity of these compounds. As such we sought potential replacements for this group that were also free of patent protection. Inspired by the conformation of the formamide group in the crystal structure, we conceived the azole-fused salicylamides shown in Figure 3. These analogues would span a range of NH pK_as[14] that would approach that of the formamide group and incorporate the biphenyl ether amides as previously described by Tokutake et al.[12a]

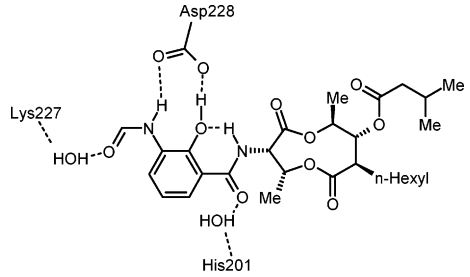

Figure 1. Structure of antimycin A_1 (**1**) showing binding interactions with the bc_1 complex.[10b]

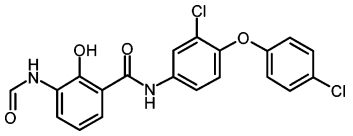

Figure 2. Structure of functional antimycin equivalent (**2**).[12a]

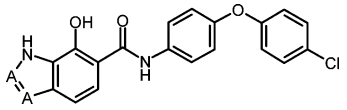

Figure 3. Designed benzazole mimics of formamidosalicylamide **2** (A = CH or N).

Chemistry

The synthesis of these targets is shown in Scheme 1. Compound **5** was prepared analogously to that described by Poupart et al.,[15] with chlorine as protection for the 5-position rather than bromine. Treatment of the amino toluene **5** with isoamyl nitrite gave the indazole **6** in good yield, which was then demethylated by use of concentrated HBr to yield the phenol-acid **7**, which could be coupled to a variety of anilines to give the targets **8**.

Using a similar sequence from the diamine **9**,[16] we prepared the benzotriazole analogue **12** (Scheme 2), and similarly, reduction of the nitro aniline **13** with Pd and formic acid gave the benzimidazole **14**, which was carried on to the final targets as described above (Scheme 3).

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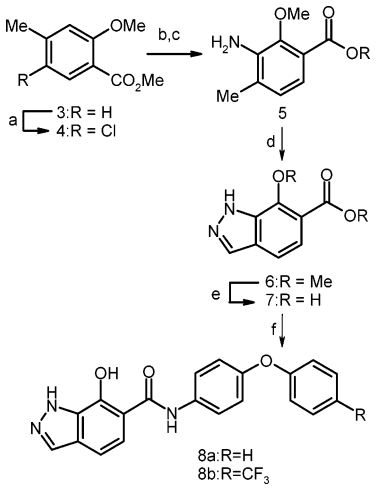
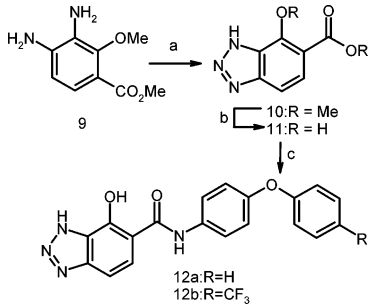
Scheme 1[a]



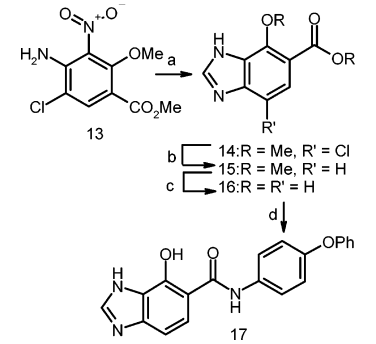
[a] Reagents and conditions: (a) NCS, DMF, 20 °C, 3 days; (b) HNO_3, Ac_2O, AcOH, 25 °C, 18 h; (c) 50 psi H_2, NEt_3, $Pd(OH_2)$/C, EtOH, 20 °C, 8 h; (d) *i*-amyl nitrite, HOAc, 20 → 117 °C, 1 h; (e) *c*HBr, 80 °C, 18 h; (f) EDC.HCl, HOBt, 4-(4*R*-phenoxy)phenylaniline, pyridine, 90 °C, 18 h.

Scheme 2[a]



[a] Reagents and conditions: (a) *i*-amyl nitrite, HOAc, 20 °C, 0.5 h; (b) *c*HBr, 80 °C, 6 h; (c) EDC·HCl, HOBt, 4-(4*R*-phenoxy)phenylaniline, pyridine, 90 °C, 7 h.

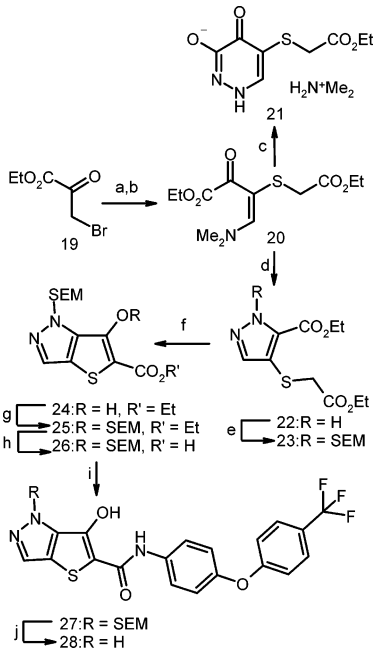
Scheme 3[a]



[a] Reagents and conditions: (a) HCO_2H, Pd/C, 100 °C, 3 days; (b) 50 psi H_2, NEt_3, Pd/C, EtOH, 20 °C, 18 h; (c) *c*HBr, 85 °C, 6 h; (d) EDC·HCl, HOBt, 4-phenoxyphenylaniline, pyridine, 85 °C, 6 h.

Since thiophenes are common bioisosteres of phenyl groups, we also targeted the pyrazolothiophene **28**, which was prepared as described in Scheme 4. Ethyl bromopyruvate was reacted with ethyl thioacetate according to a modified procedure as described by Titus and Titus.[17a] Formation of the enamine **20** was accomplished by reaction with DMFDMA, which reacted with hydrazine to give predominantly the pyridazinone **21**. Selectivity for the pyrazole **22** could be achieved under mildly acidic conditions with hydrazine hydrochloride and sodium acetate. Dieckmann condensation took place readily[18] after N-protection to give the thiophene **24**. This was followed by

Scheme 4[a]



[a] Reagents and conditions: (a) EtO_2CCH_2SH, pyridine, −35 → 15 °C; (b) DMFDMA, 25 °C, 2 h; (c) NH_2NH_2, EtOH, 20 °C; (d) NH_2NH_2·HCl, NaOAc, EtOH, 20 °C, 1.5 h; (e) NaHMDS, SEMCl, THF, 20 °C, 1 h; (f) KO-*t*-Bu, THF, 5 °C, 1 h; (g) NaHMDS, SEMCl, THF, 20 °C, 2 h; (h) NaOH, EtOH, 78 °C; (i) EDC·HCl, HOBt, 4-(4-trifluoromethylphenoxy)-phenylaniline, pyridine, 85 °C, 2 h; (j) TFA, H_2O, 20 °C, 40 min.

Table 1. Inhibitory Effect of Target Compounds against Mitochondrial Electron Transport and Cellular Growth of *Septoria nodorum*[a]

compd	FMET2−3 (I_{50}, nM)	FMET2 (I_{50}, nM)	growth inhibition (GR_{50}, nM)
8a	127 (19)	>30 000	1909 (1928)
8b	26 (7)	>30 000	343 (241)
17	>30 000	>30 000	>30 000
12a	32 (2)	>30 000	>30 000
12b	5 (1)	1815 (613)	>30 000
28	26 (4)	>30 000	11 517 (1962)
antimycin	2 (1)	>30 000	33 (20)

[a] Standard deviations are shown in parentheses. Growth inhibition data are the mean of 8 replicates.

protection of the hydroxy group to allow saponification of the ester. The conversion of **23** to **25** could be accomplished in one pot in an overall 95% yield. Amide formation under standard conditions was accompanied by loss of the SEM ether. Finally, the remaining SEM group was removed by careful treatment with TFA to give the target compound **28**.

Results and Discussion

The in vitro activity of the compounds prepared is shown in Table 1. All assays were performed on the plant pathogen *Septoria nodorum* or on submitochondrial particles derived therefrom. The first assay (FMET2−3) gives a measure of inhibition versus both complex 2 (succinate dehydrogenase) and complex 3 (bc_1 complex). The second assay (FMET2) shows inhibitory activity against complex 2 only. The third column shows the growth inhibition of cells cultured in a Petri dish.[19]

As can be seen from the table, all compounds are inactive against complex **2** and therefore activity shown in assay 1 is due solely to activity at the bc_1 complex. It can also be seen that the indazole analogues **8** have good to excellent activity in comparison with antimycin, with the 4′CF_3 substitution (**8b**) enhancing the level of activity 5-fold over that of the unsub-

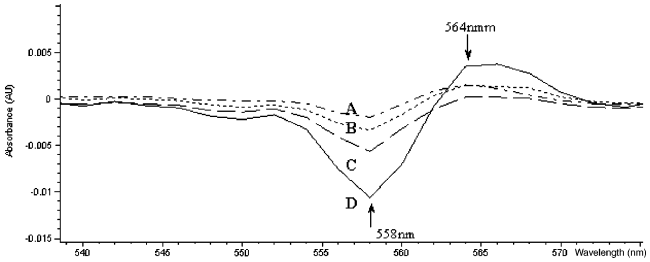


Figure 4. Difference spectrum of inhibitor binding to reduced bc_1 complex minus reduced enzyme in absence of the inhibitor: (A) 10 μM; (B) 20 μM, and (C) 40 μM compound **8a**. (D) Addition of 20 μM antimycin A. Titration mixtures contained 4 μM bc_1 complex, 0.2 mM EDTA, 2 mM NaN_3, and 0.02% Triton X-100 in 50 mM sodium phosphate, pH 7.2.

stituted compound (**8a**). The activity of the benzotriazole analogue **12b** increases a further 5-fold over **8b** and is nearly equipotent with the natural product. However, the benzimidazole analogue **17** is completely inactive, despite its apparent good electrostatic mimicry of the formamide group. The superior activity of the benzotriazole analogue might be due to the increased NH acidity versus the indazole or might be a result of increased acidity of the phenol. The activity of the thienopyrazole **28** matches that of the indazole **8b**.

The indazole compounds **8** displayed good in vivo activity against *Septoria nodorum*, with **8b** having a submicromolar GR_{50} (Table 1); however, the benzotriazole analogues **12** were inactive in this assay, possibly due to poor cell penetration of these compounds. Three membranes must be crossed to access the Qi site, and good fungicidal compounds acting at this site must therefore possess excellent translational properties. The activity of the thienopyrazole **28** was also much lower than might be expected from its intrinsic potency.

Unfortunately, none of the above compounds controlled *Septoria nodorum* grown on wheat when applied at 40 ppm.

The unsubstituted indazole (**8a**) was used as a representative analogue to confirm binding of the inhibitors to the Qi domain by use of purified bovine enzyme. The Qi domain and the Qo domain of cytochrome *b* are located near the b_H and b_L hemes, respectively. Tightly associating Qi inhibitors, such as antimycin, perturb the optical spectrum of the reduced enzyme, causing a red shift in the Soret region of the b_H heme[2]. Many, but not all, Qo domain inhibitors also elicit a red shift in the reduced optical spectrum of the b_L heme.[20] Because the wavelength transitions differ for Qi and Qo red-shifted spectra, the general localization of inhibitor binding can be distinguished. Figure 4 depicts the difference spectrum of compound **8a** binding to reduced cytochrome bc_1 upon sequential titration. Characteristic red-shift peaks and troughs were observed at 564 and 558 nm, respectively. These transitions are identical to those observed when antimycin was added to fully saturate the site, therefore confirming Qi site occupancy. It should be noted that some Qi inhibitors, such as the hydroxyquinoline N-oxides, also bind weakly to the Qo domain,[10a] and this possibility cannot be ruled out for the indazole compounds based on the current analysis.

Conclusion

A series of azole-fused salicylamides have been prepared wherein the azole NH is designed to replace the formamide NH in antimycin. These functional mimics have been shown to have excellent activity at the Qi site of the bc_1 complex. Potent control of cell growth was also observed for the indazole analogues **8**. These functional mimics might find application elsewhere such as in Bcl2 binding for cancer chemotherapy.[21]

Experimental Section

General Techniques. Reactions were carried out under an atmosphere of dry nitrogen with anhydrous solvents purchased from the Aldrich Chemical Co. Inc. where appropriate. Amine bases were dried and stored over potassium hydroxide. Reactions were monitored by thin-layer chromatography (TLC) on E. Merck silica gel plates (0.25 mm) and visualized under UV light (254 nm) and/or by heating with phosphomolybdic acid ethanol solution. Solvents used for workup and chromatography were reagent-grade from E. Merck or VWR Scientific. Flash chromatography was performed on E. Merck silica gel (60, particle size 0.040−0.063 mm). Yields refer to chromatographically and spectroscopically (^{1}H NMR) pure materials.

^{1}H NMR spectra were recorded on a Varian 400 MHz instrument at 25 °C. ^{13}C NMR spectra are recorded at 100 MHz. Chemical shifts are reported relative to TMS internal standard. Multiplicities are designated singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), or broad singlet/multiplet (br s/br m). IR samples were prepared by evaporation of a solution of the compound from $CDCl_3$ onto a NaCl plate under a stream of nitrogen. IR spectra were recorded on a Nicolet Avatar 330 spectrometer. Mass spectra were recorded on a Waters Micromass spectrometer under fast atom bombardment (FAB) conditions. HRMS were acquired on a Micromass LCT time-of-flight mass spectrometer interfaced with a Agilent 1100 liquid chromatograph. Melting points were recorded on a Thomas-Hoover Unimelt apparatus and are uncorrected. Microanalyses were performed at Quantitative Technologies Inc., Whitehouse, NJ.

7-Methoxyindazole-6-carboxylic Acid, Methyl Ester (6). To a solution of 3-amino-2-methoxy-4-methylbenzoic acid, methyl ester (**5**) (0.64 g, 3.28 mmol) in acetic acid (20 mL) was added *i*-amylnitrite (0.476 mL, 1.1 equiv). The mixture was stirred at ambient temperature for 30 min before being heated to reflux for 1 h. Upon cooling, the mixture was concentrated and purified by flash chromatography (silica gel, 30−40% ethyl acetate in hexane) to yield the indazole **6** (0.50 g, 74%) as a pale yellow solid: mp 118−121 °C; R_f 0.57 (silica, 40% ethyl acetate in hexanes). ^{1}H NMR ($CDCl_3$) δ 8.11 (s, 1H), 7.60 (d, 2H), 7.49 (d, 1H), 4.09 (s, 3H), 3.97 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 166.7, 133.5, 127.8, 123.6, 119.6, 115.7, 105.1, 62.4, 52.3. IR 3361, 2950, 2918, 1714 cm^{-1}. MS 207.3 (M + H$^+$). Anal. ($C_{10}H_{10}N_2O_3$) C,H,N.

7-Hydroxyindazole-6-carboxylic Acid (7). 7-Methoxyindazole-6-carboxylic acid, methyl ester (**6**) (0.6 g), was heated in 48% HBr (3 mL) at 80 °C for 18 h. Upon cooling, the precipitate was filtered, washed with a small amount of water, and dried in vacuo at 60 °C to give acid **7** as a white solid (0.49 g, 96%): white solid, mp 232−233 °C (decomp). ^{1}H NMR (DMSO-d_6) δ 12.4−12.0 (br s, 1H), 8.11 (s, 1H), 7.43 (d, 2H), 7.24 (d, 1H). ^{13}C NMR (DMSO-d_6) δ 173.7, 150.2, 134.4, 131.9, 128.2, 121.4, 111.5, 107.3. MS 177.1 (M − H$^+$).

7-Hydroxyindazole-6-carboxylic Acid, 4-(4-Trifluoromethylphenoxy)phenylamide (8b). To a mixture of 7-hydroxyindazole-6-carboxylic acid (**7**) (129 mg, 0.5 mmol), HOBt (71 mg, 1.05 equiv), EDC·HCl (125 mg, 1.3 equiv), and 4-(4-trifluoromethylphenoxy)aniline (152 mg, 1.2 equiv) was added pyridine (5 mL), and the mixture was heated at 90 °C for 18 h. The mixture was then cooled, acidified with 1 N HCl, extracted with methylene chloride 3×, and dried ($MgSO_4$). Upon concentration, the residue was purified by flash chromatography (silica gel, 30−40% ethyl acetate in hexane) to yield the amide **8b** (0.14 g, 68%): white solid, mp 204−205 °C; R_f 0.26 (silica, 40% ethyl acetate in hexanes). ^{1}H NMR ($CDCl_3$) δ 13.3−12.9 (br s, 1H), 8.13 (s, 1H), 8.10 (s, 1H), 7.63 (d, 2H), 7.58 (d, 2H), 7.29−7.22 (m, 3H), 7.10 (d, 2H), 7.06 (d, 2H). ^{13}C NMR ($CDCl_3$) δ 160.6, 153.2, 150.1, 135.3, 133.2, 132.5, 127.4 (q)$^+$, 127.2, 125.3, (q), 123.5, 120.8, 118.0, 117.5, 111.6, 108.7. IR 1608 cm^{-1}. HRMS Calcd. for $C_{21}H_{14}N_3O_3F_3$ (M + H$^+$): 414.1066. Found: 414.1057.

6-[2-(Trimethylsilanyl)ethoxymethoxy]-1-[2-(trimethylsilanyl)-ethoxymethyl]-1*H*-thieno[3,2-*c*]pyrazole-5-carboxylic Acid (26). To a solution of ester **25** (0.44 g) in ethanol (15 mL) was added

NaOH (1.0 N, 1.4 mL, 1.5 equiv) and the mixture was heated at reflux for 0.5 h before being cooled and concentrated in vacuo. The resulting oil was redissolved in water (10 mL) and neutralized by the dropwise addition of HCl (1.0 N). The precipated white solid was collected, washed with water, and dried in vacuo to give acid **26** (0.37 g, 89%): mp 67−68 °C; R_f 0.31 (silica, 80% ethyl acetate in hexanes). ^{1}H NMR ($CDCl_3$) δ 7.81 (s, 1H), 6.01 (s, 2H), 5.63 (s, 1H), 3.92 (t, 2H), 3.65 (t, 2H), 0.98 (m, 4H), 0.02 (s, 9H), 0.00 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 165.9, 150.4, 148.4, 123.6, 122.0, 118.4, 97.7, 83.4, 69.5, 69.0, 19.5, 19.3, 0.1, 0.0. IR 3800−2500, 2953, 1671 cm^{-1}. MS 444.91 (M + H$^+$), 443.06 (M − H$^+$). Anal. ($C_{18}H_{32}N_2O_5SSi_2$) C, H, N.

6-Hydroxy-1-[2-(trimethylsilanyl)ethoxymethyl]-1*H*-thieno-[3,2-c]pyrazole-5-carboxylic Acid, 4-(4-Trifluoromethylphenoxy)phenyl Amide (27). To a solution of acid 26 (554 mg) in pyridine (10 mL) were added HOBt (177 mg, 1.05 equiv), EDC (311 mg, 1.3 equiv), and 4-(4-trifluoromethylphenoxy)aniline (347 mg, 1.2 equiv), and the mixture was heated at 85 °C for 2 h. The mixture was then cooled, concentrated, diluted with 1 N HCl, and twice extracted with methylene chloride. The organic phase was dried ($MgSO_4$), concentrated, and purified by flash chromatography (silica gel, 20−40% ethyl acetate in hexane). The residue obtained is dissolved in ethyl ether (3 mL) and precipitated with hexanes to afford the amide 27 as an off white solid (90 mg, 13%): mp 158−160 °C; R_f 0.56 (silica, 60% ethyl acetate in hexanes). ^{1}H NMR ($CDCl_3$) δ 11.53 (br s, 1H), 7.80 (s, 1H), 7.63 (d, 2H), 7.60 (d, 2H), 7.40 (br s, 1H), 7.10 (d, 2H), 7.07 (d, 2H), 5.65 (s, 2H), 3.65 (t, 2H), 0.95 (t, 2H), 0.02 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 154.0, 151.0, 135.0, 128.6 (q), 124.1, 122.1, 119.1, 83.3, 69.1, 19.3, 0.0. IR 3300, 2917, 1648 cm^{-1}; MS 549.87 (M + H$^+$), 547.99 (M − H$^+$).

6-Hydroxy-1*H*-thieno[3,2-*c*]pyrazole-5-carboxylic Acid, 4-(4-Trifluoromethylphenoxy)phenyl Amide (28). Compound **27** (89 mg) was dissolved in TFA (3 mL) and stirred for 20 min. Water (2 mL) is then added with ice-bath cooling. After stirring for a further 5 min, an additional 1 mL of water is added and the mixture is stirred for an additional 15 min before being filtered and washed with water to give a beige solid (65 mg). The solid is triturated with methylene chloride to give clean indazole **28**. The methylene chloride extract is purified by flash chromatography (silica gel, 4080% ethyl acetate in hexane) to yield additional indazole **28** (26 mg total yield, 38%). mp 221−223 °C. R_f 0.09 (silica, ethyl acetate): ^{1}H NMR (acetone-d_6) δ 12.12 (br s, 1H), 9.35 (s, 1H), 7.93 (s, 1H), 7.84 (d, 2H), 7.72 (9d, 2H), 7.17 (d, 2H), 7.15 (d, 2H). IR 3147, 2928, 1603 cm^{-1}. HRMS Calcd. for $C_{19}H_{12}N_3O_3F_3S$ (M + H$^+$): 420.0630. Found: 420.0618.

Acknowledgment. We thank Mike Wadsley for HRMS analyses.

Supporting Information Available: Synthetic procedures, analytical data for compounds **4**, **5**, **8**, **10**−**12**, **14**−**17**, and **20**−**26**, and procedure for the characterization of bc_1 binding by red-shift titration. This material is available free of charge via the Internet at http://pubs.acs.org.

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