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Citrafungins A and B, Two New Fungal Metabolite Inhibitors of GGTase I with Antifungal Activity

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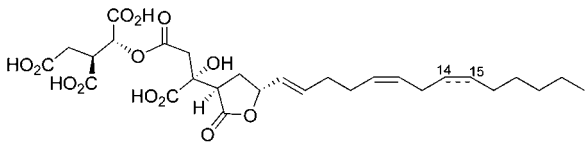
ABSTRACT



Citrafungin A (14,15-dihydro)
Citrafungin B (14,15-*cis*-olefin)

Screening of natural products extracts led to the discovery of citrafungins A and B, two new fungal metabolites of the alkylcitrate family that are inhibitors of GGTase I of various pathogenic fungal species with IC_{50} values of 2.5–15 μM. These compounds exhibited antifungal activities with MIC values of 0.40–55 μM. The isolation, structure elucidation, relative and absolute stereochemistry, and biological activities of citrafungins are described.

Proteins terminating with a CaaX motif such as Rho1p and Cdc42p[1] are post-translationally modified by geranylgeranyltransferase I (GGTase I), which transfers geranylgeranyl, a lipophilic diterpenoid unit, to the cysteine residue. This renders these proteins lipophilic and thus promotes membrane localization and biological activity.[2] Rho1p is a regulatory subunit of 1,3-β-D-glucan synthesis and a key player in cell wall biosynthesis in fungi and is essential for cell viability of *Saccharomyces cerevisiae*.[3] GGTase I has two subunits, an α (Ram2p) and a β (Cdc43p) subunit; Cdc43p is essential for viability of *S. cerevisiae*. Fungal GGTase I does not have significant homology[4] with human GGTase I. The observation that Cdc43p is essential for the survival of *S. cerevisiae* suggested that GGTase I might be a potential antifungal drug target.

To date, natural products have been the major source of antifungal drugs. An agar cell-based two-plate bioassay system using two *S. cerevisiae* strains, in which GGTase I was under- and overproduced, was used for primary screening. GGTase I selective actives would be expected to show a measurable difference in zones of inhibition. Screening of natural product extracts derived from fungal fermentations led to the identification of an extract of sterile mycelia isolated from a cow dung sample collected in Alaska that showed significant zone differential. The activity of the extract was further confirmed in a rScGGTase I enzyme assay.[4] Bioassay-guided fractionation of an organic extract led to the discovery of two new compounds named herein citrafungin A (**1a**) and B (**2a**). These polyketides belonging to the alkyl citrate class of natural products are further esterified by isocitric acid. They inhibited GGTase I activity

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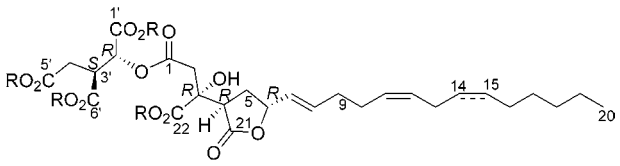
of various pathogenic fungal strains with IC_{50} values of 2.5−15 μM and exhibited antifungal activity with MIC values of 0.43−55 μM. The isolation, structure elucidation, relative and absolute stereochemistry, and details of biological activity of these two compounds are described.



1a (R = H; 14,15-dihydro) Citrafungin A
1b (R = Me; 14,15-dihydro)
2a (R = H; 14,15-*cis*-olefin) Citrafungin B
2b (R = Me; 14,15-*cis*-olefin)

The fungal sterile mycelium (MF6339) was grown on a KHCC medium.[5] The fermentation broth was extracted with methyl ethyl ketone and was chromatographed on Sephadex LH-20 followed by reverse phase HPLC (Primesphere C-8) using an aqueous CH_3CN linear gradient containing 0.1% TFA. Lyophilization of fractions afforded citrafungin A[6] (**1a**, 31 mg/L) and B (**2a**, 15 mg/L) as amorphous powders that turned into gums after storage.

Citrafungin A (1a). Positive ion ESIMS analysis of **1a** produced a pseudomolecular ion at *m*/*z* 585 $[M + H]^+$. Reaction of **1a** with diazomethane afforded a tetramethyl ester **1b** (*m*/*z* 640.3126, calcd for $C_{32}H_{48}O_{13}$ 640.3096) and indicated that it contained four free carboxyl groups, affording $C_{28}H_{40}O_{13}$ as a formula of **1a**. The UV spectrum of **1a** was nondescript, and the IR spectrum exhibited broad absorption bands for ester (1717 and 1174 cm^{-1}). The molecular formula of citrafungin A was confirmed by the ^{13}C NMR spectral analysis,[6] including DEPT indicating the presence of a methyl, 12 methylenes, 4 olefinic methines, 4 methines including 2 oxymethines, an oxygen bearing quaternary carbon, and 6 ester/acid carbonyl groups (Table 1, see Supporting Information). These assignments were fully corroborated by an HSQC experiment.[6]

The 600 MHz $^1H-^1H$ COSY NMR spectral analysis in acetone-d_6 revealed the structural fragments for C4−C20, C2′−C4′ and an isolated AB system assigned for H_2-2 (Table 1). The COSY spectrum clearly indicated that C-6 (δ_H 4.99, δ_C 79.4) was sandwiched between the olefin C-7 (δ_H 5.57, δ_C 129.1) and C-5 dissymmetric methylene (δ_H 2.39, 2.11 δ_C 30.8), which was flanked by C-4 methine (δ_H 3.07, δ_C 46.3). It was also very clear from the COSY correlations that the two olefins were separated by two methylenes C-9 and C-10.

A high-resolution HMBC (Table 1) experiment was used to connect remaining structural pieces with that of the COSY-derived structural fragments. Both H-4 and H-6 showed strong HMBC correlations to the carbonyl C-21 (δ_C 175.0), thus establishing the γ-lactone ring. Both C-5 methylene protons and H-4 displayed HMBC correlations to the oxygenated quaternary carbon C-3 (δ_C 75.5), and the latter proton also showed correlation to C-2 (δ_C 40.7). Both methylene protons of C-2 exhibited strong HMBC correlations to the C-1 carbonyl (δ_C 169.4), C-3, and C-4. In addition, one of the two protons (δ_H 3.66) showed strong and the second (δ_H 3.13) showed weak correlations to the carbonyl C-22 (δ_C 173.6), which also exhibited correlation to H-4, albeit a weak one. These correlations helped in unambiguous shift assignment of carbonyls C-1 and C-22 and fully established the structural assignment of C1−C22. The distinction of these two carbonyls was critical for the connectivity of this fragment with isocitric acid.

Both H_2-4′ showed strong HMBC correlations to two carbonyls C-5′ (δ_C 172.3) and C-6′ (δ_C 170.9) in addition to both C-2′ (δ_C 71.9) and C-3′ (δ_C 42.6). In addition, C-1′ (δ_C 168.7) and C-6′ showed correlations to H-2′ and H-3′. The strong HMBC correlation of H-2′ to C-1 proved pivotal for unambiguous ester linkage of C-1 carbonyl with C-2′ hydroxy group of isocitric acid.

The HREIMS of **1b** revealed major fragment ions at *m*/*z* 407.2404 ($C_{23}H_{35}O_6$), 364.1008 ($C_{14}H_{20}O_{11}$), and 217.0713 ($C_9H_{13}O_6$) due to cleavages of the ester linkage, C3−C4, and trimethyl isocitric acid without C-2′ oxygen, respectively.

(5) It was grown on a seed medium consisting of in (g/L) corn steep liquor (5), tomato paste (40), oat flour (10), glucose (10), trace elements [10 mL solution of $FeSO_4 \cdot 7H_2O$ (1), $MnSO_4 \cdot H_2O$ (1), $CuCl_2 \cdot 2H_2O$ (0.025), $CaCl_2$ (0.1), H_3BO_3 (0.056), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.019), $ZnSO_4 \cdot 7H_2O$ (0.2) prepared in 0.6 N HCl], agar (4) in water at pH 6.8 at 25 °C. An aliquot of the culture was transferred to a KHCC production medium consisting of in (g/L) dextrin (20), β-cyclodextrin (10), primary yeast (10), tomato paste (20), $CoCl_2 \cdot 6H_2O$ (0.005) in water at pH 7.2 grown at 25 °C.

(6) Citrafungin A (**1a**): t_R = 9.99 min (Primesphere C-8, 4.6 × 250 mm, CH_3CN-H_2O + 0.1% TFA, 1 mL/min); $[\alpha]^{25}_D$ +30.7 (*c* 2.7, CH_3OH); ^{13}C NMR (acetone-d_6) δ_C 169.4 (C-1), 40.7 (C-2), 75.5 (C-3), 46.3 (C-4), 30.8 (C-5), 79.4 (C-6), 129.1 (C-7), 134.4 (C-8), 32.2 (C-9), 26.6 (C-10), 128.7 (C-11), 130.6 (C-12), 27.2 (C-13), 29.7 (C-14), 29.6 (C-15), 29.5 (C-16), 28.8 (C-17), 31.9 (C-18), 22.7 (C-19), 13.8 (C-20), 175.0 (C-21), 173.6 (C-22), 168.7 (C-1′), 71.9 (C-2′), 42.6 (C-3′), 31.6 (C-4′), 172.3 (C-5′), 170.9 (C-6′); ^1H NMR (acetone-d_6) δ_H 3.66 (1H, d, *J* = 16 Hz, H-2), 3.13 (1H, d, *J* = 16.5 Hz, H-2), 3.07 (1H, dd, *J* = 10, 6 Hz, H-4), 2.39 (1H, ddd, *J* = 13, 7.5, 6 Hz, H-5), 2.11 (1H, m, H-5), 4.99 (1H, q, *J* = 7 Hz, H-6), 5.57 (1H, dd, *J* = 15, 7.5 Hz, H-7), 5.81 (1H, dt, *J* = 15.5, 7 Hz, H-8), 2.11 (2H, m, H_2-9), 2.13 (2H, m, H_2-10), 5.34 (1H, m, H-11), 5.37 (1H, m, H-12), 2.04 (2H, q, *J* = 6.5 Hz, H_2-13), 1.28 (12H, m, H_2-15-H_2-19), 0.86 (3H, t, *J* = 7 Hz, H_3-20), 5.47 (1H, d, *J* = 3.5 Hz, H-2′), 3.56 (1H, ddd, *J* = 8.5, 5.0, 3.5 Hz, H-3′), 2.80 (1H, dd, *J* = 17.5, 9.5 Hz, H-4′), 2.60 (1H, dd, *J* = 17, 4.5 Hz, H-4′); HMBC (C → H) C1 → H_2-2, 2′; C2 → H4; C-3 → H_2-2, 4, H_2-5; C4 → H_2-2, H_2-5, 6; C5 → H-6, 7; C6 → H-4, H_2-5, 7, 8; C7 → H_2-5, 6, 9; C8 → H-6, 9, 10; C9 → H-7, 8, 10, 11; C10 → H-8, 9, 11, 12; C11 → H-9, 10, 12, 13; C12 → H-10, 11, 13, 14; C13 → H-11, 12; C20 → H-18, 19; C21 → H-4, H_2-5, 6; C22 → H_2-2, 4; C1′ → H-2′, 3′; C2′ → H-3′, H_2-4′; C3′ → H-2′, H_2-4′; C4′ → H-2′, 3′; C5′ → H-3′, H_2-4′; C6′ → H2′, 3′, H_2-4′; UV (end absorption); IR ν_{max} (ZnSe) 3400, 2922, 1717, 1174, 965 cm^{-1}; ESIMS (*m*/*z*) 602 $[M + NH_4]^+$, 585 $[M + H]^+$; Citrafungin B (**2a**): t_R = 7.62 min (Primesphere C-8, 4.6 × 250 mm, CH_3CN-H_2O + 0.1% TFA, 1 mL/min); $[\alpha]^{23}_D$ +28.5 (*c* 2.95, CH_3OH); ^{13}C NMR (acetone-d_6) δ_C 169.4 (C-1), 40.8 (C-2), 75.5 (C-3), 46.3 (C-4), 30.8 (C-5), 79.4 (C-6), 129.2 (C-7), 134.2 (C-8), 32.1 (C-9), 26.6 (C-10), 127.9 (C-11), 130.1 (C-12), 25.6 (C-13), 128.9 (C-14), 128.8 (C-15), 27.1 (C-16), 29.3 (C-17), 31.5 (C-18), 22.5 (C-19), 13.7 (C-20), 175.0 (C-21), 173.7 (C-22), 168.7 (C-1′), 71.9 (C-2′), 42.6 (C-3′), 31.7 (C-4′), 172.2 (C-5′), 171.0 (C-6′); ^1H NMR (acetone-d_6) δ_H 3.66 (1H, d, *J* = 16.4 Hz, H-2), 3.14 (1H, d, *J* = 16.4 Hz, H-2), 3.07 (1H, dd, *J* = 10, 6 Hz, H-4), 2.40 (1H, ddd, *J* = 13.2, 7.2, 5.66 Hz, H-5), 2.08 (1H, m, H-5), 5.00 (1H, q, *J* = 7.2 Hz, H-6), 5.59 (1H, dd, *J* = 15.2, 7.2 Hz, H-7), 5.83 (1H, dt, *J* = 15.2, 6.8 Hz, H-8), 2.15 (2H, m, H_2-9), 2.18 (2H, m, H_2-10), 5.36 (1H, m, H-11), 5.36 (1H, m, H-12), 2.79 (2H, brt, *J* = 6 Hz, H_2-13), 5.36 (2H, m, H-14, H-15), 2.05 (2H, m, H_2-16), 1.29 (6H, m, H_2-17-H_2-19), 0.88 (3H, t, *J* = 6.8 Hz, H_3-20), 5.48 (1H, d, *J* = 3.6 Hz, H-2′), 3.56 (1H, ddd, *J* = 9.2, 5.2, 3.6 Hz, H-3′), 2.80 (1H, dd, *J* = 17.2, 9.2 Hz, H-4′), 2.60 (1H, dd, *J* = 17.2, 5.2 Hz, H-4′); UV (end absorption); IR ν_{max} (ZnSe) 3400, 2924, 2854, 1715 (br), 1173 (br), 962 cm^{-1}; ESIMS (*m*/*z*) 600 $[M + NH_4]^+$, 583 $[M + H]^+$.

The olefinic protons H-7 and H-8 showed vicinal coupling of 15 Hz suggesting an *E*-geometry of this olefin. The irradiation of the H-10 and H-13 methylene signals at δ_H 2.11 and δ_H 2.04 revealed a vicinal coupling of 10 Hz between H-11 and H-12 indicating a *Z*-geometry of this olefin.

The relative stereochemistry at C-4 and C-6 was elucidated by NOEDS spectroscopy of **1a**. Irradiations of H-6 showed a NOE to H_β-5 (δ_H 2.39) and to the H-7 and H-8; H_α-5 (δ_H 2.11) to H-4, H-7, and H-8 indicating that H-4 and H-6 are on the opposite face of the molecule (Figure 1).

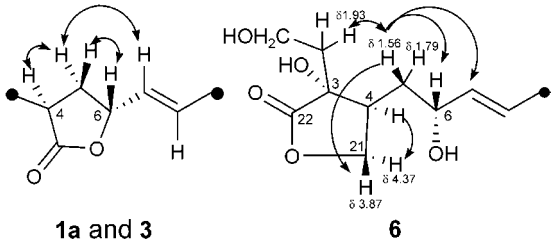
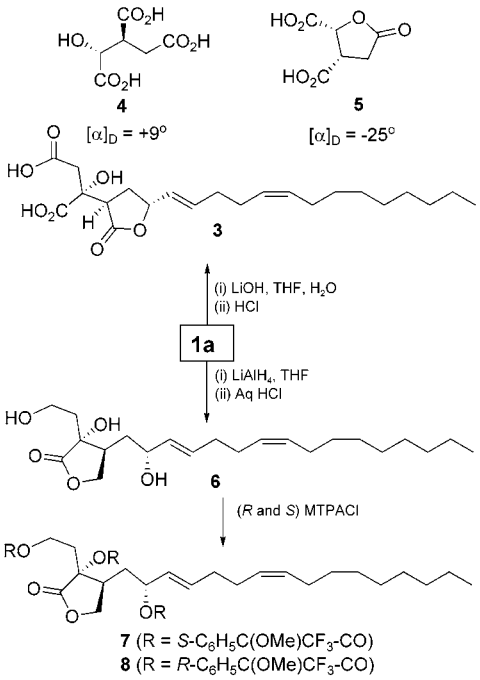


Figure 1. Structural fragments of **1a**, **3**, and **6** in acetone-d_6 arrows showing NOE.

Basic hydrolysis (LiOH) of **1a** followed by extraction with ethyl acetate and RPHPLC provided compound **3** (72% yield, ESIMS (*m*/*z*) 433 $[M + Na]^+$, $[\alpha]^{25}_D$ +9.4 (*c* 0.85, CH_3OH) (Scheme 1). An amide-80 (TSKgel, TosoHaas, normal phase mode) chromatography of the water-soluble portion of the reaction mixture yielded isocitric acid **4** ($[\alpha]_D$ +9) and

Scheme 1



corresponding γ-lactone **5** ($[\alpha]_D$ −25.2) leading to its identification as *threo* (2*R*,3*S*)-isocitric acid.[7] Compound **3** provided NOEs similar to those of **1a**, further supporting the relative stereochemistry at C-4 and C-6.

$LiAlH_4$ reduction of **1a** provided the reduced γ-lactone **6**, which was reacted with excess (*R* and *S*)-2,2-methoxy-trifluoromethyl-benzoyl chloride (Mosher chloride) using standard reaction conditions and furnished *S*- and *R*-triesters **7** and **8**. Full assignment of the ^1H NMR spectra of both compounds followed by calculation of $\Delta\delta(S\text{-}R)$ (Figure 2)

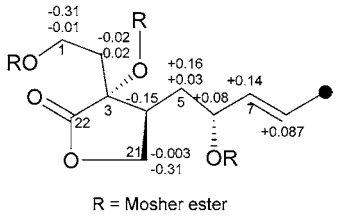


Figure 2. $\Delta\delta$ (*S*-*R*) of Mosher esters (**7** and **8**).

led to the absolute stereochemistry of citrafungin A (**1a**) as 3*R*,4*R*,6*R*,2′*R*,3′*S*.

Citrafungin B (2a). ESIMS analysis of **2a** showed a pseudomolecular ion at *m*/*z* 583 $[M + H]^+$ and a molecular formula of $C_{28}H_{38}O_{13}$. Like **1a**, methylation with diazomethane afforded tetramethyl ester **2b**. The ^1H and ^{13}C NMR spectral comparison of **2a** with **1a** indicated that they only differed by an olefin, which was assigned at C14−C15 by the COSY correlations of the downfield shifted triplet H_2-13 with H-12 and H-14. Like citrafungin A, the NMR spectrum of citrafungin B was fully assigned by using HSQC and HMBC experiments (Table 1). The decoupling of H_2-13 and H_2-16 provided a *J* value of 10 Hz between H-14 and H-15 and accordingly established a *Z*-geometry to the new olefin.

CJ-15,183 a metabolite of *Aspergillus aculeatus*, which is identical to citrafungin B except for the difference of the ester linkage at C-22, was reported by Watanabe et al.[8] in 2001 as an inhibitor of rat liver squalene synthase (IC_{50} 5.2 μM) and *Candida albicans* squalene synthase (IC_{50} 8.6 μM). The ^1H and ^{13}C NMR spectra of citrafungin B recorded in CD_3OD (see Supporting Information) was identical to the spectral data published for CJ-15,183. The establishment of the ester linkage of CJ-15,183 relied on SINEPT-based distinction of C-1 and C-22, which could be potentially erroneous as a result of the proximal shifts of H-4 and one of H_2 at C-2 as observed with **1a** (see Supporting Information). Therefore, CJ-15,183 and citrafungin B may potentially be identical.

(7) Although optical activity of the isolated isocitric acid is lower than the reported values for (1*R*,2*S*)-isocitric acid, $[\alpha]_D = +30.6$, corresponding γ-lactone $[\alpha]_D = -61.1$, this is the only isomer of the four isocitric acids that showed a positive optical rotation of the acid and negative value of corresponding γ-lactone; Chapman and Hall database.

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Table 2. GGTase I Inhibitory Activities of Citrafungins **1**−**3**

	GGTase I inhibition (IC_{50}, μM)				
compd	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*A. fumigatus*	human
1a	10.0	5.0	3.0	2.5	0.46
2a	15.0	5.0	3.0	2.6	0.90
3		15.0			

Table 3. Antifungal Activities of Citrafungins **1**−**3**

	MIC (μM)			
compd	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*A. fumigatus*
1a	0.43	0.43	6.8	13.7
2a	13.7	6.9	55.0	55.0
3		>312		

Biological Activities. Citrafungins A (**1a**), B (**2a**), methyl esters (**1b** and **2b**), and the hydrolytic product **3** were evaluated against recombinant *S. cerevisiae* GGTase I and purified enzymes from *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. Ras-CVIL was used as a substrate using a general procedure described earlier.[9] To determine selectivity, the more active of these compounds were tested for their activity against recombinant human GGTase I (Table 2) and rScFTase.[10] Details of the purification and assay procedures of many of these enzymes have been described previously.[4,11] Citrafungin A inhibited the GGTase I of *S. cerevisiae*, *C. albicans*, *C. neoformans*, and *A. fumigatus* with IC_{50} values of 10, 5.0, 3.0, and 2.5 μM, respectively (Table 2). Citrafungin B showed essentially similar activity and indicated that the additional olefin does not have significant effect on the activity (Table 2). The hydrolysis product **3** was 3-fold less active against *C. albicans* GGTase I and showed an IC_{50} of 15 μM (it was not tested against other enzymes). Both trimethyl esters were completely inactive against all GGTase I enzymes (IC_{50} >200 μM) indicating the critical importance of the free carboxyl groups.

Citrafungin A (**1a**) exhibited moderate to potent antifungal activity and showed minimum inhibitory concentration (MIC) values of 0.43, 0.43, 6.8, and 13.7 μM against *S. cerevisiae*, *C. albicans*, *C. neoformans*, and *A. fumigatus*, respectively. While the presence of the extra olefin in citrafungin B had no effect on GGTase I activity, it had significant effect in its ability to inhibit the fungal growth leading to 4- to 32-fold reductions in MIC values (Table 3). The MIC value of compound **3** against *C. albicans* was >312 μM. Citrafungin A showed a level of antifungal activity against *S. cerevisiae* and *C. albicans* that could not be easily explained by the mere inhibition of GGTase I, suggesting multiple modes of action. The antifungal activity of citrafungins was unlikely due to inhibition of ScFTase due to poor inhibition (IC_{50} 103 and 174 μM). These compounds did not inhibit sphingolipid biosynthesis despite having structural resemblance to sphingolipid inhibitory antifungal agents, viridiofungins.[12] It is possible that these compounds are inhibitors of squalene synthase just like CJ-15,186 (e.g., IC_{50} 8.6 μM, vide infra), which contributes to the antifungal activity. They were potent inhibitors of recombinant human GGTase I exhibiting IC_{50} values of 0.46 and 0.90 μM, respectively, indicating lack of selectivity.

Unfortunately, it was subsequently found that the null mutants of GGTase I β-subunit (*Cacdc43*) of *C. albicans*, the major human pathogen, were morphologically abnormal but viable, and the validity of the target became questionable. It was hypothesized that the inhibition of GGTase I activity causes buildup of Rho1p and Cdc42p, which enables them as substrates of farnesyltransferase, another prenylation enzyme, resulting in morphological changes but viability of *C. albicans*.[11] However, this hypothesis remains to be fully validated.

In summary, the isolation and GGTase I and antifungal activity of citrafungin A and B, two new polyketides of the alkyl citrate family, have been described. These inhibitors are only the third reported examples[13] of natural product inhibitors of fungal GGTase I. The other two are marine natural products polyacetylenic compound (IC_{50} range 1.9−7.3 μM)[13a] and massadine (IC_{50} 3.9 μM).[13b]

Acknowledgment. We would like to thank Mr. Gerald E. Bills of St. Marys, WV for collection of the cow dung from Alaska for isolation of producing organism.

Supporting Information Available: Copies of ^1H, ^{13}C NMR, HSQC, COSY, HMQC, selected HMBC and SINEPT spectra of **1a**, **1b**, **3**, **6**, **7**, **8** and Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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