Notes

Circumdatin A, B, and C: Three New Benzodiazepine Alkaloids Isolated from a Culture of the Fungus *Aspergillus ochraceus*

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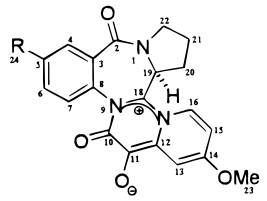
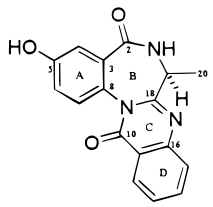
Three new benzodiazepine alkaloids, circumdatin A (**1**), B (**2**), and C (**3**) have been isolated from a terrestrial isolate of the fungus *Aspergillus ochraceus*. Recently developed ^{1}H-detected INEPT2- and HMBC-INADEQUATE NMR experiments were used to solve the structures of the two zwitterionic benzodiazepines **1** and **2**.

Benzodiazepines constitute a widely prescribed class of psychoactive drugs,[1] and numerous compounds have been synthesized and tested. The majority of naturally occurring benzodiazepines is reported from filamentous fungi and actinomycetes of the genera *Penicillium*, *Aspergillus*, and *Streptomyces*. Benzodiazepines from *Aspergillus* sp. include aszonalenin from *A. zonatus* and another *Aspergillus* sp.,[2] a benzodiazepinedione from *A. flavipes*,[3] and from *A. alliaceus* the asperlicins,[4] of which asperlicin is used for treatment of gastrointestinal and CNS disorders.[5] We wish to report the UV-guided isolation and structure elucidation of further three benzodiazepines from this genus.

The development of standardized methods for identification of secondary metabolites not only constitutes a powerful tool in chemotaxonomy,[6] but also in discovery of new metabolites in fungal extracts. The crude extract of a small-scale fermentation of a terrestrial isolate of *A. ochraceus* was subjected to analytical HPLC coupled with photodiode array detection. Knowledge of retention times and appearance of the UV spectra of known compounds from the genus *Aspergillus* served to confirm the presence of several known compounds: penicillic acid, 4-hydroxymellein, mellein, viomellein, vioxanthin, xanthomegnin, and various compounds previously unknown from this group of fungi. UV-guided separation of the extract obtained from a large-scale fermentation led to the isolation of three new metabolites. These have now been classified as benzodiazepines and named circumdatin A (**1**), B (**2**), and C (**3**), as the fungal species is taxonomically placed in *Aspergillus* subgenus *Circumdati* section *Circumdati* (formerly the *Aspergillus ochraceus* group).



1: R = OMe
2: R = H



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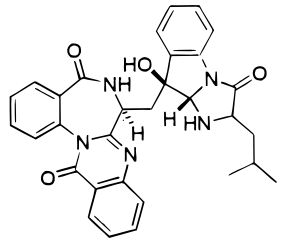

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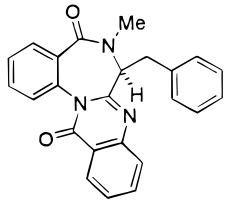

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Spectroscopic analysis indicated that the structures of **1** and **2** were identical except for the substitution of a methoxy group in **1** with hydrogen in **2**, and only the structure elucidation of **1** will be discussed in detail. The molecular formula of **1**, $C_{21}H_{19}N_3O_5$, was established by NMR analysis in combination with HREIMS (M^+, 393.1333, $C_{21}H_{19}N_3O_5$, Δ +0.9 mmu). The ^{1}H and ^{13}C NMR spectra implied the presence of two 1,2,4-trisubstituted aromatic rings, two methoxy, one methine, three methylene, and nine quaternary, sp^2-hybridized carbon atoms. The aliphatic CH and CH_2 groups were combined to a $CH(CH_2)_3$ moiety by COSY correlations, and comparison with literature data suggested the presence of a proline ring. Owing to the inability of the HMBC technique to discriminate between two-, three-, and, in some cases, four-bond correlations, the experiment provided, in this case, insufficient information for unambiguous structure assignment. To solve the problem, recourse was taken to recently developed ^{1}H-detected INEPT2- and HMBC-INADEQUATE NMR experiments (see Figure 1

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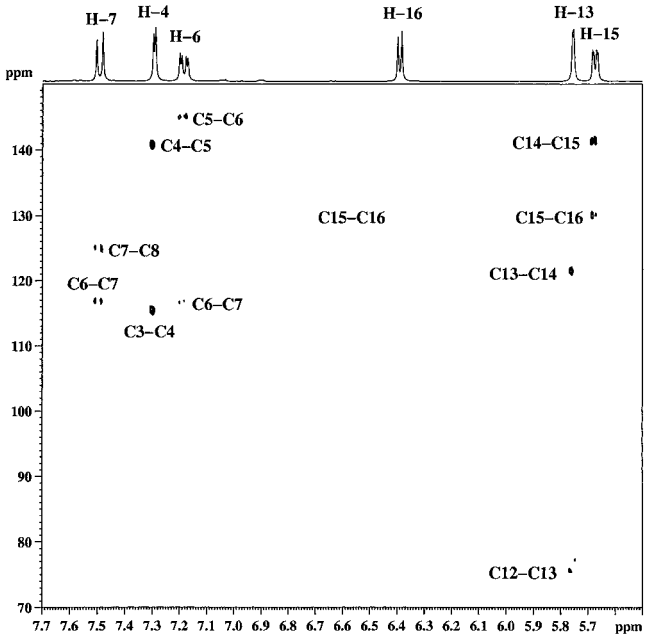

Figure 1. INEPT2-INADEQUATE spectrum for circumdatin A (**1**).

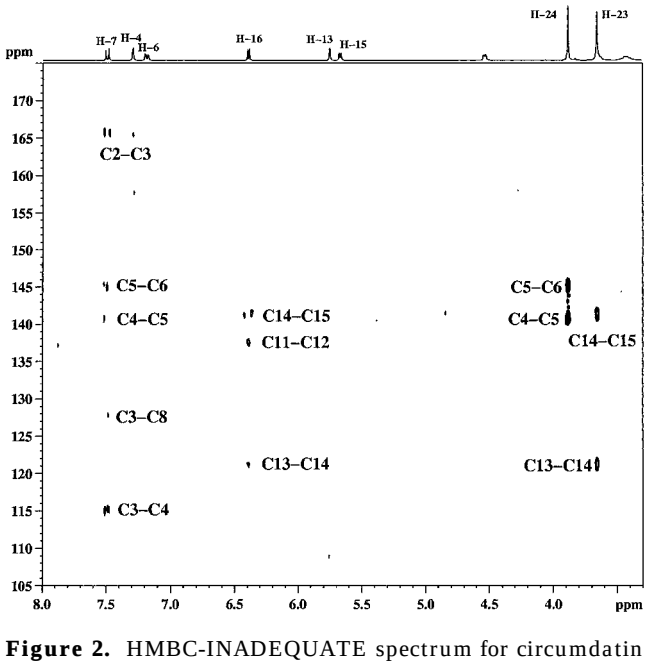

Figure 2. HMBC-INADEQUATE spectrum for circumdatin A (**1**).

and Figure 2).[7,8] The INEPT2-INADEQUATE experiment allows detection of coupling between pairs of carbon atoms of which at least one is protonated, whereas the HMBC-INADEQUATE technique correlates pairs of *J*-coupled carbon atoms of which at least one exhibits long-range coupling to a proton. Thus, the $^1J_{CC}$-INEPT2-INADEQUATE experiments optimized for a one-bond

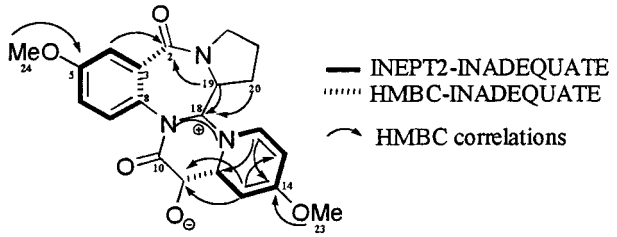

Figure 3. Connections determined from INADEQUATE experiments and diagnostic HMBC correlations for circumdatin A (**1**).

$^{13}C-^{13}C$ coupling constant of $^1J_{CC} = 55$ Hz established the C3−C4, C4−C5, C5−C6, C6−C7, C7−C8, C12−C13, C13−C14, C14−C15, and C15−C16 connections in **1** (Figure 3 bold bonds). Confirmatory information was obtained by the $^1J_{CC}$-HMBC-INADEQUATE ($^1J_{CC} = 55$ Hz) experiment, which furthermore provided evidence for connections between C2 and C3, C3 and C8, and C11 and C12 (Figure 3, broken bonds). HMBC correlations linked the anthranilic acid to the proline moiety (from C2 to H19), placed OMe23 on C14 and OMe24 on C5, and supported the substructures resulting from the INADEQUATE experiments, e.g. from C2 to H4, from C10 and C11 to H13, from C12 and C14 to H16, and from C15 to H13 (Figure 3). The substructure represented by C11 to C16 was assumed to be attached to N9 and C18 through carbonyl C10 and N17 as shown in Figure 3, as the structural fragment carbonyl-C10−N9−C18−N17 is observed in several naturally occurring benzodiazepines[9,10] and supported by biosynthetic considerations. The remaining oxygen was placed on C11 in accordance with the low field carbon shift (158.1 ppm). The presence of the heterocyclic ring system conforms with a characteristic, large, one-bond $^1H-^{13}C$-coupling constant, $^1J_{CH} = 199$ Hz, observed for CH16.[11] Thus the structure of **1** was determined to be a zwitterion as shown in Figure 3.

Analyzing the hydrolysis products of **1** by ^1H NMR and ESIMS identified major degradation products as methoxyanthranilic acid (*m/z* at 190 $[M + Na]^+$), proline (*m/z* at 138 $[M + Na]^+$), and methoxyanthranilic acid coupled with proline (*m/z* at 269 $[M + Na]^+$). The absolute configuration of the proline moiety of **1** was determined by reaction of the hydrolysis products with Marfey's reagent (N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide). The resulting product contained L-proline as shown by HPLC comparison with references of known configuration.

Compound **3** revealed itself also to be derived from anthranilic acid by the characteristic δ_H value at 8.24 ppm (H12) and the carbon shifts for C11 and C16 (123 and 148 ppm, respectively).,[8,1213] ESIMS (*m/z* at 308 $[M + H]^+$, 330 $[M + Na]^+$, and 306 $[M - H]^-$), NMR analysis, and the pseudomolecular ion peak (MH^+) observed in the HRFABMS at *m/z* = 308.1036 ($C_{17}H_{14}N_3O_3$, Δ −0.1 mmu) established the molecular formula of **3**, $C_{17}H_{13}N_3O_3$. The ^1H, ^{13}C NMR, APT, and COSY experiments revealed **3**

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(8) F2- and F1-frequencies in the INADEQUATE-experiments represent ^1H-chemical shifts and ^{13}C double quantum frequencies (sum of ^{13}C chemical shifts relative to the ^{13}C carrier frequency offset (132 ppm)) for the *J*-coupled $^{13}C-^{13}C$−pair, respectively.

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Table 1. ^{1}H and ^{13}C NMR Data for Circumdatin A (1), B (2), and C (3)

	1 (DMSO-d_6)		**2** (DMSO-d_6)		**3** (MeOH-d_4)	
no.	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	163.0		163.2		170.2	
3	133.1		132.5[a]		133.3	
4	112.4	7.26 (d, 2.9)	130.5[b]	7.82 (ddd, 7.0, 1.5, 0.5)	116.2	7.22 (d, 3.0)
5	158.8		129.0[b]	7.58 (ddd, 8, 8, 2)[c]	160.6	
6	117.0	7.19 (dd, 9.2, 2.9)	128.7[b]	7.64 (ddd, 7.5, 7.5, 2.0)[c]	120.2	7.05 (dd, 9, 3)
7	130.2	7.49 (d, 9.2)	129.1[b]	7.57 (dd, 8.0, 2.0)	131.1	7.43 (d, 9.0)
8	125.3		131.9[a]		125.9	
10	161.9		161.9		163.9	
11	158.1		158.2		122.6	
12	109.8		110.0		128.1	8.24 (ddd, 8.0, 1.0, 0.5)
13	95.2	5.73 (br d, 1.8)	95.1	5.74 (br d, 1.8)	128.7	7.56 (ddd, 8.0, 7.0, 1.0)
14	156.6		156.7		136.2	7.84 (ddd, 8, 8, 2)
15	115.3	5.68 (dd, 5.9, 2.0)	115.4	5.70 (dd, 5.9, 1.9)	128.8	7.76 (ddd, 8.0, 1.0, 0.5)
16	145.1	6.40 (dd, 5.9, 0.4)	145.2	6.40 (d, 5.9)	148.0	
18	157.2		157.3		157.9	
19	58.0	4.55 (dd, 6.6, 1.8)	58.0	4.53 (dd, 6.7, 2.0)	51.4	4.45 (q, 7.0)
20	26.2	2.66 (m)	26.2	2.66 (m)	15.4	1.66 (d, 7.0)
		2.0 (m)[d]		2.0 (m)[d]		
21	23.2	2.0 (m)[d]	23.3	2.0 (m)[d]		
		1.93 (m)		1.94 (m)		
22	46.2	3.6 (m)[e]	46.2	3.6 (m)[e]		
		3.39 (m)		3.41 (m)		
23	55.0	3.64 (s)	55.1	3.65 (s)		
24	55.7	3.86 (s)				

[a] Signals are interchangeable. [b] Signals are interchangeable. [c] Signals are interchangeable. [d] Average value of unresolved signals from two protons. [e] Signal is overlapping with methoxy signal (C23).

to contain seven aromatic protons attached to 1,2-disubstituted and 1,2,4-trisubstituted benzene moieties, a CH−CH$_3$ group, and eight quarternary sp^2 carbon atoms. HMBC correlations established the substructures C18−C19−C20, C3 through C8, and C10 through C16, where the low field δ_C values of C5 and C18 indicated the presence of an OH group attached to C5 and a C−N double bond in position 18. Several structures composed of one 4-hydroxyanthranilic acid, one anthranilic acid, and one alanine moiety were possible, differing only in the position of the hydroxy group on an A−B ring system or a C−D ring system. Comparative analysis of δ_C values from A−B and C−D ring systems of asperlicin (**4**)[9] and benzomalvin A (**5**)[8] with those of **3**, demanded ring system C−D to be unsubstituted as in **4** and **5**, leading to the structure of **3**.[14] The incorporation of a 5-hydroxyanthranilic acid rather than a 4-hydroxyanthranilic acid was substantiated from ^{13}C chemical shift calculations.[15] Hydrolysis of **3** and analysis of the resulting amino acids by chiral TLC identified the alanine substructure as derived from L-alanine.

The seven-membered benzodiazepine ring is part of a cyclic dipeptide which involves anthranilic acid and an α−amino acid.[16] Compounds **1** and **2** consist of anthranilic acid and L-proline and represent the first naturally occurring zwitterionic benzodiazepines. L-Alanine is part of the seven-membered ring of **3**. Biosynthetic experiments with {^{13}C-carboxy}anthranilic acid are warranted in order to determine if anthranilic acid is a precursor for the pyridine skeleton in **1** and **2**.

A. ochraceus very consistently produces circumdatin A, B, and C,[17] penicillic acid, 4-hydroxymellein, mellein, viomellein, vioxanthin, and xanthomegnin. Furthermore, some strains yield ochratoxin A. *A. ostianus* and *A. petrakii* both produce circumdatin A and B,[18] but no other fungi belonging to the section *Circumdati* express the circumdatins. Therefore the circumdatins are good chemotaxonomic markers for the three species. Circumdatin B (**2**) was tested in the NCI's 60 cancer cell line panel but was not found active.

Experimental Section

General. NMR spectra were recorded in DMSO-d_6 or MeOH-d_4 solution at 400 or 500 MHz, and 100.6 or 125.7 MHz for ^{1}H and ^{13}C NMR, respectively. The HMBC and the ^{1}H-detected INEPT2- and HMBC-INADEQUATE NMR experiments of **1** were recorded on a Bruker DRX400 instrument equipped with a selective inverse 2.5 mm probe head. The pulse sequences were essentially as described by Meissner et al.[7] The NMR sample for the INADEQUATE experiments was prepared by dissolving **1** (ca. 10 mg) in 50 μL of DMSO-d_6. Marfey's reagent (N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) was purchased from Sigma.

Collection and Fermentation. The fungus *Aspergillus ochraceus* (IBT 12704) was isolated from sterilized milo sorghum seeds buried in the soil for 1−4 months at Sevilleta National Wildlife Refuge, Socorro County, New Mexico. Subsequently the seeds were surface sterilized, incubated on potato dextrose agar for one week, and isolated. The fungus was cultivated in larger

(14) See Supporting Information Available.

(15) Advanced Chemistry Development (ACD version 2.51), Advanced Chemistry Department Inc.: 133 Richmond St. West, Suite 605, Toronto, ON M5H 2L3 Canada, 1994−1997.

(16) Roos, W. *Alkaloids (Academic Press)* **1990**, *39*, 63−97.

(17) Following isolates were examined: NRRL 5221, NRRL 3174, NRRL 5175, NRRL 5063, NRRL 5228, NRRL 2864, NRRL 5220, NRRL 402, NRRL 410, NRRL 5222, NRRL 6319, IBT 10136, CBS 263.67, CBS 108.08, CBS 420.80, CBS 624.78, CBS 748.7, RMF 7884, RMF 7943. NRRL = Northern Regional Research Laboratory, Peoria, IL. IBT = Department of Biotechnology, Technical University of Denmark. CBS = Centraalbureau voor Schimmelscultures, Baarn, Holland. RMF = Rocky Mountain Fungi, Department of Botany, University of Wyoming, Laramie.

(18) Following strains were examined. *A. ostianus*: CBS 103.07, CBS 101.23, CBS 311.80, NRRL 422, IMI 177964b, IMI 348937, CCRC 32240. *A. petrakii*: CBS 105.57. For CBS and NRRL see ref 17. IMI = Commonwealth Agricultural Bureau International Mycological Institute, Kew, Great Britain. CCRC = Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan.

scale in Petri dishes with Czapek yeast extract agar (total 900 mL) with an incubation time of 22 days at 25 °C.

Extraction and Separation. The culture and media were extracted with EtOAc, giving 852 mg of crude extract. A portion of the extract was subjected to purification by liquid chromatography on a Lobar LiChroprep Si 60 (40−63 μm) size B column from Merck (EtOAc:EtOH:heptane 80:2.5:17.5, 270 nm, 6 mL/min) giving eight fractions. One of the fractions (52 mg) was further purified on a HPLC column (LiChroCART LiChrospher Si 60 (10 μm) 250−10, EtOAc:EtOH:heptane 80:2.5:17.5, 270 nm, 6 mL/min), giving pure **1** (33 mg). A second fraction (22 mg) gave pure **2** (14 mg) and a third fraction gave **3** (4 mg) on separation with the same HPLC column and solvent system. Compound **3** was separated again (HPLC, LiChroCART LiChrospher Si 60 (10 μm) 250−10, EtOAc:EtOH:hexanes 60:5:35, 270 nm, 6 mL/min), giving an almost pure fraction (1.6 mg). Unfortunately **2** was decomposed in $CDCl_3$ and had to be reisolated from the crude extract (6 mg).

Hydrolysis of Circumdatin A (1). **1** (1.7 mg) was hydrolyzed with 1 mL 6 N HCl for ca. 18 h at 115 °C. The hydrolysis products were treated with Marfey's reagent (N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) according to the directions given by Marfey.[19] The resulting diastereomers were analyzed by HPLC using the method described except for the flow rate where 1 mL/min was used instead of the recommended 2 mL/min. L-proline was identified by comparison (t_R = 25.9 min) with the Marfey derivatives prepared from L-proline (t_R = 25.2 min) and D,L-proline (t_R = 28.0 min and t_R = 25.4 min), respectively.

Another 10 mg of **1** was hydrolyzed with 4 mL of 6 N HCl for ca. 18 h at 115 °C. After reaction, the products were cooled to room temperature, a small amount of water was added, and the sample was freeze-dried. After repeating the treatment with water and lyophilization, the remnants were dissolved in MeOH, subjected to centrifugation to remove a dark precipitate, and concentrated in vacuo. The resulting amino acids were separated on a Prep Nova-Pak HR C18 (6 μm, 60 Å) 25 × 100 mm from Waters (MeCN:H_2O 1:9, 10 mL/min). 5-Methoxyanthranilic acid was identified from a ESIMS (*m*/*z* at 190 $[M + Na]^+$) and the ^1H NMR spectrum in MeOH-d_4: δ 7.41 (d, J = 3.1 Hz, H3), 6.98 (dd, J = 9.0, 3.1 Hz, H5), 6.79 (d, J = 9.0, H6), 3.77 (s, Me). Because of the small amount available from the hydrolysis experiment, the ^{13}C NMR spectra only revealed the position of the protonated carbon atoms: δ 121.7, 117.9, 113.0, 54.3 (Me). The presence of proline was confirmed by a ESIMS *m*/*z* peak at 138 $[M + Na]^+$ and by comparison of the ^1H NMR spectrum with an authentic sample: DMSO-d_6 δ 3.97 (1H, bt, J = 7.3), 3.21 (1H, m), 3.10 (1H, m), 2.16 (1H, m), 1.95 (1H, m), 1.82 (2H, m).

Hydrolysis of Circumdatin C (3). About 0.8 mg of **3** was hydrolyzed with 0.5 mL of 6 N HCl for ca. 4 h at 120 °C. The products were cooled to room temperature, water was added, and the sample was freeze-dried. Chiral TLC analysis (Chiralplates, Macherey-Nagel) with acetone:MeOH:H_2O (10:2:2) against authentic D and L standards (Aldrich, R_f = 0.53 and 0.57, respectively) showed that alanine has the L configuration (R_f = 0.57).

Circumdatin A (1). Red-orange solid; $[\alpha]^{22}_D$ −421° (*c* 0.018, EtOH); UV (EtOH) λ_{max} nm (log ϵ) 357 (3.30) 290sh (3.33), 238sh (3.84); CD, λ_{ext} (*c* 0.006, EtOH) ($\Delta\epsilon$) 298 (−4.32), 262 (5.42), 220 (−2.06), 204 (8.43); EIMS *m*/*z* 393; $C_{21}H_{19}N_3O_5$, ^1H and ^{13}C NMR see Table 1.

Circumdatin B (2). Red-orange solid; $[\alpha]^{22}_D$ −163° (*c* 0.040, EtOH); UV (EtOH) λ_{max} nm (log ϵ) 358 (2.76), 284sh (2.73); CD, λ_{ext} (*c* 0.040, EtOH) ($\Delta\epsilon$) 283 (−14.01), 244 (19.01), 232 (10.51), 219 (26.26); EIMS *m*/*z* 363; $C_{20}H_{17}N_3O_4$, ^1H and ^{13}C NMR see Table 1.

Circumdatin C (3). Solid; $[\alpha]^{22}_D$ −75° (*c* 0.160, MeOH); UV (MeOH) λ_{max} (log ϵ) 312 (3.50), 272 (3.85), 229 (4.31); CD, λ_{ext} (*c* 1.35 10^{-3}, MeOH) ($\Delta\epsilon$) 307 (−4.31), 263 (8.04), 233 (−22.86), 213 (29.76); ESIMS *m*/*z* 308 $[M + H]^+$, $C_{17}H_{14}N_3O_3$, ^1H and ^{13}C NMR see Table 1. Following HMBC correlations were observed: from C3 to H7, from C4 to H6, from C5 to H7, from C7 to H4, from C8 to H6, from C10 to H12, from C11 to H13 and H15, from C14 to H12, from C16 to H12 and H14, from C18 to H19 and H20, and from C19 to H20.

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Supporting Information Available: Copies of ^1H and ^{13}C NMR spectra of compounds **1**, **2**, and **3**; INEPT2- and HMBC-INADEQUATE spectra of **1**; and material supporting the structure elucidation of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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