New Triterpenoid Alkaloid Cholinesterase Inhibitors from *Buxus hyrcana*

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Three new triterpenoid alkaloids, (+)-*N*-benzoylbuxahyrcanine [(20*S*)-3β-benzoylamino-20-dimethylaminobux-9(11)-ene-10α-ol] (**1**), (+)-*N*-tigloylbuxahyrcanine [(20*S*)-20-(dimethylamino)-3β-(2′-methyl-2′-butenoylamino)bux-9(11)-en-10α-ol] (**2**), and (+)-*N*-isobutyroylbuxahyrcanine [(20*S*)-20-(dimethylamino)-3β-(2′-methylpropanoyl)bux-9(11)-en-10α-ol] (**3**), have been isolated from the leaf extracts of *Buxus hyrcana* collected in Iran. Their structures were determined using spectroscopic methods. The structures of compounds **1** and **2** were unambiguously confirmed by single-crystal X-ray diffraction techniques. Compounds **1**−**3** were evaluated for their acetylcholinesterase and butyrylcholinesterase inhibitory activities, and compound **2** was found to be active against both enzymes.

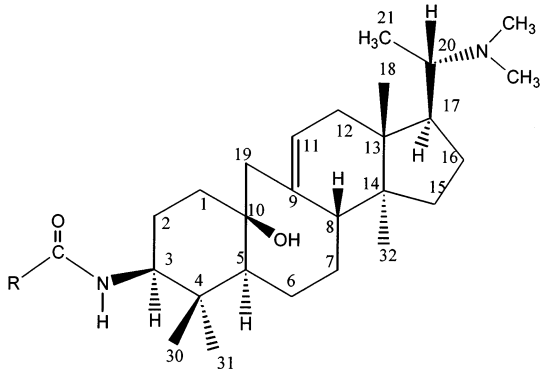
The *Buxus* plants are a rich source of triterpenoid alkaloids. Previous phytochemical studies on *Buxus* species have resulted in the isolation of more than 200 such compounds.[1] In the indigenous system of medicine, extracts of genus *Buxus* have been used for the treatment of various disorders such as malaria, rheumatism, and skin infections.[2] Continuing our studies on alkaloids of various *Buxus* species, work was started on the leaves of *Buxus hyrcana* Pojark. (Buxaceae). Seven compounds have been previously reported from this species.[3−6] Continuing our studies on *B. hyrcana*, we report here the isolation, structure elucidation, and cholinesterase inhibitory activities of three new alkaloids, (+)-*N*-benzoylbuxahyrcanine (**1**), (+)-*N*-tigloylbuxahyrcanine (**2**), and (+)-*N*-isobutyroylbuxahyrcanine (**3**). The structures of these compounds were deduced from their spectral and crystallographic data.

Results and Discussion

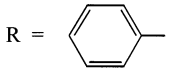
The crude alkaloids were isolated from the methanolic extract of the leaves of *B. hyrcana* by extraction at different pH values. The fractions obtained at pH 7.0 were combined and evaporated to a gum, which was then subjected to column chromatography to yield alkaloids **1**−**3**.

(+)-*N*-Benzoylbuxahyrcanine (**1**) was isolated as a colorless amorphous material, which exhibited a peak at *m/z* 506 (HREIMS *m/z* 506.3812, $C_{33}H_{50}N_2O_2$, calcd 506.3872). The base peak at *m/z* 72.0814 ($C_4H_{10}N$, calcd 72.0813) indicated the presence of an *N*,*N*-dimethylamino group at C-20.[7] Another peak at *m/z* 105.0380 (C_7H_5O, calcd 105.0340) was due to a benzoyl moiety. The UV spectrum showed absorption at 223 nm, characteristic of a secondary benzamide chromophore.[8] The IR spectrum displayed intense bands at 3618 (OH), 3409 (NH, amide), 1660 (C=O, amide), and 1602 (C=C) cm^{-1}.

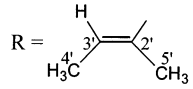
The ^1H NMR ($CDCl_3$) spectrum of **1** displayed four 3H singlets at δ 0.66, 0.96, 0.98, and 0.72 for the C-18, C-30, C-31, and C-32 tertiary methyl groups, respectively, while the secondary methyl resonated as a doublet at δ 0.82 ($J_{20,21}$ = 6.5 Hz). A 6H broad singlet at δ 2.17 was assigned to the N_b-dimethyl protons. In turn, the proton resonating at



1 R =



2 R =



3 R = $(CH_3)CH$—

δ 3.91 (ddd, $J_{3\alpha,2\beta}$ = 12.5 Hz, $J_{3\alpha,NH}$ = 9.5 Hz, $J_{3\alpha,2\alpha}$ = 3.5 Hz) was assigned to the C-3 methine proton, while the amide NH resonated as a doublet at δ 6.05 ($J_{NH,3\alpha}$ = 9.5 Hz). A doublet at δ 2.22 ($J_{19\alpha,\ 19\beta}$ = 15.7 Hz) was due to H-19α, while H-19β appeared as a multiplet at δ 2.58. Finally, a downfield multiplet at δ 5.30 was ascribed to the C-11 olefinic proton, while the aromatic protons of the benzoyl moiety appeared as three sets of multiplets centered at δ 7.39, 7.45, and 7.72.

The ^{13}C NMR spectra of **1** showed resonances for all 33 carbons including seven CH_3, eight CH_2, eleven CH, and seven quaternary carbons. The olefinic C-11 resonance appeared at δ 124.9, while its adjacent quaternary carbon resonated at δ 136.5. The C-3 signal was observed at δ 56.9, and its downfield shift suggested that a benzamide chromophore may be attached to this carbon. A quaternary carbon resonating at δ 73.0 suggested that **1** contains a tertiary OH group, whose probable position is C-10. Com-

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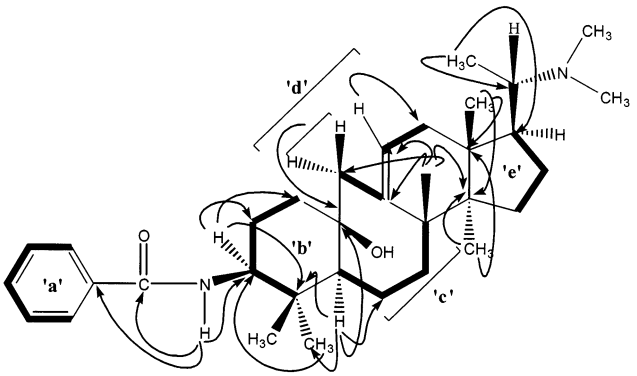

Figure 1. Important HMBC correlations and ^{1}H spin systems (bold lines) in **1**.

Table 1. ^{13}C and ^{1}H NMR Chemical Shift Assignments of **1**

carbon	δ ^{13}C	δ ^{1}H (*J*, Hz)
1	26.9	1.45 m
2	25.4	1.65 m, 1.9 m
3	56.9	3.9 ddd (12.5, 9.5, 3.5)
4	38.9	
5	56.1	1.2 dd (12.5, 4.0)
6	27.1	1.52 m
7	28.2	1.3 m, 1.8 m
8	49.3	2.1 m
9	136.5	
10	73.00	
11	124.9	5.3 m
12	38.1	1.9 m
13	43.1	
14	49.7	
15	32.9	1.37 m
16	42.0	1.55 m, 1.65 m
17	49.2	1.8 m
18	16.0	0.66 s
19	52.7	2.22 d (15.9), 2.52 m
20	61.6	2.49 m
21	9.6	0.82 d (6.5)
30	16.2	0.96 s
31	27.4	0.98 s
32	16.2	0.72 s
$N(CH_3)_2$	39.9	2.17 s
1′	167.9	
2′	135.3	
3′	126.8	7.76 m
4′	128.5	7.40 m
5′	131.2	7.45 m
6′	128.5	7.40 m
7′	126.8	7.76 m

plete ^{13}C NMR chemical shift assignments and ^{1}H−^{13}C direct one-bond connectivities of all protonated carbon atoms of **1**, as determined by the HMQC spectrum, are presented in Table 1.

The COSY-45° and HOHAHA (20, 60, and 100 ms) NMR spectra of **1** revealed the presence of five major spin systems (Figure 1). The cross-peaks in the HMBC spectrum were used to join the individual spin systems to obtain structure **1**. The NH (δ 6.05) and H-3 (δ 3.90) showed HMBC cross-peaks with the carbonyl carbon (δ 167.9), showing that the benzamidic group is attached to C-3. H-3 (δ 3.90) and H-5 (δ 1.2) showed long-range correlations with C-4 (δ 38.9), while H-5 also showed HMBC interactions with C-10 (δ 73.0). This information suggested that spin systems b and e are connected to each other through C-4. The H-19α and β signals (δ 2.22 and 2.58) showed long-range interactions with the C-10 (δ 73.0) and C-9 (δ 136.5) resonances, thereby indicating the presence of the OH group at C-10. Important HMBC interactions are shown in Figure 1.

A subsequent single-crystal X-ray diffraction analysis (Figure 2) unambiguously confirmed the structure **1** for this compound. The structure was solved by the direct methods[9] and expanded by Fourier techniques.[10] The figures were plotted with the aid of ORTEPII.[11] Most of the non-hydrogen atoms were refined anisotropically. A few atoms were found to be disordered. The tertiary OH group present at C-10 was found to be β oriented, showing that the A−B ring junction was trans. Phenyl rings were constrained as regular hexagons with C−C 1.39 Å and C−C−C 120°. Hydrogen atoms were included at geometrically idealized positions and were not refined. The refinement converged at $R_1 = 0.068$ and $wR_2 = 0.177$ with a goodness of fit of 1.02 for 6695 reflections with $F_o > 2\sigma(F_o)$. The largest peak on the final difference map was 0.26 e/Å^3. The bond lengths and bond angles are within the usual ranges. Figure 2 shows a computer-generated perspective of the final X-ray model of **1**. The NOESY spectrum supported the stereochemistry determined by X-ray, in which cross-peaks were observed between H-19α (δ 2.2), H-5α (δ 1.2), and H_3-32α (δ 0.72), which are only possible when ring B is disposed in *equatorial* position and the OH group is in *axial* position.

N-Tigloylbuxahyrcanine (**2**) ($C_{31}H_{52}N_2O_2$, *m/z* 484.3891, calcd 484.4028) was quite similar to **1** except that instead of a peak at *m/z* 105, an ion was observed at *m/z* 83 (composition C_5H_7O), which indicated the presence of a tigloyl group instead of a benzoyl group at C-3. The UV spectrum showed only terminal absorption at 206 nm, indicating the lack of any conjugated chromophore in the molecule. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1** except for signals from the tigloyl moiety which resonated at δ 168.9 (C-1′), 132.5 (C-2′), 129.0 (C-3′), 13.8 (C-4′), and 15.9 (C-5′) and the absence of signals for the benzoyl substituent. In the NOESY spectrum, the H-3′ signal showed interaction only with the C-4′ methyl signal. The C-4′ and C-5′ methyls showed interactions with each other, thereby suggesting that the two methyls are in close vicinity to each other. The structure of **2** was then determined by single-crystal X-ray diffraction, which showed that 10-OH was β oriented. Figure 2 shows a computer-generated perspective of the final X-ray model of **2**. Therefore, the structure of compound **2** was determined as *N*-tigloylbuxahyrcanine.

N-Isobutyroylbuxahyrcanine (**3**) gave a M^+ at *m/z* 472.4012 ($C_{30}H_{52}N_2O_2$, calcd 472.4028) in the HREIMS. The UV spectrum of **3** showed end absorption indicating the absence of any chromophore. The ^{1}H NMR spectrum of **3** showed a distinct resemblance with those of **1** and **2** except that it showed the presence of an isobutyroyl group (δ 1.13 and 1.15, d, each 3H, $J = 5.1$ Hz, C-3′ and C-4′ methyls, δ 2.3, m, 1H, C-2′, compared with literature).[12] Several 2D ^{1}H NMR experiments (COSY-45°, HOHAHA, HMBC, and HMQC) further supported the chemical shift assignments for various protons, and in this way structure **3** was determined as *N*-isobutyroylbuxahyrcanine.

Extracts of several plants of the family Buxaceae have exhibited antiacetylcholinesterase properties.[13] Furthermore, buxaminol E, a steroidal alkaloid isolated from *B. sempervirens*, has also been reported as a good inhibitor of the enzyme.[14] We have previously reported the antiacetylcholinesterase activity of some *Buxus* alkaloids.[6] Inhibition of acetylcholinesterase (AChE) is considered to be a promising approach for the treatment of Alzheimer's (AD) and related neurodegrative diseases.[15,16] The role of butyrylcholinesterase (BChE) in normal aging and brain diseases is still unclear, but recently BChE has been found in significantly higher quantities in Alzheimer's plaques

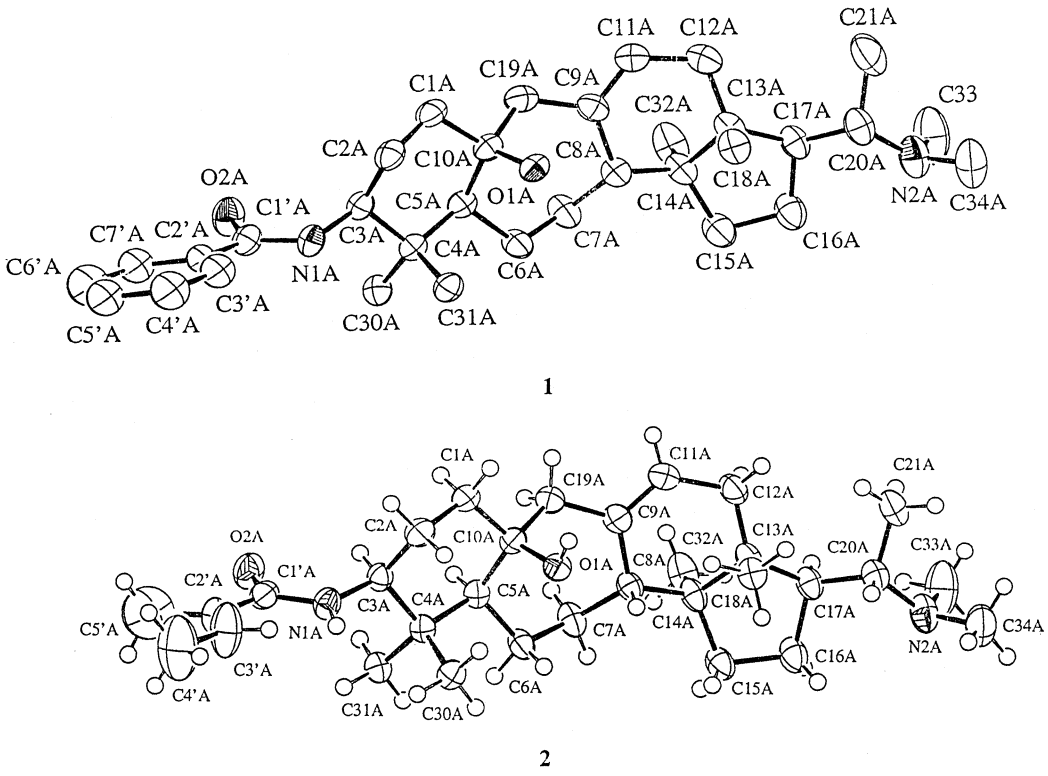

Figure 2. ORTEP representation of the X-ray structure of compounds **1** and **2**.

Table 2. *In Vitro* Quantitative Inhibition of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) by Compounds **1**−**3**

	IC_{50} (μM) ± SEM		selectivity	
compound	AChE	BChE	AChE	BChE
N-benzoylbuxahyrcanine (**1**)	>1000	310.6 ± 0.1		3.2
N-tigloylbuxahyrcanine (**2**)	443.6 ± 15.1	31.2 ± 3.0		14.2
N-isobutyroylbuxahyrcanine(**3**)	>1000	53.7 ± 3.7		18.6
eserine (standard)	0.041	0.857		0.047

than in plaques of normal age-related nondemented brains. Moreover the inclusion of cymserine, which is a very potent selective BChE inhibitor, indicates that BChE inhibition may be an effective tool for the treatment of AD and related dementias.[17]

Alkaloids **1**−**3** were tested for AChE and BChE inhibition. The concentrations of compounds **1**−**3** that inhibited the enzyme activity by 50% (IC_{50} values) are presented in Table 2. As shown in Table 2, all three alkaloids were found to be better inhibitors of BChE as compared to AChE. Compound **2** was found to be the most potent against both enzymes. This could be due to the longer side chain at C-3, while compound **1** was found to be the least potent inhibitor of both. This may be due to the presence of a benzoyl group at C-3. However, we assume that the selectivity of these compounds toward BChE could be due to the larger active site of BChE as compared to AChE, which enables these bulky compounds to be easily accommodated.[17]

Experimental Section

General Experimental Procedures. The melting points are uncorrected and were recorded on a Buchi 535 melting point apparatus. Optical rotations were measured on a JASCO DIP-360 digital polarimeter. UV and IR spectra were recorded on Shimadzu UV-240 and Shimadzu IR-240 spectrophotometers, respectively. The ^{1}H NMR spectra were recorded in $CDCl_3$ at 500 MHz on a Bruker AMX-500 NMR spectrometer. The ^{13}C NMR spectra were recorded at 125 MHz on a Bruker AMX-500 NMR spectrometer. The LREI and HREIMS were recorded on a JEOL JMS HX 110 mass spectrometer. Column chromatography was carried out using SiO_2 gel (E. Merck, type 60, 70−230 mesh), and purity of compounds was checked on precoated SiO_2 gel (GF-254 TLC plates, 20 × 20 cm, 0.25 mm thick).

Plant Material. The leaves (28.5 kg) of *B. hyrcana* were collected by one of us from Tehran, Iran, during March−April 1997. A voucher specimen (No. B-530) was deposited in the herbarium of the Shaheed Beheshti University, Tehran.

Extraction and Isolation. Extraction was carried out with methanol (30 L × 3) at 25 °C for 3 days. The solvent was evaporated in vacuo to afford a gum (3.0 kg), which was then suspended in H_2O. This aqueous layer was defatted with hexane (10 L × 3) and extracted with $CHCl_3$ (10 L × 3) at different pH values. The fractions obtained at pH 7.0 (263.0 g) were subjected to column chromatography (SiO_2 gel, 70−230 mesh, 1.25 kg). The column was eluted with gradients of mixtures of $CHCl_3$−MeOH. Elution with $CHCl_3$−MeOH (95:05) afforded a fraction (26.8 g), which was again subjected to flash column chromatography (SiO_2 gel, 240−300 mesh, 400 g) employing $CHCl_3$−C_6H_{14}−Et_2NH as the eluting solvent. Two fractions obtained at $CHCl_3$−C_6H_{14}−Et_2NH (2.5:7.4:0.1) after evaporation showed the presence of some solid portion. When these fractions were repeatedly washed with acetone, some part remained insoluble. The acetone-soluble portion was removed, and the insoluble portion was dissolved in $CHCl_3$ and subjected to TLC separation (SiO_2 gel GF-254, $(CH_3)_2CO$−C_6H_{14}−Et_2NH (2:7.9:0.1)) to obtain pure compounds *N*-benzoylbuxahyrcanine (**1**) and *N*-tigloylbuxahyrcanine (**2**).

The fractions eluted with increasing polarities of $CHCl_3$ in MeOH were combined (20 g) and rechromatographed (SiO_2 gel, 240−300 mesh, 300 g) employing $(CH_3)_2CO$−C_6H_{14}−Et_2NH mixtures for elution. The fractions obtained on elution with

$(CH_3)_2CO-C_6H_{14}-Et_2NH$ (2:7.9:0.1) were evaporated, and the solid mass thus obtained was repeatedly washed with acetone to yield *N*-isobutyroylbuxahyrcanine (**3**).

***N*-Benzoylbuxahyrcanine (1):** colorless amorphous powder; 91.8 mg (% yield 3.22×10^{-4}) ($CHCl_3$); crystallized from MeOH; mp 239.7 °C; $[\alpha]^{29}_D$ +15° (*c* 0.136, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 223 (4.20) nm; IR ($CHCl_3$) ν_{max} 3648 (OH), 3409 (NH), 1660 (C=O), 1602 (C=C) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; EIMS *m/z* 506 (0.1), 491 (0.2), 105 (5), 72 (100); HREIMS *m/z* 506.3812 (calcd for $C_{33}H_{50}N_2O_2$, 506.3872).

***N*-Tigloylbuxahyrcanine (2):** colorless amorphous powder; 150 mg (% yield 5.26×10^{-4}) ($CHCl_3$); crystallized from MeOH−C_6H_6 (1:1); mp 245.5 °C; $[\alpha]^{29}_D$ +62° (*c* 0.096,$CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 206 (4.33) nm; IR ($CHCl_3$) ν_{max} 3547 (OH), 3452 (NH), 2839 (CH, aliphatic), 1640 (C=O, amide), 1622 (C=C) cm^{-1}; EIMS *m/z* 484 $[M]^+$(8), 469 (6), 83.0 (38), 72 (100); HREIMS *m/z* 484.3891 (calcd for $C_{31}H_{50}N_2O_2$, 484.4028).

***N*-Isobutyroylbuxahyrcanine (3):** colorless amorphous powder; 169 mg (% yield 3.22×10^{-4}) ($CHCl_3$); mp 234−235 °C; $[\alpha]^{29}_D$ +14° (*c* 0.124, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 205.6 (4.33) nm; IR ($CHCl_3$) ν_{max} 3408 (OH), 2838 (CH, aliphatic), 1660 (C=O, amide) cm^{-1}; EIMS *m/z* 472 (8), 457 (6), 73 (50), 72 (100); HREIMS *m/z* 472.4012 (calcd for $C_{30}H_{52}N_2O_2$, 472.4028).

Crystal Data for 1: $C_{33}H_{50}N_2O_2$, mol wt = 506, monoclinic, space group $P2_1$, a = 14.2824(4) Å, b = 10.7080(3) Å, c = 20.2068(7) Å, V = 3037.21(16) $Å^3$, Z = 4, D_c = 1.108 Mg/m^3, $F(000)$ = 1112, μ(Mo Kα) = 0.068 mm^{-1}. A single crystal of approximate dimensions 0.22 × 0.22 × 0.08 mm^3 was used for all measurements. The intensity data of all unique reflections within the θ range 3.2−27.4° were collected at 295(2) K in a Nonius Kappa CCD diffractometer, using Mo Kα radiation on a graphite monochromator. A total of 7186 reflections were recorded, of which 6695 reflections with F_o [$I > 2\sigma(I)$] were taken into account for structure solution and refinements. The intensities were corrected for Lorentz and polarization factors and for absorption using the multiscan method.

Crystal Data for 2: $C_{31}H_{52}N_2O_2$, mol wt = 484, monoclinic, space group $P2_1$, a = 14.0960(3) Å, b = 10.7512(2) Å, c = 19.6728(5) Å, V = 2908.32(11) $Å^3$, Z = 4, D_c = 1.107 Mg/m^3, $F(000)$ = 1072, μ(Mo Kα) = 0.068 mm^{-1}. A single crystal of approximate dimensions 0.25 × 0.22 × 0.08 mm^3 was used for all measurements. The intensity data of all unique reflections within the θ range 3.7−27.5° were collected at 175(2) K in a Nonius Kappa CCD diffractometer, using Mo Kα radiation on a graphite monochromator. A total of 6901 reflections were recorded, of which 5156 reflections with F_o [$I > 2\sigma$(I)] were taken into account for structure solution and refinements. The intensities were corrected for Lorentz and polarization factors and for absorption using the multiscan method. Crystallographic data of **1** and **2**, including atomic coordinates, have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: 44-(0) 1223-306033 or e-mail: deposit@ccdc.cam.ac.uk].

Cholinesterase Inhibition Assay. Acetylcholinesterase and butyrylcholinesterase inhibition were determined spectrophotometrically, using acetylthiocholine and butyrylthiocholine as substrates, respectively, by modifying the method of Ellman.[18] The protocol has already been described in this journal.[19]

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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