

## Isolation and Characterization of Tirandamycins from a Marine-Derived *Streptomyces* sp.

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The novel dienoyl tetramic acids tirandamycin C (**1**) and tirandamycin D (**2**) with activity against vancomycin-resistant *Enterococcus faecalis* were isolated from the marine environmental isolate *Streptomyces* sp. 307-9, which also produces the previously identified compounds tirandamycins A (**3**) and B (**4**). Spectroscopic analysis of **1** and **2** indicated structural similarity to **3** and **4**, with differences only in the pattern of pendant oxygenation on the bicyclic ketal system. The isolation of these putative biosynthetic intermediates was enabled by their sequestration on an adsorbent resin during early stationary-phase fermentation.

Tetramic acids comprise a growing set of natural products containing a 2,4-pyrrolidinedione ring system formed from the condensation of an amino acid to a polyketide-derived acyl chain (Figure 1).<sup>1</sup> These compounds continue to generate significant interest due to their broad structural diversity and breadth of biological activities, with representative examples including the HIV-1 integrase inhibitor equisetin,<sup>2</sup> the mycotoxin cyclopiazonic acid,<sup>3</sup> and the first discovered tetramic acid antibiotic streptolydigin.<sup>4,5</sup> Structurally similar to streptolydigin, the antibiotic tirandamycin A (**3**) was first isolated from the culture broth of the terrestrial bacterium *Streptomyces tirandis* in 1971<sup>6</sup> and again in 1976 from *Streptomyces flaveolus* along with tirandamycin B (**4**).<sup>7</sup> These compounds exhibited antimicrobial activity against Gram-positive bacteria and *in vitro* activity against bacterial RNA polymerase.<sup>8,9</sup> The related compounds tirandalydigin,<sup>10</sup> BU-2313,<sup>11</sup> and nocamycin II<sup>12</sup> have since been characterized, all of which share a bicyclic ketal system and dienoyl tetramic acid moiety characteristic of streptolydigin and tirandamycin. Interest in the biosynthesis of these compounds prompted feeding studies in the streptolydigin system, the results of which support a hybrid polyketide-nonribosomal peptide origin.<sup>13</sup> The biogenesis of these compounds would be further informed by the identification of pathway intermediates, but to date these efforts have been quite limited. We report here the use of established resin capture methods to isolate and identify the two new natural products tirandamycin C (**1**) and tirandamycin D (**2**), presumed to be key biosynthetic intermediates in the pathway to **3** and **4**.

During a screen to discover new natural products from marine-derived actinomycetes with activity against vancomycin-resistant *Enterococcus faecalis* (VRE), we isolated from marine sediments a bacterium of *Streptomyces*-like morphology designated as strain *Streptomyces* sp. 307-9. Analysis of extracts from shake-flask fermentation identified the major anti-VRE components in the culture broth as the previously reported **3** and **4**. Varying the duration of culture prior to extraction indicated a gradual change in the ratio of the two major products, with **4** accumulating over time at the expense of **3**. This suggested that **3** is a transiently secreted or diffusible intermediate en route to the biosynthesis of the final product **4**. Isolation of **3** was likely enabled by the capture of this intermediate from the culture broth via early harvest. We imagined that an adsorbent resin might be used to capture and observe previously undetected biosynthetic intermediates, protecting them from bioconversion and facilitating their isolation.

We and others have previously used XAD-16 resin in microbial fermentation to increase the yield of a desired final product

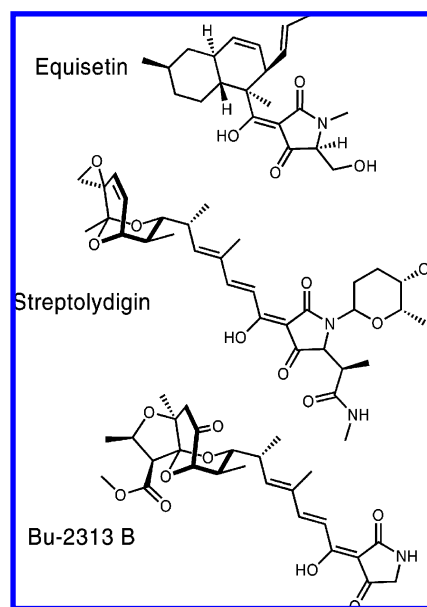


Figure 1. Tetramic acid natural products.

(sequestration of the final product presumably evades feedback repression mechanisms), facilitate convenient extraction from culture broth,<sup>14–17</sup> or stabilize a desired fermentation product against spontaneous degradation and/or enzymatic conversion.<sup>18,19</sup> We added sterile XAD-16 resin bags to shake flask cultures of *Streptomyces* sp. 307-9 entering stationary phase and allowed fermentation to proceed for several days, after which the mesh bags were removed and the resin extracted. Reversed-phase HPLC analysis of the resulting crude extracts revealed several new peaks that shared the characteristic UV spectrum of **3** and **4** and that were not observed if resin was omitted from the culture flasks. Compounds **1** and **2** were observed reproducibly following resin extraction and were purified by semipreparative HPLC.

Molecular formulas for **1** and **2** were established by high-resolution ESIMS, which indicated that **2** (C<sub>22</sub>H<sub>27</sub>NO<sub>6</sub>) differs from **3** (C<sub>22</sub>H<sub>27</sub>NO<sub>7</sub>) by the absence of a single oxygen atom and that **1** (C<sub>22</sub>H<sub>29</sub>NO<sub>5</sub>) differs further by the absence of an additional oxygen and the presence of two additional protons. As shown in Table 1, <sup>1</sup>H NMR spectra of **1** and **2** supported a close structural relationship to **3** and **4**, demonstrated by the consistent appearance of the olefinic and methyl signals of the dienoyl acyl chain and the geminal protons of the tetramic acid methylene carbon. Correlations observed in COSY, HSQC, and HMBC experiments further confirmed the identical composition of this region in **1–4** and highlighted

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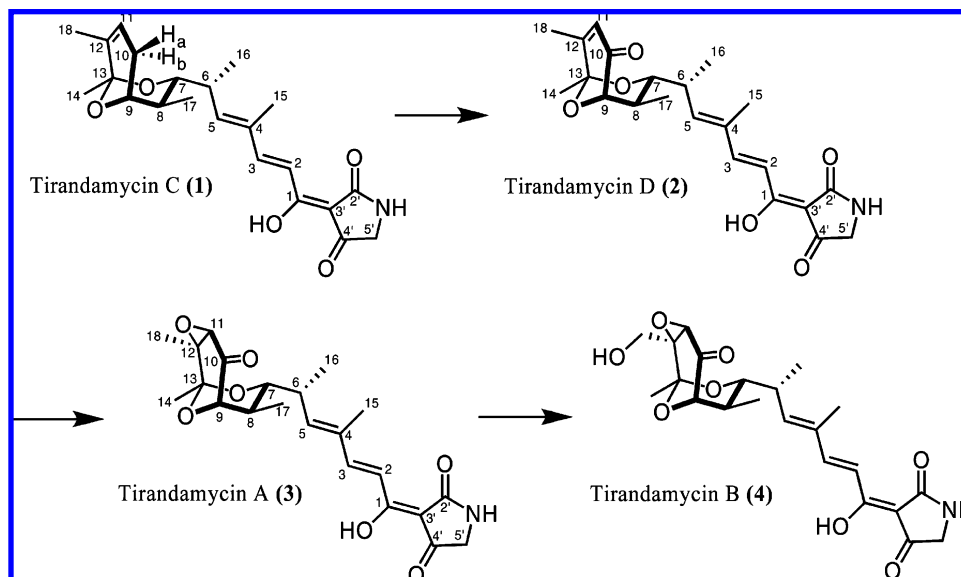
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**Table 1.**  $^1\text{H}$ ,  $^{13}\text{C}$ , and 2D NMR Data for Tirandamycin A (**3**), Tirandamycin D (**2**), and Tirandamycin C (**1**) in  $\text{CD}_2\text{Cl}_2$ 

Nno.	tirandamycin A ( <b>3</b> )			tirandamycin D ( <b>2</b> )			tirandamycin C ( <b>1</b> )					
	$\delta_{\text{C}}^a$	$\delta_{\text{H}}$ (J Hz)		$\delta_{\text{C}}^a$	$\delta_{\text{H}}$ (J Hz)	COSY <sup>d</sup>	HMBC <sup>e</sup>	$\delta_{\text{C}}^a$	$\delta_{\text{H}}$ (J Hz)	COSY <sup>d</sup>	HMBC <sup>e</sup>	NOESY <sup>f</sup>
1	175.5			175.5				175.5				
2	117.1	7.15, dd (15.8, 0.4)		116.7	7.15, dd (15.8, 0.4)	3	1, 4	116.1	7.12, dd (15.7, 0.4)	3	1	3, 15
3	150.0	7.58, dd (15.8, 0.8)		150.2	7.61, dd (15.8, 0.7)	2	1, 5, 15	150.4	7.62, dd (15.7, 0.8)	2	1	2, 5
4	135.3			135.5				134.9				
5	144.5	6.24, d (9.9)		145.8	6.30, d (10.1)	6, 15	3, 15	147.5	6.32, d (10.2)	6, 15	3, 15	3, 6, 8, 16
6	34.9	2.87, m		34.8	2.89, m	5, 16	4, 5, 16	34.9	2.83, m	5, 7, 16		5, 7, 15, 16, 17
7	77.4	3.58 <sup>c</sup>		77.7	3.44, dd (11.3, 2.1)	8	5	77.0	3.49, dd (11.0, 2.1)	6, 8		6, 8, 16, 17
8	35.0	1.97, m		33.9	1.97, m	7, 9, 17	7, 9	35.5	1.84, m	7, 9, 17		5, 7, 9, 17
9	79.4	3.98, d (6.1)		79.5	3.97, d (5.8)	8	7, 8, 10, 13	71.4	3.90, bd (6.5)	8, 10a	7, 8, 11, 13	8, 10a, 17
10a	203.2			195.7				24.5	2.33, m	9, 10b, 11, 18		9, 10b
10b								24.5	1.96, m	10a, 11, 18		10a, 11, 17
11	61.6	3.25, s		127.4	6.08, s	18	13, 18	123.6	5.70, bs	10b, 10b, 18		10b, 18
12	57.5			156.7				133.2				
13	97.4			96.7				96.1				
14	22.8	1.53, s		24.6	1.54, s		12, 13	24.5	1.38, s		12, 13	
15	12.5	1.91, d (1.3)		12.4	1.91, d (1.1)	5	3, 4, 5	12.4	1.91, d (1.3)	5	3, 4, 5	2, 6
16	17.2	1.14, d (6.8)		17.0	1.07, d (7.0)	6	5, 6, 7	17.2	1.05, d (7.0)	6	5, 6, 7	5, 6, 7
17	11.6	0.71, d (7.0)		11.6	0.69, d (7.2)	8	7, 8, 9	13.2	0.68, d (7.0)	8	7, 8, 9	6, 7, 8, 9, 10b
18	15.8	1.46, s		19.4	1.92, d (1.5)	11	11, 12, 13	18.3	1.61 <sup>c</sup>	10a, 10b, 11	11, 12, 13	11
1'(N)				5.70, s		5'						
2'	176.9			177.0				177.1				
3'	N/A <sup>b</sup>			N/A <sup>b</sup>				N/A <sup>b</sup>				
4'	193.1			193.0				193.1				
5'	52.1	3.78, s		51.9	3.78, s	1'	2', 4'	51.9	3.78, s		2', 4'	

<sup>a</sup> Shift values for  $^{13}\text{C}$  assignments taken from HSQC, HMBC, and  $^{13}\text{C}$  data. <sup>b</sup> Signal not observed in HSQC and HMBC of **1**, **2**, and **3**, but seen in the  $^{13}\text{C}$  spectrum of **4**. <sup>c</sup> Signal obscured in the  $^1\text{H}$  spectrum but assigned by 2D spectra. <sup>d</sup> Correlations are between protons at positions indicated. <sup>e</sup> Correlations are from proton to indicated carbon positions. <sup>f</sup> NOESY data reported for 0.4–6.0 ppm.

### Scheme 1. Structures and Putative Biosynthetic Scheme for Secondary Metabolites of the Tirandamycin Pathway



structural differences in the pendant oxygenation patterns on the bicyclic ketal system. When compared to **3**, **2** was determined to contain a C-11/C-12 *Z* double bond in place of the epoxide, indicated by the chemical shift values for positions 11 ( $\delta_{\text{H}}$  6.08,  $\delta_{\text{C}}$  127.4) and 12 ( $\delta_{\text{C}}$  156.7), and the adjacent carbonyl at C-10 ( $\delta_{\text{C}}$  195.7). This assignment was further supported by 2D correlations including (a) a long-range COSY coupling between H-11 and H-18 and (b) HMBC correlations from H-11 to C-13 and C-18, H-18 to C-11 and C-12, and H-14 to C-12. Compound **1** was also determined to contain this double bond, but presented a methylene carbon ( $\delta_{\text{C}}$  24.5) in place of the C-10 carbonyl of **2**, demonstrated by the appearance of new multiplet signals for H-10b ( $\delta_{\text{H}}$  1.96) and H-10a ( $\delta_{\text{H}}$  2.33) and the reduced deshielding at positions 9 ( $\delta_{\text{H}}$  3.90,  $\delta_{\text{C}}$  71.4) and 11 ( $\delta_{\text{H}}$  5.70,  $\delta_{\text{C}}$  123.6). This feature was confirmed by 2D COSY correlations including (a) a geminal coupling between H-10b and H-10a and (b) couplings between the H-10 protons and the H-11 olefin signal and the H-9 methine signal (coupled to H-10a only) and a long-range coupling to the H-18

methyl signal (see Supporting Information for a complete mapping of 2D correlations). Assignment of the diastereotopic protons of the C-10 center of **1** was achieved through analysis of a NOESY spectrum, which indicated (a) proximity between H-10b and H-17 and (b) proximity between H-10a and H-9. With this information, H-10b was determined to be the *pro-S* proton and H-10a was assigned as *pro-R*. To confirm the absolute configuration of **1**–**4**, we obtained optical rotation measurements for tirandamycins A and B from both *Streptomyces* sp. 307-9 and *S. tirandis* NRRL 3689. Tirandamycin A was originally isolated from *S. tirandis* NRRL 3689, and the absolute configuration of this compound was established by X-ray crystallography of a *p*-bromophenacyl ester derivative.<sup>6,20</sup> The  $[\alpha]_{\text{D}}^{25}$  values of **3** and **4** from *Streptomyces* sp. 307-9 are consistent with those for tirandamycins A and B from *S. tirandis*, indicating the same absolute configuration. The configurations of **1** and **2** were presumed to correspond to those of **3** and **4**.

We compared the anti-VRE activity of **1**–**4** in a microtiter plate format with a consecutive 2-fold dilution series of each compound

spanning a concentration range of 6 orders of magnitude. Compound **3** exhibited the highest activity (MIC 2.25  $\mu$ M), with all others presenting diminished inhibitory activity: **1** MIC 110  $\mu$ M, **4** MIC 100  $\mu$ M, **2** MIC > 9  $\mu$ M. The MIC for **2** can only be expressed as a lower limit because of a minor presence of **3** in this sample that accounts for the apparent MIC. A comparison of these activities suggests that the C-10 ketone and C-11/C-12 epoxide (present in **3** but not in **1**) confer increased potency, but this effect can be attenuated by the hydroxy group at C-18 (as in **4**). Previous studies have shown that inhibition of bacterial RNA polymerase (RNAP) is the primary mechanism behind the antibacterial activity of tirandamycin and streptolydigin.<sup>9,21–23</sup> The crystal structures of *E. coli* and *Thermus thermophilus* RNAP complexed with streptolydigin have been solved and reveal an abundance of key contacts in the streptolol ring system (structurally analogous to the bicyclic ketal system of tirandamycin).<sup>24,25</sup> This is consistent with the bioassay results presented here, which demonstrate that the substituents of this moiety are key determinants of potency, assuming that *in vivo* anti-VRE activity reflects inhibition of RNAP.

The structures of **1** and **2** also suggest possible biosynthetic routes to **3** and **4**. The oxidative modifications of **3** and **4** are typical of tailoring steps found in other secondary metabolite pathways,<sup>26,27</sup> suggesting that **1** and **2** are transiently secreted intermediates that were sequestered, via resin capture, and protected from complete oxidative modification. This implies a biosynthetic route composed of a series of oxygenation steps (Scheme 1), and this enzymology is the subject of ongoing investigation.

In summary, we have identified novel tirandamycins C (**1**) and D (**2**) with structures that suggest their role as intermediates toward the biosynthesis of **3** and **4**. Key to the isolation of these compounds was the inclusion of the adsorbent XAD-16 resin during fermentation as a means of protecting intermediates from bioconversion or breakdown prior to harvest. We suggest that this technique may be useful for situations in which previously undetected biosynthetic intermediates are anticipated to exist transiently in culture broth, yet their isolation is precluded by rapid bioconversion or degradation. Access to such intermediates enriches biosynthetic hypotheses and facilitates further experimental work toward this end. Indeed, our isolation of **1** and **2** has enabled ongoing biochemical studies utilizing these compounds as substrates that will be reported in due course.

## Experimental Section

**General Experimental Procedures.** Optical rotation measurements were obtained on an AUTOPOL III polarimeter. UV spectra were obtained on a UV–visible 300 Bio (Cary) spectrophotometer at room temperature in a solvent of MeOH supplemented with 0.1% TFA. All NMR spectra were acquired on a Varian INOVA 400 MHz and a Varian INOVA 600 MHz spectrometer at the Center for Chemical Genomics, University of Michigan. NMR spectra were processed using MestReNova software. High-resolution ESIMS spectra were measured at the University of Michigan core facility in the Department of Chemistry using a Waters Micromass AutoSpec Ultima. RP-HPLC purification was performed using Waters XBridge 5  $\mu$ m ODS columns and a solvent system of MeCN and H<sub>2</sub>O supplemented with 0.1% TFA. LC-MS analysis of HPLC fractions was performed on a Shimadzu 2010 EV ESI spectrometer.

**Biological Materials.** *Streptomyces* sp. 307-9 was isolated from marine sediments collected at a depth of 15 m during a scuba expedition in Salt Cay, U.S. Virgin Islands. The procedure for the isolation of actinomycetes from these samples has been described previously.<sup>16</sup> Maintenance and propagation of cultures was performed using standard media and protocols.<sup>28</sup> On 2xYT agar, this strain grew as substrate-adherent mycelia that secreted a dark pigment into the media and produced a top layer of white and purple spores. Light microscopy images of stained cells from agar plate cultures revealed Gram-positive, filamentous cells as well as spore chains. These characteristics are consistent with the morphology of *Streptomyces* species. A phylogenetic analysis was performed using a fragment of the 16S rRNA gene amplified from genomic DNA of *Streptomyces* sp. 307-9 using a

previously described protocol.<sup>29</sup> BLAST analysis revealed the highest sequence identity (97–99%) with previously characterized *Streptomyces* species. This strain is preserved and available from an in-house collection (database index DHS1549). See Supporting Information for microscopy images and 16S rRNA gene sequence analysis. The 16S rRNA partial gene sequence was deposited with GenBank under accession number EU679113. *Streptomyces tirandis* NRRL 3689 was obtained from the USDA Agricultural Research Service.

**Culture Maintenance and Fermentation.** Seed cultures of 10 mL of 2xYT media were inoculated with a loopful of vegetative cells from an ISP2 agar plate culture of *Streptomyces* sp. 307-9 and incubated with shaking (150 rpm) at 30 °C for 2 days. Six 2 L baffled flasks each containing 1 L of autoclaved production media Md2 (10 g dextrose, 2 g NZ-Amine A, 1 g yeast extract, 0.77 g meat extract, 30 g NaCl per 1 L H<sub>2</sub>O) were each inoculated with 1 mL of seed culture and grown for 2 days under identical conditions. To each flask was then added two autoclaved resin bags containing 10 g of XAD-16 resin (Supelco, Bellefonte, PA) enclosed in Unitherm 100 fabric (Midwest Filtration, Cincinnati, OH) by heat sealing. Production cultures were grown for an additional 4 days, after which the resin bags were collected for extraction.

**Isolation.** Resin bags were cut open, and the free resin was rinsed with water, filtered, and batch extracted with 90% CH<sub>2</sub>Cl<sub>2</sub>/10% MeOH (3  $\times$  1.5 L). The combined extract was washed twice with an equal volume of 1 M NaCl (adjusted to pH 4.0 with TFA). The CH<sub>2</sub>Cl<sub>2</sub> layer was collected and dried by rotary evaporation to give a dark yellow oil that was dissolved into 2 mL of DMSO. This extract was purified by RP-HPLC on a gradient of 30–100% MeCN and followed by UV/vis photodiode array detection. Compounds exhibiting the characteristic absorption spectrum of the tirandamycin tetramic acid moiety ( $\lambda_{\max}$  356 nm) were collected. The purest fractions (as determined by LC-MS analysis) were combined and dried to afford (in order of elution) **4** (3.0 mg), **3** (9.0 mg), **2** (1.4 mg), and **1** (1.9 mg).

**Tirandamycin C (1):** yellow oil;  $[\alpha]_D^{25}$  –59 (*c* 0.11, EtOH); UV (MeOH + 0.1% TFA)  $\lambda_{\max}$  (log  $\epsilon$ ) 213 (4.09, sh), 353 (4.23) nm; for complete NMR data see Table 1; HRESIMS *m/z* [M + Na]<sup>+</sup> 410.1949 (calcd for C<sub>22</sub>H<sub>29</sub>NO<sub>5</sub>Na, 410.1944).

**Tirandamycin D (2):** yellow oil;  $[\alpha]_D^{25}$  –60 (*c* 0.02, EtOH); UV (MeOH + 0.1% TFA)  $\lambda_{\max}$  (log  $\epsilon$ ) 213 (4.23, sh), 353 (4.04) nm; for complete NMR data see Table 1; HRESIMS *m/z* [M + Na]<sup>+</sup> 424.1737 (calcd for C<sub>22</sub>H<sub>27</sub>NO<sub>6</sub>Na, 424.1736).

**Tirandamycin A (3):** yellow oil;  $[\alpha]_D^{25}$  +15 (*c* 0.3, EtOH) from *Streptomyces* sp. 307-9,  $[\alpha]_D^{25}$  +4 (*c* 0.5, EtOH) from *S. tirandis*; UV (MeOH + 0.1% TFA)  $\lambda_{\max}$  (log  $\epsilon$ ) 213 (4.29, sh), 353 (4.32) nm; for complete NMR data see Table 1, match reported values.<sup>30</sup>

**Tirandamycin B (4):** yellow oil;  $[\alpha]_D^{25}$  –14 (*c* 0.5, EtOH) from *Streptomyces* sp. 307-9,  $[\alpha]_D^{25}$  –8 (*c* 0.55, EtOH) from *S. tirandis*; UV (MeOH + 0.1% TFA)  $\lambda_{\max}$  (log  $\epsilon$ ) 213 (4.16, sh), 353 (4.24) nm; NMR data match reported values.<sup>31</sup>

**Bioassay.** A 2  $\mu$ L sample of 20 mM DMSO solutions of **1–4** was added to 100  $\mu$ L LB broth (to give 400  $\mu$ M final concentrations), and 2-fold serial dilutions of this stock were prepared in LB. All cultures of vancomycin-resistant *Enterococcus faecalis* were grown in LB supplemented with 10  $\mu$ g/mL vancomycin at 37 °C with shaking (150 rpm). An overnight seed culture of VRE was diluted to an OD<sub>600</sub> of 0.05, grown to an OD<sub>600</sub> of 0.4, and back diluted to an OD<sub>600</sub> of 0.005, and 50  $\mu$ L of this dilution was added to each well of a microtiter plate that contained 50  $\mu$ L of a given dilution of compound in LB. Plate cultures were grown for 16 h, and OD<sub>600</sub> measurements were taken. MIC values were taken as the lowest concentration of compound for which no growth was observed. Concentrations of compounds **1–3** were adjusted to correct for impurities observed in NMR spectra.

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**Supporting Information Available:** <sup>1</sup>H, COSY, HSQC, and HMBC spectra for **1–3**, NOESY of **1**, <sup>1</sup>H and <sup>13</sup>C of **4**; maps of 2D NMR correlations; anti-VRE bioassay data, representative HPLC chromatograms of tirandamycin metabolite profiles; microscopy images and 16S

rRNA gene sequence derived phylogenetic analysis of strain *Streptomyces* sp. 307-9. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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