Pseudotheonamides, Serine Protease Inhibitors from the Marine Sponge *Theonella swinhoei*[1]

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Abstract: Six new peptides with serine protease inhibitory activity have been isolated from the marine sponge *Theonella swinhoei*. Their structures including absolute stereochemistry were unambiguously established by interpretation of spectral data and chemical degradation. Pseudotheonamides A_1 (**1**), A_2 (**2**), and B_2 (**3**) are linear pentapeptides embracing the rare piperazinone and piperidinoiminoimidazolone ring systems. Pseudotheonamide C (**4**) contains v-Tyr (vinylogous tyrosine) instead of a piperazinone ring. Pseudotheonamide D (**5**) is a tetrapeptide which lacks a *C*-terminal k-Arg (α-ketohomoarginine) unit. Dihydrocyclotheonamide A (**6**) is a reduction product of the known cyclotheonamide A (**7**).[2]

The cyclotheonamides were isolated from the marine sponge *Theonella swinhoei* collected off Hachijo-jima Island, 300 km south of Tokyo, and are unusual cyclic peptides containing two new amino acids, α-ketohomoarginine (k-Arg) and vinylogous tyrosine (v-Tyr).[2] Significantly, they possess potent inhibitory activity against serine proteases including thrombin, trypsin, and plasmin. Their mode of action was well elucidated by X-ray crystallography of the complex between cyclotheonamide A (**7**) and human α-thrombin or trypsin, which disclosed the binding of cyclotheonamide A to the catalytic triad of the enzymes by forming a covalent bond between the α-keto group of the k-Arg residue of cyclotheonamide A and the hydroxyl group of the serine residue of one of the triad residues.[3] Since the cyclotheonamides have become important model compounds for serine protease inhibitors, we further examined the extract of *T. swinhoei*, which has resulted in isolation of six new peptides related to the cyclotheonamides: pseudotheonamides A_1 (**1**), A_2 (**2**), B_2 (**3**),[4] C (**4**), D (**5**), and dihydrocyclotheonamide A (**6**) (Chart 1). The first three compounds possess the rare piperazinone and piperidinoiminoimidazolone ring systems. This paper describes the isolation, structure elucidation, and activity of these compounds.

Result and Discussion

Frozen sponge samples of the 1993 collection (30 kg, wet weight) were extracted with EtOH, and the extract was concentrated and partitioned between ether and *n*-BuOH. The BuOH layer was repeatedly fractionated by chromatographies on ODS (aqueous MeOH), Sephadex LH-20 (MeOH), and ODS (aqueous MeCN with 0.05% TFA), followed by reversed-phase HPLC to yield pseudotheonamides A_1 (**1**, 44 mg, 1.5×10^{-4}% yield based on wet weight), A_2 (**2**, 1.2 mg, 4.0×10^{-6}%), B_2 (**3**, 4.5 mg, 1.5×10^{-5}%), D (**5**, 4.4 mg, 1.5×10^{-5}%), and dihydrocyclotheonamide A (**6**, 6.2 mg, 2.1×10^{-5}%). Samples of the 1996 collection (52 kg, wet weight) were similarly processed to yield 11.4 mg of pseudotheonamide C (**4**).

Pseudotheonamide A_1 (**1**) had a molecular formula of $C_{36}H_{45}N_9O_8$ as established by HR-FABMS, which was the same as that of cyclotheonamide A (**7**). Interpretation of the COSY, HMQC,[5] and HOHAHA[6] spectra allowed the assignment of spin systems for component amino acids, and for a formamide which was also present in **7**. Chemical shift values (Table 1) for the Phe and Pro residues in **1** were essentially identical with those in **7**, whereas those for the Dpr (2,3-diaminopropionic acid) and k-Arg residues were significantly different. The β-methylene protons of the Dpr residue resonated much closer to each other (δ 3.40 and 3.27) in **1** than in **7** (δ 4.24 and 2.95), whereas ϵ-methylene protons of the k-Arg residue in **1** were not equivalent [δ 3.83 and 3.24 in **1**; 3.17 (2H) in **7**]. More significantly, pseudotheonamide A_1 (**1**) revealed no olefinic signals of the v-Tyr residue in the ^{1}H and ^{13}C NMR spectra. However, signals for a para-substituted phenol and the benzylic δ-methylene (2.89 and 2.61 ppm) adjacent to a nitrogenous γ-methine (3.49 ppm) of v-Tyr were present. The nitrogenous γ-methine was further coupled to another nitrogenous methine (3.02 ppm; Hβ) which was in turn coupled to a pair of methylene protons (2.66 and 2.24 ppm; $H_2\alpha$), thus delineating the backbone of the v-Tyr residue; this unit is now called e-Tyr (ethylene-inserted Tyr) for convenience's sake. Presumably, the v-Tyr residue has undergone an intramolecular Michael addition with an amino group of Phe.[7]

(1) Bioactive Marine Metabolites. Part 86. Part 85: Tsukamoto, S.; Matsunaga, S.; Fusetani, N. *J. Nat. Prod.*, in press.

(2) (a) Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053−7054. (b) Nakao, Y.; Matsunaga, S.; Fusetani, N. *Bioorg. Med. Chem.* **1995**, *3*, 1115−1122. (c) Nakao, Y.; Oku, N.; Matsunaga, S.; Fusetani, N. *J. Nat. Prod.* **1998**, *61*, 667−670.

(3) (a) Maryanoff, B. E.; Qiu, X.; Padmanabhan, K. P.; Tulinsky, A.; Almond, Jr. H. R.; Andrade-Gordon, P.; Greco, M. N.; Kauffman, J. A.; Nicolaou, K. C.; Liu, A.; Brungs, P. H.; Fusetani, N. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8048−8052. (b) Lee, A. Y.; Hagihara, M.; Karmacharya, R.; Albers, M. W.; Schreiber, S. L.; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 12619−12620. (c) Maryanoff, B. E.; Greco, M. N.; Zhang, H.; Andrade-Gordon, P.; Kauffman, J. A.; Nicolaou, K. C.; Liu, A.; Brungs, P. H. *J. Am. Chem. Soc.* **1995**, *117*, 1225−1239. (d) Ganesh, V.; Lee, A. Y.; Clardy, J.; Tulinsky, A. *Protein Sci.* **1996**, *5*, 825−835.

(4) Although pseudotheonamide B_1 has not been isolated, we named compound **3** as pseudotheonamide B_2 based on the same stereochemistry of the piperazinone moiety as in **2**.

(5) Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, *108*, 4285−4294.

(6) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.

(7) March, J. *Advanced Organic Chemistry*; Willey-Interscience: New York, 1992; p 768.

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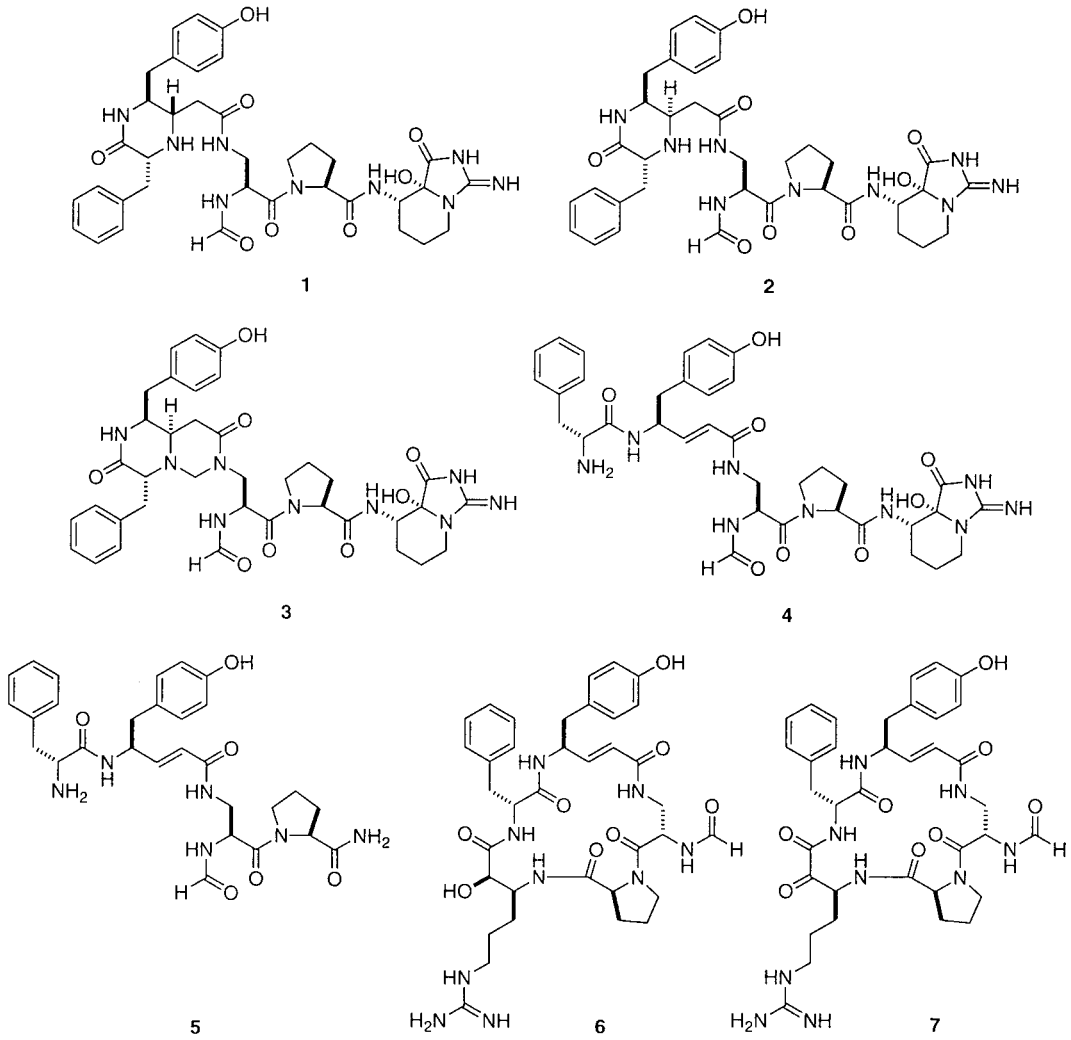
Chart 1



Further structural assignment and sequencing of the amino acid residues was accomplished with the aid of HMBC[8] data. The Hβ and NH (7.21 ppm) in the e-Tyr residue were correlated with the Cα (59.8 ppm) and CO (172.3 ppm) of the Phe residue, respectively, thereby indicating the presence of a piperazinone ring. HMBC cross-peaks (8.39, 3.40, and 3.27/173.6 ppm) demonstrated that the Dpr residue was linked to the e-Tyr residue through the β-amino group (8.39 ppm); the α-amino group (8.15 ppm) was formylated as in **7**. The k-Arg residue was also modified; the characteristic ϵ-NH signal of the guanidyl group was missing, whereas the α-carbon resonated at 85.0 ppm, indicative of the presence of an aminal moiety rather than a hydrated ketone (δ 97.4 in **7**). Splitting patterns of Hβ (J = 12.7, 9.2, 3.9 Hz), Hϵ (J = 13.8, 5.0 Hz), and Hϵ' (J = 13.8, 13.1, 3.3 Hz) indicated that these protons were located in a six-membered ring. An HMBC cross-peak between Hϵ (3.83 ppm) and Cα (85.0 ppm) led to the connectivity of the ϵ-nitrogen atom and Cα. There were a guanidyl carbon (δ 159.5) and a carbonyl carbon (δ 178.3), both of which could be accommodated in the modified k-Arg residue; this carbonyl carbon must form a five-membered ring with a guanidyl nitrogen atom to satisfy the molecular formula. A similar ring closure is observed in the formation of creatinine from creatine.[9]

Relative stereochemistry in the piperazinone ring was assigned on the basis of coupling constant arguments and ROESY data (Figure 1). Hβ (3.02 ppm) and Hγ (3.49 ppm) in the e-Tyr residue were in a trans-diaxial relationship as evidenced by a coupling constant of 10.2 Hz as well as by the absence of a ROESY correlation between them. Stereochemistry of e-Tyr Hβ could be deduced from a ROESY cross-peak between D-Phe Hα and e-Tyr Hβ (vide infra). Though the stereochemistry of Cα in the modified k-Arg residue was not determined spectroscopically, it was presumed that the hydroxyl group was axial, reflecting the anomeric effect.[10]

Absolute stereochemistry of both Pro and Dpr residues were determined to be L by Marfey analysis[11] of the acid hydrolysate. Interestingly, amino acid analysis of the acid hydrolysate revealed a distinctive peak for Phe which was likely to be formed by retro-Michael reaction followed by hydrolysis; Marfey analysis showed D-configuration for Phe. Absolute stereochemistry of the modified k-Arg was determined as follows. Acid hydrolysis of **1** released k-Arg which was detected by amino acid analysis. Although we needed both isomers of k-Arg for Marfey analysis, only the (3*S*) isomer, which had been isolated from the acid hydrolysate of cyclotheonamide A (**7**), was available. To overcome this problem, a chromatographic equivalent of (3*R*)-k-Arg was prepared by reacting the (3*S*)-k-Arg with the Marfey's reagent prepared from D-Ala.[12] With both

(8) Bax, A.; Azolos, A.; Dinya, Z.; Sudo, K. *J. Am. Chem. Soc.* **1986**, *108*, 8056−8063.

(9) Edgar, G.; Hinegardner, W. S. *J. Biol. Chem*. **1923**, *56*, 881−886.

(10) Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*, Pergamon: Oxford, 1983; pp 4−21.

(11) Marfey, P. *Carlsberg Res. Commun*. **1984**, *49*, 591−596.

Table 1. NMR Data for Pseudotheonamide A_1 (**1**) in CD_3OH

		^{13}C	^1H	HMBC	ROESY
Phe	CO	172.3			
	α	59.8	3.68 dd 9.8, 3.8	172.3, 138.3, 37.5	7.24, 3.02, 2.76
	β	37.5	3.37	172.3, 138.3, 130.3, 59.8	7.24, 2.76
			2.76 dd 14.6, 9.8	172.3, 138.3, 130.3, 59.8	7.24, 3.68, 3.37
	1	138.3			
	2,6	130.3	7.24 d 7.7	130.3, 127.9, 37.5	3.68, 3.37, 2.76
	3,5	129.7	7.29 dd 7.7, 7.3	138.3, 129.7	
	4	127.9	7.22	130.3	
	NH				
e-Tyr	CO	173.6			
	α	37.0	2.66 dd 15.8, 2.9	173.6, 58.1, 54.9	8.39, 7.04, 3.49, 3.02, 2.89, 2.20
			2.24	173.6, 58.1, 54.9	8.39, 3.49, 2.66
	β	54.9	3.02 ddd 10.2, 9.6, 2.9	173.6, 59.8, 58.1, 38.2, 37.0	7.04, 3.68, 2.66, 2.61
	γ	58.1	3.49 ddd 10.2, 6.7, 4.4	127.2, 54.9, 38.2, 37.0	7.21, 7.04, 2.89, 2.66, 2.24
	δ	38.2	2.89 dd 14.4, 4.4	131.9, 127.2, 58.1, 54.9	7.21, 7.04, 3.49, 2.66, 2.61
			2.61 dd 14.1, 6.7	131.9, 127.2, 58.1, 54.9	3.02, 2.89
	1′	127.2			
	2′,6′	131.9	7.04 d 8.5	157.8, 131.9, 116.7, 38.2	7.21, 3.49, 3.02, 2.89, 2.66
	3′,5′	116.7	6.73 d 8.5	157.8, 127.2, 116.7	
	4′	157.8			
	NH		7.21 bs	172.3, 59.8, 58.1, 54.9	7.04, 3.49, 2.89
formyl	CO	163.5	7.99 bd 1.0	50.1	8.15
Dpr	CO	169.9			
	α	50.1	4.84		3.69, 3.64
	β	41.8	3.40 ddd 13.9, 6.0, 5.8	173.6, 169.9, 50.1	8.39, 8.15, 3.27
			3.27	173.6, 169.9, 50.1	8.39, 8.15, 3.40
	αNH		8.15 dd 9.2, 1.0		7.99, 3.40, 3.27
	*β*NH		8.39 dd 6.2, 6.0	173.6, 41.8	3.40, 3.27, 2.66, 2.24
Pro	CO	173.6			
	α	61.5	4.50 dd 8.5, 4.2	173.6, 48.9, 30.7, 25.7	8.17, 2.27, 2.22, 1.96
	β	30.7	2.27	173.6, 61.5, 48.9, 25.7	4.50, 3.69, 3.64
			2.22	173.6, 61.5, 48.9, 25.7	4.50, 3.69, 3.64
	γ	25.7	2.07	61.5, 48.9, 30.7	3.69, 3.64
			1.96	61.5, 48.9, 30.7	4.50, 3.69, 3.64
	δ	48.9	3.69 bd 7.9	61.5, 30.7, 25.7	4.84, 2.27, 2.22, 2.07, 1.96
			3.64 dd 7.9, 2.7	61.5, 30.7, 25.7	4.84, 2.27, 2.22, 2.07, 1.96
k-Arg	CO	178.3			
	α	85.0			
	β	51.4	4.03 ddd 12.7, 9.2, 3.9	173.6, 26.3	1.74, 1.57
	γ	26.3	1.90	51.4	8.17, 3.24, 1.74
			1.74 dddd 16.5, 3.9, 3.8, 3.3		4.03, 1.90
	δ	24.9	1.79 dddd 13.9, 3.3, 3.3, 3.1		3.83, 3.24, 1.57
			1.57 ddddd 13.9, 13.1, 13.1, 5.0, 3.8		4.03, 3.83, 1.79
	ϵ	38.9	3.83 dd 13.8, 5.0	159.5, 85.0, 26.3, 24.9	3.24, 1.79, 1.57
			3.24 ddd 13.8, 13.1, 3.3		3.83, 1.90, 1.79
	guanidine	159.5			
	NH		8.17 d 9.2	173.6	4.50, 1.90

standards in hand, we carried out Marfey analysis for the acid hydrolysate which disclosed the 3*S*-stereochemistry for the modified k-Arg residue in **1** (Scheme 1).

Pseudotheonamide A_1 (**1**) revealed minor NMR signals with intensity of approximately 20% of the major signals. Virtually all signals were doubled. Though it was not possible to assign all minor signals, they were characterized on the basis of ^{13}C NMR data. All minor ^{13}C signals appeared close to the major signals except for those of the Pro residue; chemical shifts of the major peaks corresponded well with those reported for the *trans*-isomer (C*β* 30.7 and C*γ* 25.7 ppm), whereas the minor peaks (C*β* 32.9 and C*γ* 23.5 ppm) corresponded with those of the *cis*-isomer. Therefore, it was concluded that the dichotomy of the signals observed in **1** was due to geometrical isomerism in the prolyl peptide bond.

The molecular formula of pseudotheonamide A_2 (**2**) was determined as $C_{36}H_{45}N_9O_8$ which is identical with that of **7**, on the basis of HR-FABMS [$(M + H)^+$ *m/z* 732.3466 (Δ −0.3 mmu)]. The HOHAHA spectrum of **2** revealed the presence of all spin systems found in **1**. HMQC, HMBC, and ROESY data unambiguously disclosed that **1** and **2** had the same gross structure. However, ^1H and ^{13}C chemical shifts of the piperazinone moiety were different between **1** and **2** (Table 2). The *J* value of 3.3 Hz between H*β* and H*γ* in e-Tyr and ROESY cross-peaks between $H_2\alpha$ (2.43 and 2.34 ppm) of e-Tyr and Hα (3.69 ppm) of Phe indicated that the stereochemistry of **2** at C*β* was opposite to that in **1** (Figure 1). Marfey analysis of acid hydrolysate of **2** showed D-Phe, L-Dpr, L-Pro, and (3*S*)-k-Arg; therefore, pseudotheonamide A_2 was an epimer of **1**.

Pseudotheonamide B_2 (**3**) had a molecular formula of $C_{37}H_{45}N_9O_8$, one carbon atom larger than **1**. The ^1H and ^{13}C NMR spectra of **3** were similar to those of **1**, except for more prominent isomerism; the intensity ratio of the major and minor peaks was 3:2, as well as for signals of a new methylene group [*δ*H 4.39 (d, *J* = 12.5 Hz), 4.29 (d, *J* = 12.5 Hz); *δ*C 68.6] (Table 3). The deshielded chemical shifts for this methylene group and lack of signals for the *β*-NH of the Dpr residue and NH of the Phe residue indicated that the additional methylene group must be inserted between the *β*-nitrogen atom of the Dpr residue and the amino nitrogen of the piperazinone moiety.

(12) Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1995**, *60*, 1177−1181.

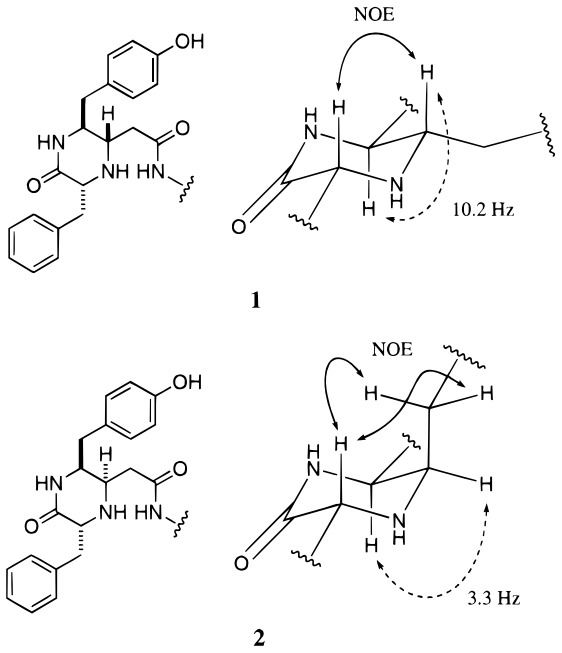


Figure 1. Stereochemistry of the piperazinone moiety in **1** and **2**.

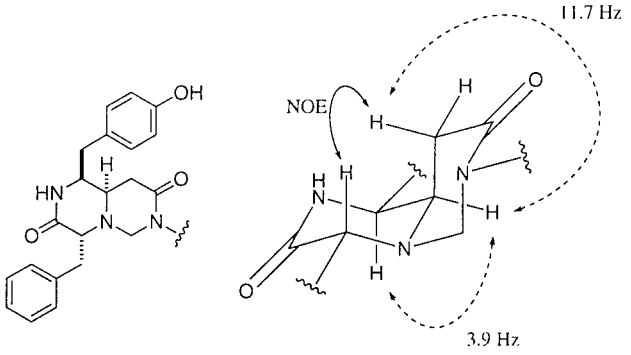
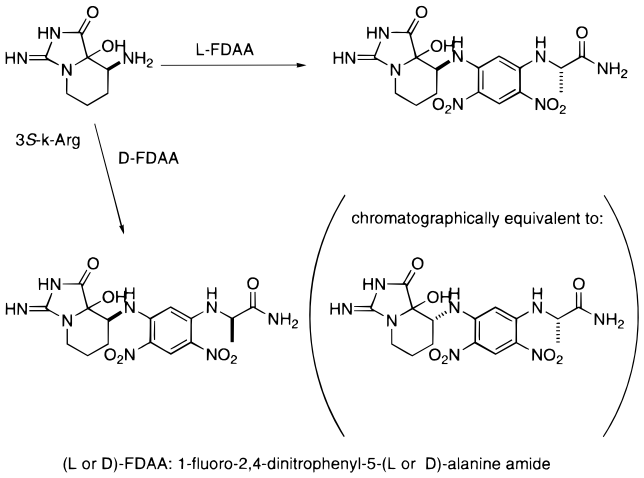


Figure 2. Stereochemistry of the piperazinone moiety in **3**.

Scheme 1



Therefore, the gross structure of **3** was determined as shown. Stereochemistry of the piperazinone ring was again assigned by interpretation of ROESY data. A *J* value of 3.9 Hz between Hβ (3.14 ppm) and Hγ (3.25 ppm) of e-Tyr and a ROESY cross-peak between e-Tyr H$_2\alpha$ (2.39 and 2.27 ppm) and Phe Hα (3.67 ppm) implied the stereochemistry of e-Tyr Cβ in **3** to be the same as that in **2**. Absolute stereochemistry of the component amino acids was determined by Marfey analysis of the acid hydrolysate: all component amino acids had the same absolute stereochemistry as in **2**.

The molecular formula of pseudotheonamide C (**4**) was again identical with that of cyclotheonamide A (**7**). The ^{1}H NMR spectrum contained olefinic protons (6.78 and 5.98 ppm) characteristic of v-Tyr residue which were missing in **1**−**3**. Analysis of 2D NMR data including HOHAHA, HMQC, and HMBC revealed the presence of v-Tyr, α-formyl-Dpr, Phe, Pro, and k-Arg units. These residues accounted for 18 of 19 unsaturations, thereby suggesting cyclization of the k-Arg residue, which was in fact evident from NMR data. Each residue could be connected on the basis of HMBC and ROESY data to construct the gross structure **4**. Marfey analysis of the acid hydrolysate of **4** resulted in the absolute stereochemistry of the Pro, Dpr, Phe, and k-Arg residues as in the case of **1**. The absolute stereochemistry at Cγ of v-Tyr was determined to be *S* which was evident from the fact that L-Asp was detected in the acid hydrolysate of the ozonolysis product of **4**.

Pseudotheonamide D (**5**) had a molecular formula of $C_{29}H_{36}N_6O_6$ as determined by HR-FABMS. Amino acid analysis as well as ^{1}H NMR data showed the presence of Phe, v-Tyr, α-formyl-Dpr, and Pro. HMBC and ROESY data led to the sequence of Phe/v-Tyr/α-formyl-Dpr/Pro; the C-terminus was blocked by an amide (7.69 and 6.97 ppm). Stereochemistry of each residue was similarly determined as δ-Phe, 4*S*-v-Tyr, L-Dpr, and L-Pro.

Dihydrocyclotheonamide A (**6**) displayed a ^{1}H NMR spectrum very similar to that of cyclotheonamide A (**7**), except for the presence of an additional singlet in **6** at 4.08 ppm. The molecular formula of $C_{36}H_{47}N_9O_8$ determined by HRFABMS as well as the replacement of the ^{13}C signal of the hydrated ketone by a secondary alcohol indicated that **6** was dihydrocyclotheonamide A, in which the α-keto group of the k-Arg residue was reduced. This was supported by 2D NMR data. Marfey analysis revealed D-, L-, and L-stereochemistry for Phe, Pro, and Dpr residues, respectively. Reduction of cyclotheonamide A with $NaBH_4$ gave **6** as the major product; therefore, the stereochemistry of v-Tyr residue and Cβ of the k-Arg residue was shown to be identical in the two compounds. The remaining Cα stereocenter in the reduced k-Arg unit was determined as follows: dihydrocyclotheonamide A, which was prepared from **7**, was hydrolyzed and subjected to ion-exchange chromatography to yield α-hydroxyhomoarginine. This was converted to a cyclic carbamate, whose ^{1}H NMR spectrum revealed a 5.0 Hz coupling constant between H2 and H3, thus suggesting *syn* stereochemistry (Scheme 2). Therefore, the reduced-k-Arg unit in **6** had 2*R*,3*S* stereochemistry.[13]

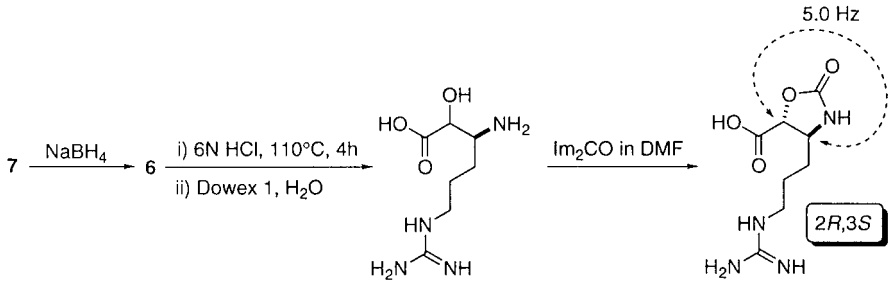
Pseudotheonamides A$_1$ (**1**), A$_2$ (**2**), B$_2$ (**3**), C (**4**), D (**5**), and dihydrocyclotheonamide A (**6**) inhibited thrombin with IC$_{50}$ values of 1.0, 3.0, 1.3, 0.19, 1.4, and 0.33 μM, respectively, while they inhibited trypsin with IC$_{50}$ values of 4.5, >10, 6.2, 3.8, >10, and 6.7 μM, respectively. As revealed by the X-ray crystallography and structure−activity relationship study of synthetic derivatives, potent inhibition of serine proteases by cyclotheonamides is associated with the presence of the α-keto group in the k-Arg residue.[3] It is therefore not surprising that compounds **1**−**6**, in which the α-keto group was either modified or missing, showed moderate activity. Similarly, a mixture of diastereomeric aminals obtained by base treatment of cyclotheonamide A was less active.[14] Our compounds **1**−**4** are related to these products by the cyclic aminal structure of the k-Arg residue.

(13) Dondoni, A.; Fantin, G.; Fogagnolo, M.; Pedrini, P. *J. Org. Chem.* **1990**, *55*, 1439−1446.

Table 2. NMR Data for Pseudotheonamide A_2 (**2**) in CD_3OH

		^{13}C	^1H	HMBC	ROESY
Phe	CO	174.0			
	α	57.9	3.69 dd 9.8, 3.3	174.0, 139.6, 39.0	7.27, 7.23, 3.13, 2.43, 2.34
	β	39.0	3.13 dd 13.9, 3.3	174.0, 139.6, 130.5	7.27, 7.23, 3.69, 2.85
			2.85 dd 13.9, 9.8	174.0, 139.6, 130.5, 57.9	7.23, 3.47, 3.13
	1	139.6			
	2,6	130.5	7.23 d 7.1	130.5, 127.6, 39.0	3.69, 3.13, 2.85
	3,5	129.5	7.27 dd 7.7, 7.1	139.6, 129.5	3.69, 3.13
	4	127.6	7.18 t 7.7	130.5	
	NH		-		
e-Tyr	CO	174.1			
	α	36.2	2.43 dd 14.6, 10.6	174.1, 57.9, 50.0	8.24, 3.69, 3.47, 2.34
			2.34 dd 14.6, 10.2	174.1, 50.0	8.24, 7.02, 3.69, 3.65, 2.43
	β	50.0	3.47 ddd 10.6, 10.2, 3.3	174.1, 57.9	7.02, 3.65, 2.85, 2.68, 2.64
	γ	57.9	3.65 m		7.20, 7.02, 3.47, 2.34
	δ	37.0	2.68 dd 13.9, 6.5	131.3, 128.9, 57.9, 50.0	7.20, 7.02, 3.47
			2.64 dd 13.9, 8.1	131.3, 128.9, 57.9, 50.0	7.20, 7.02, 3.47
	1′	128.9			
	2′,6′	131.3	7.02 d 8.5	157.4, 131.3, 37.0	2.68, 2.64
	3′,5′	116.6	6.71 d 8.5	157.4, 128.9, 116.6	
	4′	157.4			
	NH		7.20 d 2.7	174.0, 57.9, 50.0	3.65, 2.68, 2.64
formyl	CO	163.5	8.01 bs	50.6	4.91
Dpr	CO	170.3			
	α	50.6	4.91 m		8.26, 8.01
	β	41.7	3.49 m	174.1, 170.3, 50.6	8.26, 8.24
			3.35 ddd 13.1, 6.2, 3.5	174.1, 50.6	8.26, 8.24
	αNH		8.26 d 8.5	163.5	4.91, 3.49, 3.35
	*β*NH		8.24 dd 6.5, 6.2	174.1	3.72, 3.49, 3.35, 2.43, 2.34
Pro	CO	173.6			
	α	61.7	4.52 dd 8.1, 4.2	173.6, 49.0, 30.7, 25.7	7.83
	β	30.7	2.27 m	173.6, 61.7, 49.0, 25.7	7.83, 2.05, 1.94
			2.23 m	173.6, 61.7, 25.7	2.05, 1.94
	γ	25.7	2.05 m	61.7, 30.7	3.72, 3.67
			1.94 m	61.7, 30.7	3.72, 3.67
	δ	49.0	3.72 m	30.7, 25.7	8.24, 2.05, 1.94
			3.67 m	25.7	2.05, 1.94
k-Arg	CO	-			
	α	85.6			
	β	52.9	3.87 ddd 12.7, 9.0, 4.1		7.83, 1.78, 1.59
	γ	26.9	1.87 dddd 13.1, 13.1, 12.7, 3.3		7.83, 3.18
			1.78 dddd 12.7, 4.1, 3.6, 3.5	25.4	7.83, 3.87
	δ	25.4	1.74 dddd 13.9, 4.2, 3.6, 3.3		3.78, 3.18, 1.59
			1.59 ddddd 13.9, 13.5, 13.1, 3.7, 3.5		3.87, 3.78, 1.74
	ϵ	38.8	3.78 dd 13.3, 3.7	167.3, 85.6, 26.9, 25.4	3.18, 1.74, 1.59
			3.18 ddd 13.5, 13.3, 3.1		3.78, 1.87, 1.74
	guanidine	167.3			
	NH		7.83 d 9.0	173.6	4.52, 3.87, 2.27, 1.87

Scheme 2



Experimental Section

General. NMR spectra were recorded either on a Bruker AM 600 or JEOL α-600 spectrometer. Mass spectral data were obtained using a JEOL SX102/SX102 tandem mass spectrometer. Optical rotation of all compounds was measured by a JASCO DIP-1000 digital polarimeter. UV spectra were recorded on a Hitachi 330 spectrophotometer. Amino acid analyses were carried out using a Hitachi 8500 amino acid analyzer.

Sponge Specimens and Isolation. The sponge was collected by hand using SCUBA at a depths of 10−25 m off Hachijo-jima Island, 300 km south of Tokyo, and identified as *T. swinhoei* by Professor Rob van Soest. The 1993 collection (30 kg wet weight) was extracted with EtOH. The concentrated EtOH extract was partitioned between Et_2O and H_2O, and the aqueous layer was further extracted with *n*-BuOH. Fractionation was monitored by inhibition assay against thrombin and trypsin. The *n*-BuOH extract was separated by ODS flash chromatography using aq MeOH as mobile phase. The active fractions were eluted with 50, 70, and 100% MeOH, which were combined and further

(14) Maryanoff, B. E.; Zhang, H.; Greco, M. N.; Zhang, E.; Vanderhoff-Hanaver, P.; Tulinsky, A. *Tetrahedron Lett.* **1996**, *37*, 3667−3670.

Table 3. NMR Data for **3**−**6** in CD_3OH

		3		**4**		**5**		**6**	
		^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Phe	CO	172.9		169.5		168.4		172.0	
	α	59.5	3.67 t 4.2	55.2	4.28 dd 7.3, 6.6	55.2	3.98 dd 7.9, 6.0	55.4	4.62 ddd 8.4, 6.8, 5.4
	β	38.2	3.09 d 4.2	38.7	2.97 dd 14.2, 6.6	37.8	2.96 dd 14.2, 6.0	40.9	2.83 dd 13.1, 6.8
					2.83 dd 14.2, 7.3		2.81 dd 14.2, 7.9		2.72 dd 13.1, 5.4
	1	138.8		135.3		134.6		137.5	
	2,6	131.6	7.20	130.4	7.06 d 6.9	129.7	7.07 d 8.0	130.7	6.80 dd 8.1, 1.9
	3,5	128.9	7.20	129.9	7.28 t 6.9	129.3	7.29 m	129.2	7.16 m
	4	127.6	7.20	128.6	7.25 t 6.9	128.1	7.27 m	127.6	7.13 m
	NH								7.88 d 8.4
	NCH_2N	68.6	4.39 d 12.5						
			4.29 d 12.5						
e-Tyr	CO	171.3		168.3		167.8		167.8	
or	α	27.4	2.39 dd 17.5, 11.7	124.1	5.98 d 15.4	124.3	5.96 dd 15.6, 1.1	124.5	6.06 dd 15.8, 2.7
v-Tyr			2.27 dd 17.5, 4.8						
	β	53.6	3.14 ddd 11.7, 4.8, 3.9	144.5	6.78 dd 15.4, 5.0	143.8	6.69 dd 15.3, 6.2	143.9	6.76 dd 15.8, 2.3
	γ	55.5	3.25	53.4	4.72	53.7	4.69 m	53.4	4.57 m
	δ	36.1	2.54 dd 14.2, 6.9	40.3	2.78 dd 14.1, 6.2	39.4	2.79 dd 14.0, 6.4	39.7	2.82 dd 13.8, 6.2
			2.37 dd 14.2, 9.1		2.56 dd 14.1, 8.8		2.58 dd 14.0, 8.7		2.47 dd 13.8, 9.6
	1′	127.7		129.0		128.4		129.7	
	2′,6′	130.8	6.87 d 8.5	131.3	6.99 d 8.5	130.8	6.99 d 8.5	131.3	7.06 d 8.5
	3′,5′	116.6	6.68 d 8.5	116.3	6.69 d 8.5	115.5	6.70 d 8.5	116.4	6.75 d 8.5
	4′	157.6		157.4		156.8		157.4	
	NH		7.29 bs		8.46 d 8.5		8.41 d 8.4		8.18 d 8.1
formyl	CO	163.7	8.05 s	163.5	8.05 s	162.8	8.06 s	163.3	8.03 s
Dpr	CO	169.8		170.6		169.9		171.2	
	α	49.9	5.11 ddd 8.5, 8.5, 5.0	50.6	4.86 ddd 7.6, 7.3, 6.7	50.7	4.92 ddd 7.2, 6.7, 6.5	49.9	4.70 m
	β	36.1	3.81	41.6	3.71 ddd 13.1, 7.3, 6.7	40.9	3.60 dd 13.6, 6.7	41.1	4.23 m
			3.38		3.39 ddd 13.1, 7.3, 5.2		3.56 dd 13.6, 6.5		2.79 m
	αNH		8.32 d 8.5		8.37 d 7.6		8.29 d 7.2		8.44 br
	*β*NH				8.28 dd 7.3, 5.2		8.09 dd 6.1, 6.1		8.61 d 10.0
Pro	CO	173.4		173.6		176.4		174.1	
	α	61.7	4.48 dd 8.5, 3.9	61.7	4.51 dd 7.7, 5.8	60.7	4.43 dd 8.8, 4.7	61.8	4.49 dd 8.5, 5.4
	β	30.6	2.30	30.6		29.8	2.23 m	31.5	2.26 m
			2.18 dddd 12.7, 8.9, 8.9, 7.3				1.96 m		2.01 m
	γ	25.8	2.10	25.8	2.03	24.8	2.02 m	26.0	1.93 quint 6.5
			1.96		1.95		1.96 m		
	δ	48.8	3.85	49.0	3.74 ddd 10.0, 6.9, 6.9	48.2	3.77 ddd 10.1, 6.8, 6.5	49.1	3.78 ddd 10.0, 6.9, 6.9
			3.68		3.62 ddd 10.0, 6.5, 6.5		3.69 ddd 10.1, 7.1, 6.1		3.50 ddd 10.0, 6.9, 6.9
	NH						7.69 bs		
	NH						6.97 bs		
k-Arg	CO	186.4		186.9				173.1	
	α	85.5		85.5				73.3	4.08 bs
	β	52.0	4.07 ddd 12.7, 10.0, 3.9	51.9	3.87 ddd 13.1, 9.2, 3.9			53.1	4.21 m
	γ	26.8	1.91	26.6	1.90 dddd 13.1, 13.1, 13.1, 3.5			29.7	1.73 m
			1.73		1.75 dddd 13.1, 3.9, 3.7, 3.5				1.66 m
	δ	25.3	1.73	25.3	1.78			26.5	1.64 m
			1.61		1.56 ddddd 13.1, 13.1, 13.1, 4.4, 3.7				1.62 m
	ϵ	38.9	3.82	38.7	3.81 dd 13.9, 4.4			41.9	3.13 bt 6.2
			3.24		3.20 ddd 13.9, 3.7, 3.5				
	guanidine	163.2		165.8				158.5	7.27
	NH		8.07 d 8.5		8.03 d 9.2				7.95 d 10.0

separated by gel-filtration on a Sephadex LH-20 column (MeOH) and by ODS flash chromatography (aq MeCN containing 0.05% TFA). The active fractions eluted with 20 and 25% MeCN containing 0.05% TFA were combined and then subjected to ODS column chromatography (24% MeCN containing 0.05% TFA) affording 20 fractions. Fraction 3 (119.4 mg) was separated by ODS HPLC (COSMOSIL-5C_{18}AR; 15% MeCN containing 0.05% TFA) to yield pseudotheonamide A_1 (**1**: 44.0 mg) and an impure pseudotheonamide A_2 fraction (190.0 mg). The latter was separated in three steps by HPLC [COSMOSIL 5C_{18}-AR with 12% MeCN containing 0.05% TFA; capcell pak UG120 with 17% MeCN containing 0.05% TFA; and YMC−Pack A-624 S-5 120A NH_2 with $CHCl_3$/MeOH (1:1)] to yield 1.2 mg of pseudotheonamide A_2 (**2**). Fraction 11 (250.5 mg) was separated in two steps by ODS HPLC (COSMOSIL-5C_{18}AR with 23% MeCN containing 0.05% TFA; and COSMOSIL-5C_{18}MS with 21% MeCN containing 0.05% TFA), and finally on Asahipak GS320 column (10% MeCN containing 0.05% TFA) yielding pseudotheonamide B_2 (**3**: 4.5 mg) and dihydrocyclotheonamide A (**6**: 6.2 mg). Fraction 6 (156.4 mg) was fractionated by ODS HPLC (COSMOSIL-5C_{18}MS with 18% MeCN containing 0.05% TFA) and HPLC on Asahipak GS320 column (10% MeCN containing 0.05% TFA) to afford pseudotheonamide D (**5**: 4.4 mg). Pseudotheonamide C (**4**) was isolated from *T. swinhoei* collected in 1996. The aqueous layer of EtOH extract of the sponge (52 kg wet weight) was chromatographed on an ODS column [three steps: (1) aqueous MeOH, (2) aqueous MeCN, and (3) 15% MeCN with 0.05% TFA] and finally purified by ODS HPLC [capcell pak UG120 with 15% MeCN containing 0.05% TFA] to yield 11.4 mg of **4**.

Pseudotheonamide A_1 (1): colorless amorphous solid, $[\alpha]^{29}_D$ −28° (*c* 0.085, MeOH), UV (MeOH) 227 nm (*ϵ* 14000 sh), 243 (4500 sh), 278 (2000 sh), HR-FABMS $(M + H)^+$ *m*/*z* 732.3452 for $C_{36}H_{46}N_9O_8$ (Δ −1.8 mmu).

Pseudotheonamide A_2 (2): colorless amorphous solid, $[\alpha]^{29}_D$ −34° (*c* 0.065, MeOH), UV (MeOH) 227 nm (*ϵ* 17000 sh), 243 (7100 sh), 278 (2300 sh), HR-FABMS $(M + H)^+$ *m*/*z* 732.3466 for $C_{36}H_{46}N_9O_8$ (Δ −0.3 mmu).

Pseudotheonamide B_2 (3): colorless amorphous solid, $[\alpha]^{29}_D$ −17° (*c* 0.050, MeOH), UV (MeOH) 226 nm (ϵ 15000 sh), 243 (4500 sh), 278 (1800 sh), HR-FABMS $(M + H)^+$ *m*/*z* 744.3447 for $C_{37}H_{46}N_9O_8$ (Δ −2.2 mmu).

Pseudotheonamide C (4): colorless amorphous solid, $[\alpha]^{29}_D$ −16° (*c* 0.47, MeOH), UV (MeOH) 227 nm (ϵ 19000 sh), 242 (11000 sh), 278 (2300 sh), HR-FABMS $(M + H)^+$ *m*/*z* 732.3452 for $C_{36}H_{46}N_9O_8$ (Δ −1.8 mmu).

Pseudotheonamide D (5): colorless amorphous solid, $[\alpha]^{29}_D$ −11° (*c* 0.085, MeOH), UV (MeOH) 225 nm (ϵ 15000 sh), 243 (5100 sh), 278 (1600 sh), HR-FABMS $(M + H)^+$ *m*/*z* 565.2780 for $C_{29}H_{37}N_6O_6$ (Δ +0.6 mmu).

Dihydrocyclotheonamide A (6): colorless amorphous solid, $[\alpha]^{29}_D$ +37° (*c* 0.25, MeOH), UV (MeOH) 225 nm (ϵ 15000 sh), 243 (4800 sh), 278 (1400 sh), HR-FABMS $(M + H)^+$ *m*/*z* 734.3647 for $C_{36}H_{48}N_9O_8$ (Δ +2.2 mmu).

Amino Acid Analysis. For standard amino acid analysis, 100 mg portion of each peptide was dissolved in 6 N HCl and heated at 110 °C for 5 h in a sealed tube. After the solvent was removed in a stream of N_2, the residue was dissolved in 1% HCl and subjected to amino acid analysis. Retention times in the amino acid analysis were as follows: Pro (32.32 min), Phe (54.18 min).

Marfey Analysis. A portion (100 μg) of each peptide was hydrolyzed with 6 N HCl at 105 °C for 12 h. After the solution was dried in a stream of N_2, to the residue was added 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone (1 mg/mL) and 100 μL of 0.1 M $NaHCO_3$ and kept at 80 °C for 5 min. The reaction mixture was neutralized by adding 50 μL of 0.2 N HCl and diluted with 100 μL of 50% MeCN containing 0.05% TFA. A portion of this solution was subjected to ODS HPLC (COSMOSIL $5C_{18}$-MS, linear gradient from 0 to 50% MeCN containing 0.05% TFA). Peaks were identified by coinjection with standard amino acid derivatives.

Ozonolysis. A solution of each peptides **4**−**6** (100 μg each) in 1 mL of MeOH was cooled to 0 °C. A gentle stream of of ozone was bubbled into the solution for 10 min. After removal of excess ozone by N_2, the solution was warmed to room temperature. After addition of 100 μL of AcOH, the solution was concentrated. To the residual solution were added 50 μL of AcOH, 50 μL of H_2O, 30 μL of 30% hydrogen peroxide, and 1.2 μL of H_2SO_4, and the mixture was heated at 90 °C for 1 h. After cooled to room temperature, the mixture was stirred over Pd/C overnight. The reaction mixture was filterd and concentrated before acid hydrolysis (5 N HCl, 105 °C), and the hydrolysate was subjected to amino acid and Marfey analyses.

Isolation of k-Arg from 1. Cyclotheonamide A (20 mg) was hydrolyzed with 5 N HCl at 105 °C for 16 h. After the solution was dried under N_2 gas, the hydrolysate was subjected to anion-exchange chromatography (Dowex 1) with H_2O, followed by cation-exchange chromatography (COSMOGEL SP, 20 mM NH_4OAc, pH 6.6) to yield k-Arg (2.0 mg) in a cyclic form.

α-Keto-homoarginine (k-Arg; cyclic form): ^{1}H NMR (D_2O) δ 3.68 (dd 13.8, 4.2; Hβ), 1.90 (m; Hγ), 1.85 (m, Hγ), 1.78 (m, Hδ), 1.55 (m, Hδ), 3.32 (dd 12.6, 4.8; Hϵ), 3.16 (ddd 13.8, 12.6, 3.6 Hϵ). FAB-MS *m*/*z* 185 $(M + H)^+$.

Reduction of Cyclotheonamide A. Cyclotheonamide A (10 mg) was reduced with $NaBH_4$ in a mixture of THF and DMF. The reaction mixture was stirred at room temperature for 30 min and was subjected to ODS HPLC (COSMOSIL $5C_{18}$-MS, 17% MeCN containing 0.05% TFA) to yield dihydrocyclotheonamide A (8.6 mg), which gave the same ^{1}H NMR spectrum as that for **6**.

Reaction of Dihydrocyclotheonamide A. Dihydrocyclotheonamide A (8.6 mg) prepared from **1** was hydrolyzed with 6 N HCl at 110 °C for 4 h. After the hydrolysate was dried in a stream of N_2, it was subjected to anion-exchange chromatography on Dowex 1 (OH^- form) with H_2O to yield reduced k-Arg (2.4 mg). To the mixture of reduced k-Arg (1.2 mg) and Im_2CO (5 mg dissolved in 40 μL of DMF), 500 μL of DMF was added, and the mixture was stirred at room temperature for 5 h. The reaction mixture was lyophilized and subjected to NMR analysis without purification. ^{1}H NMR signals were assigned by means of HOHAHA data.

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Supporting Information Available: NMR spectra for **1**−**6** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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