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Thionation of Segetalins A and B, Cyclic Peptides with Estrogen-like Activity from Seeds of *Vaccaria segetalis*[1]

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Abstract—Thionation of estrogen-like active cyclic peptides, segetalins A (**1**) and B (**2**), with Lawesson's reagent provided each two thiosegetalins; thiosegetalin A1 [Gly-1-ψ(CS-NH)-Val-2; Trp-5-ψ(CS-NH)-Ala-6]segetalin A, thiosegetalin A2 [Gly-1-ψ(CS-NH)-Val-2; Ala-6-ψ(CS-NH)-Gly-1]segetalin A, thiosegetalin B1 [Gly-1-ψ(CS-NH)-Val-2; Ala-3-ψ(CS-NH)-Trp-4]segetalin B, and thiosegetalin B2 [Gly-1-ψ(CS-NH)-Val-2; Trp-4-ψ(CS-NH)-Ala-1]segetalin B. Thiosegetalin A2 only showed estrogen-like activity against ovariectomized rats. On the basis of their conformations analysed by NMR experiments, the backbone conformation was considered to play an important role in estrogen-like activity for segetalins.

Introduction

Modification of the peptide bonds has attracted great interest for changing structural and biological properties of peptide. Among them thionation, that is, replacement of amide bonds with thioamide bonds, can strongly influence the secondary structure in spite of a minimal variation of isosteric replacement and they may be helpful in the understanding of conformationally induced structure–activity relationships of bioactive compounds.[2] Lawesson's reagent as a thionating reagent is usually limited to the size of peptides owing to problems of regioselectivity and yields.[3] However, recently thionation of relatively large cyclic peptides such as cyclosporin A,[4] RA-VII[5] and astin[6] with Lawesson's reagent has been reported.

We have isolated cyclic peptides, named segetalins A (**1**) and B (**2**), with estrogen-like activity from the seeds of *Vaccaria segetalis* (Caryophyllaceae) (Fig. 1).[7,8] By conformational studies using NMR spectroscopy, X-ray diffraction, and computational methods, **1** and **2** took similar conformations as each other in the common region of Trp-Ala-Gly-Val.[9,10]

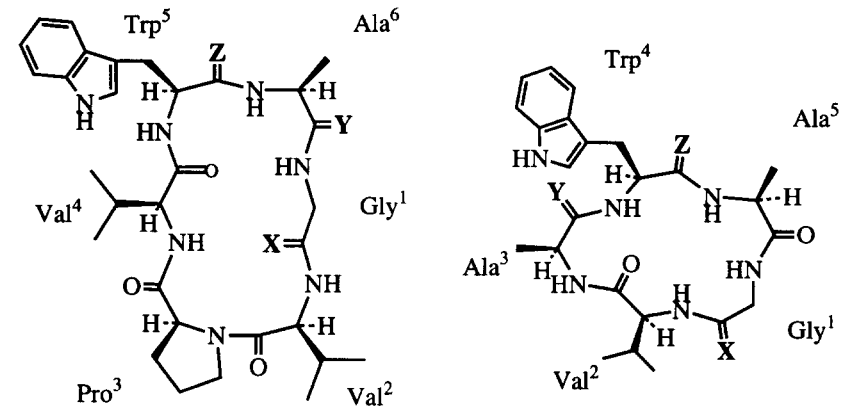
Conformation of cyclic peptides, which might be related to their biological functions, is quite interesting since it showed that many cyclic peptide backbones are not rigid in solution and have limited conformational flexibility. We have focused the influence of backbone modification of **1** and **2** as well as of conformational change by backbone modification on estrogen-like activity. In order to investigate the relationship between the conformations of segetalins and their activity, we carried out thionation of segetalins A (**1**) and B (**2**). In this paper, we describe thionation of **1** and **2** with Lawesson's reagent,[11] the structure elucidation, estrogen-like activity and conformational analysis of the produced thionated segetalins by NMR spectroscopy.

Results and Discussion

Thionation of segetalins A and B

Thionation of segetalin A (**1**) with 3 mol equiv of Lawesson's reagent [2,4-bis(4-methoxyphenyl)-1,3-dithio-2,4-diphosphetane-2,4-dithione] in dioxane at 50 °C for 30 min afforded two dithionated segetalins, named thiosegetalins A1 (**3**) and A2 (**4**) in 20 and 5% yield, respectively.

The structures of **3** and **4** were suggested to be dithionated by their molecular ion peaks in their FAB mass spectra [*m/z* 664 $(M+Na)^+$ for **3** and 642 $(M+H)^+$ for **4**]. Thionated position of each product was deduced from the comparison of thioamide proton and amide proton chemical shifts, and further that of thiocarbonyl carbon and carbonyl carbon shifts in NMR spectra. It is known that the thioamide protons and thiocarbonyl carbons in thiopeptides resonate in lower fields than those in parent peptides.[11] 2D NMR analysis using ^{1}H-^{1}H COSY, HMQC[12], HMBC[13] and ROESY[14] spectra revealed the ^{1}H and ^{13}C signal assignments of the thionated products (**3** and **4**) as listed in Tables 1 and 2. In thiosegetalin A1 (**3**), Val^2- and Ala^6-NH assignable to δ 9.44 and 10.31 in thioamide protons were resonated at δ 7.42 and 8.93, respectively, in segetalin A (**1**) in DMSO-d_6. In addition, the thioamide proton signals of Gly^1-NH (δ 9.41) and Val^2-NH (δ 10.26) of thiosegetalin A2 (**4**) were resonated at a lower



segetalin A (**1**) X=O Y=O Z=O
thiosegetalin A1 (**3**) X=S Y=O Z=S
thiosegetalin A2 (**4**) X=S Y=S Z=O

segetalin B (**2**) X=O Y=O Z=O
thiosegetalin B1 (**5**) X=S Y=S Z=O
thiosegetalin B2 (**6**) X=S Y=O Z=S

Figure 1. Structures of segetalins A, B, and thiosegetalins A1, A2, B1, and B2.

Table 1. ^{1}H NMR chemical shifts (ppm) of thiosegetalins (**3**, **4**, **5** and **6**)

	Proton	3	4		5	6
Gly^1				Gly^1		
	Hα	4.54(dd,6.8,16.0)	4.66(dd, 4.6,13.4)		4.51(dd,5.7,15.4)	4.60(dd,6.7,14.8)
	Hα	3.49(m)	4.05(dd,4.6,13.4)		3.81(dd,5.0,15.4)	3.72(dd,5.2,14.8)
	NH	7.73(dd, 5.0, 6.8)	9.41(t, 4.6)		8.52(dd, 5.0,5.7)	9.18(dd,5.2,6.7)
Val^2				Val^2		
	Hα	4.92(m)	5.32(dd,6.4,8.7)		4.79 (dd,8.5,8.7)	4.73(m)
	Hβ	2.14(m)	2.15(m)		2.19(m)	2.18(m)
	Hγ	0.89(d,6.9)	0.92(d,6.6)		0.93(d,6.7)	0.93(d,6.6)
	Hγ	0.87(d,6.8)	0.88(d,6.8)		0.91(t, 6.9)	0.91(d,6.8)
	NH	9.44(d,6.4)	10.26(d,6.4)		9.41(d, 8.7)	9.75(d,9.2)
Pro^3				Ala^3		
	Hα	4.56(m)	4.74(m)		4.56(m)	3.97(m)
	Hβ	2.14(m)	2.01(m)		1.37(d,7.3)	1.19(d,7.2)
	Hβ	1.86(m)				
	Hγ	1.86(m)	1.92(m)			
	Hγ	1.63(m)	1.76(m)			
	Hδ	3.46(m)	3.76(m)			
			3.65(m)			
	NH				8.83(d, 8.3)	8.25(d,8.3)
Val^4						
	Hα	4.02(t,6.0)	3.82(m)			
	Hβ	1.86(m)	2.32(m)			
	Hγ	0.71(d,6.2)	0.83(d,6.8)			
		0.70(d,6.2)	0.82(d,6.8)			
	NH	7.22(d,6.0)	7.06(d,7.7)			
Trp^5				Trp^4		
	Hα	4.61(ddd,6.1,6.8,6.9)	4.38(m)		5.04(ddd,6.9,7.2,7.8)	4.73(m)
	Hβ	3.41(m)	3.09(m)		3.35(dd,6.9,14.4)	3.31(m)
					3.29(dd,7.2,14.4)	
	NH	8.22(brs)	8.41(d,7.6)		9.78(d,7.8)	8.19(d,7.9)
	1(NH)	10.88(d,1.8)	10.08(d,2.0)		10.84(d,2.3)	10.85(d,2.0)
	2	7.10(d,1.8)	7.18(d,2.0)		7.10(d,2.3)	7.14(d,2.0)
	4	7.56(d,7.7)	7.56(d,7.7)		7.61(d,7.8)	7.60(d,8.0)
	5	7.09(t,7.7)	7.06(t,7.7)		7.06(t,7.8)	7.06(t,8.0)
	6	6.99(t,7.7)	6.97(t,7.7)		6.97(t,7.8)	6.99(t,8.0)
	7	7.34(d,7.7)	7.32(d,7.7)		7.32(d,7.8)	7.33(d,8.0)
Ala^6				Ala^5		
	Hα	4.91(brm)	3.95(m)		4.10(m)	4.97(m)
	Hβ	1.72(d,6.8)	1.36(d,7.0)		1.22(d,6.8)	1.31(d,6.8)
	NH	10.31(brs)	8.64(d,7.1)		8.43(d,7.3)	9.61(d,7.2)

Measurements were performed in DMSO-d_6 at 400 MHz.
Multiplicity and coupling constants (J/Hz) are in parentheses.

Table 2. ^{13}C NMR chemical shifts (ppm) of thiosegetalins (**3**, **4**, **5** and **6**)

Proton	3	4	5	6
Gly^1			Gly^1	
Cα	50.35	53.85	50.90	51.43
C=O	200.23	196.96	200.64	200.91
Val^2			Val^2	
Cα	61.64	61.09	65.13	64.97
Cβ	31.40	28.78	30.00	30.44
Cγ	18.57	18.28	19.06	18.80
Cγ	17.91	18.24	18.64	18.39
C=O	169.59	169.77	168.69	168.57
Pro^3			Ala^3	
Cα	60.49	61.14	58.69	49.98
Cβ	30.66	31.58	18.20	16.67
Cγ	21.76	21.40		
Cδ	46.43	45.95		
C=O	171.62	170.53	203.07	170.67
Val^4				
Cα	59.74	59.82		
Cβ	31.76	28.92		
Cγ	18.91	18.41		
Cγ	18.81	18.36		
C=O	171.51	170.53		
Trp^5			Trp^4	
Cα	64.45	55.15	61.05	63.84
Cβ	27.49	27.89	26.20	29.30
2	123.51	123.48	123.64	123.63
3	109.40	109.25	109.01	110.13
4	118.07	118.32	118.26	118.11
5	121.05	120.89	120.94	120.93
6	118.39	118.21	118.44	118.33
7	111.43	111.22	111.30	111.38
8	136.14	136.03	136.00	136.05
9	126.95	127.22	127.31	127.09
C=O	202.73	170.64	169.36	202.54
Ala^6			Ala^5	
Cα	53.80	55.81	49.03	54.19
Cβ	14.97	18.68	16.26	15.70
C=O	170.43	200.80	171.65	171.27

Measurements were performed in DMSO-d_6 at 100 MHz.

field than those (δ 7.45 and 7.42, respectively) of **1**. In ^{13}C NMR spectra, the corresponding thiocarbonyl carbon of Gly^1 (δ 200.23) and Trp^5 (δ 202.73) in **3** and Gly^1 (δ 196.96) and Ala^6 (δ 200.80) in **4** were resonated at a lower field than those in segetalin A (Gly^1 δ 169.70, Trp^5 δ 172.95, Ala^6 δ 171.04). Therefore, the structures of **3** and **4** were elucidated to be [Gly-1-ψ(CS-NH)-Val-2; Trp-5-ψ(CS-NH)-Ala-6]segetalin A and [Gly-1-ψ(CS-NH)-Val-2; Ala-6-ψ(CS-NH)-Gly-1]segetalin A, respectively.

Segetalin B (**2**) was also treated with Lawesson's reagents under the same condition as mentioned above and two thionated products (**5** and **6**) were obtained in 34 and 8% yield, respectively. The mass spectral parent ions [517 $(M+H)^+$ for **5** and **6**] indicated that both are dithionated compounds. On the basis of ^1H and ^{13}C assignments (Tables 1 and 2), it was apparent that compound **5** was thionated at both Gly^1 and Ala^3, and **6** at both Gly^1 and Trp^4. Therefore, the structures of **5** and **6** were elucidated to be [Gly-1-ψ(CS-NH)-Val-2; Ala-3-ψ(CS-NH)-Trp-4] segetalin B, and [Gly-1-ψ(CS-NH)-Val-2; Trp-4-ψ(CS-NH)-Ala-5] segetalin B, respectively.

It is known that the sulfur atom is not liable to introduce to sterically hindered amide carbonyl groups.[15] For this reason, sulfur atoms were considered to be introduced to Gly residues in **1** and **2**, whereas the difference in conformational state between **1** and **2** in the reaction mixture may account for the varying sulfur atom position of each product.

Estrogen-like activity

The estrogen-like activity was examined using five ovariectomized S.D. rats of four weeks old for each compound. The increment in the weight of uterus when the thiosegetalins (**3–6**) were administrated at a dosage of 2.5 mg kg^{-1} to ovariectomized rats consecutively for 14 days, is shown in Table 3. The weight of uterus was increased greater than that of the control, when only the thiosegetalin A2 was administrated, however the other ones did not show the activity. Therefore, thiosegetalin

Table 3. Effect of segetalins A and B from *V. segetalis* and their thioderivatives on ovariectomized rats uterine weight

Drug	Dose	Animal (*n*)	Uterus weight (mg)
Control		5	36.4 ± 1.85
Segetalin A (**1**)	2.5 mg/kg	5	54.4 ± 6.81*
Segetalin B (**2**)	2.5 mg/kg	5	56.3 ± 6.45*
Thiosegetalin A1 (**3**)	2.5 mg/kg	5	45.6 ± 1.58
Thiosegetalin A2 (**4**)	2.5 mg/kg	5	52.0 ± 2.67*
Thiosegetalin B1 (**5**)	2.5 mg/kg	5	45.8 ± 1.61

The values are mean ± S.E.
*Difference significantly from control by multiple comparison of Dunnett ($p<0.05$).

Table 4. Temperature coefficients, $-d\delta/dT$ (10^{-3} ppm/K), of NH protons of segetalins A and B (**1** and **2**), and thiosegetalins A1–B2 (**3–6**) in ten intervals over the range 300–330 K in DMSO-d_6

	Gly^1	Val^2	Val^4	Trp^5	Ala^6
Segetalin A (**1**)	2.8	−1.5[a]	0.3	5.0	9.4
Thiosegetalin A1 (**3**)	5.4	6.2	−2.4	9.7	6.4
Thiosegetalin A2 (**4**)	2.8	7.5	−0.7	3.5	5.9
	Gly^1	**Val^2**	**Ala^3**	**Trp^4**	**Ala^5**
Segetalin B (**2**)	4.6	5.0	3.3	3.3	5.0
Thiosegetalin B1 (**5**)	2.8	1.4	2.9	1.1	3.5
Thiosegetalin B2 (**6**)	7.6	3.3	3.0	3.3	−0.3

[a]The negative temperature coefficient was presumed to result from conformational fluctuation around Val^2-NH proton.[8]

A2 possessed the similar estrogen-like activity to the parent compound, segetalin A.

Conformation of thiosegetalins A1, A2, B1, and B2

Conformational analysis of segetalins A and B in solid and solution have been reported previously,[9,10] indicating that the conformation of segetalin A was similar to that of segetalin B in the region of -Trp-Ala-Gly-Val-. Solution forms of thiosegetalins **3–6** were deduced on the basis of NMR spectral data including ROE experimental results and temperature effect on NH protons, and the comparison with those of their parent compounds.

The ^{1}H and ^{13}C NMR sharp signals of thiosegetalins **4–6** indicated that each one was shown to exist in a single stable conformational state in DMSO-d_6, whereas the ^{1}H signals around Ala^6 of thiosegetalin A1 (**3**) were observed as slightly broad signals, indicating conformation mobility around Ala^6 residue. Variation of the secondary structure of cyclic peptides in solution is well reflected by the temperature dependence of the NH proton chemical shifts.[16] Low temperature dependence of NH protons (<3 ppb T^{-1}) in such a solvent as DMSO-d_6 implies that the NH protons are shielded from the solvent through hydrogen bonding. The temperature coefficient ($d\delta/dT$) of NH protons of **3–6** in 10 intervals over the range 300–330 K are compared with those of **1** and **2** (Table 4). As can be seen from Table 4, the coefficients between **3** and **4**, and between **5** and **6** were slightly different each other. Hydrogen donor activity of the nitrogen next to the thiocarbonyl group is known to increase, comparing to that of an amide bond.[17] The low temperature coefficients of NH protons of Gly^1 and Val^4 in thiosegetalin A2 (**4**), which is located next to the thiocarbonyl group, indicated that these amide protons are involved in strong intramolecular hydrogen bonds in DMSO-d_6. Position of the amide protons of **4** involved in intramolecular hydrogen bonds is the same as that in segetalin A (**1**).[8] However, the coefficient (5.4 ppb K^{-1}) of Gly^1 in **3** indicated that it was not shielded from the solvent. Therefore, the amide protons in **1** and **4** exhibited the same shielding

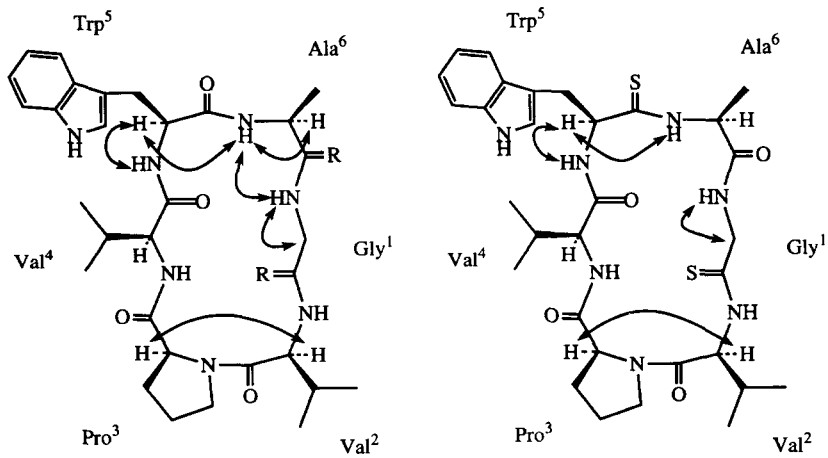


Figure 2. Selected ROE correlations of **1**, **3**, and **4** by phase sensitive ROESY spectra in DMSO-d_6.

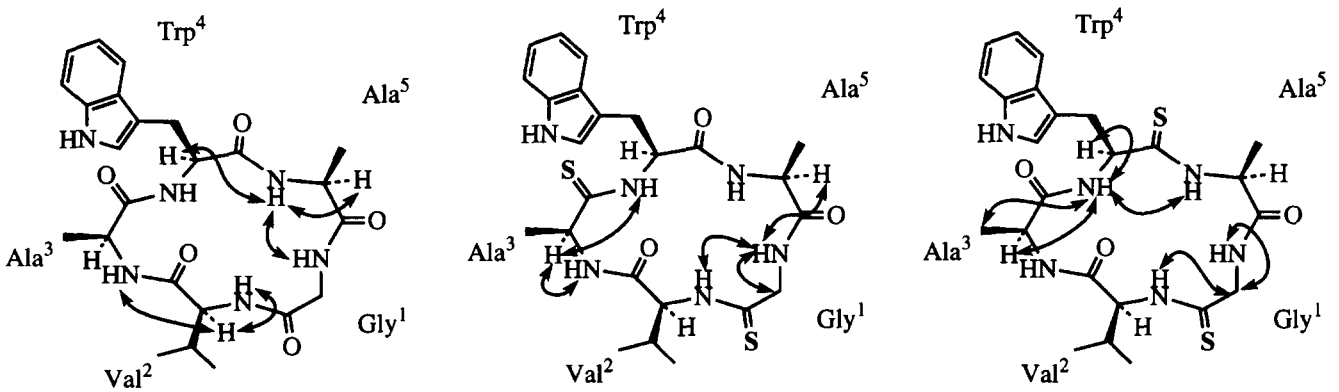


Figure 3. Selected ROE correlations of **2**, **5**, and **6** by phase sensitive ROESY spectra in DMSO-d_6.

pattern in DMSO-d_6. All of the amide protons in segetalin B (**2**) were not involved in intramolecular hydrogen bonds.[9] However, the amide protons of Val^2 and Trp^4 in thiosegetalin B1 (**5**) and that of Trp^4 in thiosegetalin B2 (**6**) are involved in strong intramolecular hydrogen bonds in DMSO-d_6, indicating that the solution conformations of **2**, **5** and **6** are different to each other.

The similarity of solution conformation between segetalin A (**1**) and thiosegetalin A2 (**4**) was also implied by the strong ROE enhancements of **1**, **3** and **4** in Figure 2 observed by phase-sensitive ROESY spectra[14] in DMSO-d_6. The strong ROEs between Trp^5-Hα and Ala^6-NH, between Ala^6-NH and Ala^6-Hα, and between Ala^6-NH and Gly^1-NH in **1** and **4** were observed, indicating the presence of type II β-turn structure between Trp^5 and Ala^6. However, in thiosegetalin A1 (**3**), the latter two correlations as indicated above were not observed, and both Ala^6-NH and Ala^6-Hα were observed as broad signals. The observation of a strong ROE cross-peak between Val^2-Hα and Pro^3-Hα in **1**, **3**, and **4** provided strong evidence for the occurrence of a *cis* peptide bond and was shown to commonly take a characteristic type VI β-turn.

Conformational change between segetalin B and thiosegetalins B1 and B2 was also suggested by the difference of ROE relationship. Segetalin B takes a type II-like β-turn at Trp^4 and Ala^5 residues in DMSO-d_6.[9] Selected ROE correlations of thiosegetalins B1 and B2 are shown in Figure 3. In the thiosegetalin B1 (**5**), strong ROE correlations between Ala^5-Hα and Gly^1-NH, between Gly^1-NH and Gly^1-Hα, and between Gly^1-NH and Val^2-NH suggest the presence of β-turn at Ala^5 and Gly^1 residues. On the other hand, in the thiosegetalin B2 (**6**), the presence of β-turn at Ala^3 and Trp^4 residues was implied by the ROEs between Trp^4-NH and Ala^3-Hα, between Trp^4-NH and Ala^3-Hβ, between Trp^4-NH and Trp^4-Hα, and between Trp^4-NH and Ala^5-NH.

From the foregoing evidence, the thiosegetalin A2 (**4**) only took the similar solution conformation to that of parent segetalin A and showed estrogen-like activity. The conformation of thiosegetalins A1, B1 and B2 were considered to be changed by thionation. In this manner, thionation is a useful and convenient method to investigate the conformational structure–activity relationship, and the conformation of segetalins may play an important role in their biological activity.

Further attempts to obtain the relation between conformational homology and estrogen-like activity of segetalins and the related ones as well as details of their mode of action are currently in progress.

Experimental

General details

Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. FAB and high-resolution mass spectra were taken with a VG Autospec spectrometer. IR spectrum was recorded on a Perkin–Elmer 1710 spectrophotometer. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm, GL Science Inc.) packed with 10 mm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent[18] and iodine. ^1H and ^{13}C NMR spectra were recorded on a Bruker (AM400 and AM500) and Varian Unity 400 spectrometers at 303 K. ROESY experiments were made with a mixing time of 100 ms. The NMR coupling constants (J) are given in Hz.

Materials

Segetalins A and B were prepared according to the previous procedure.[6,8]

Thionation of 1 and 2

Solutions of **1** and **2** (12.2 and 9.7 mg, respectively; 0.02 mmol) in 1,4-dioxane (1 ml) and Lawesson's reagent (24.3 mg, 0.06 mmol) were stirred at 50 °C. After 30 min, water (3 ml) was added to the mixture which was left standing for 3 h. Each reaction mixture was concentrated to dryness and the residue was chromatographed on alumina with $CHCl_3$:MeOH (10:0–9:1). Finally, Segetalin A gave thiosegetalins A1 (**3**, 2.5mg) and A2 (**4**, 0.6mg) by reversed phase HPLC using 50% CH_3CN followed 65% MeOH as eluent, in the case of segetalin B, reversed phase HPLC using 35% CH_3CN afforded thiosegetalins B1 (**5**, 3.5mg) and B2 (**6**, 0.8mg).

Thiosegetalin A1(3)

Colourless powder, $[\alpha]_D$ −155.7° (c 0.12, MeOH); *m/z* 664 $(M+Na)^+$ [Found: $(M+Na)^+$, 664.2715. $C_{31}H_{43}N_7O_4S_2Na$ requires, 664.1607]; ν_{max} (KBr) cm^{-1} 3279, 1667,; λ_{max} (MeOH) nm 224 (log ε 4.50) and 271 (log ε 4.24).

Thiosegetalin A2(4)

Colourless powder, $[\alpha]_D$ −65.0° (c 0.20, MeOH); *m/z* 642 $(M+H)^+$ [Found: $(M+H)^+$, 642.2896. $C_{31}H_{44}N_7O_4S_2$ requires, 642.2861]; ν_{max} (KBr) cm^{-1} 3254, 1667; λ_{max} (MeOH) nm 220 (log ε 4.5), 271 (log ε 4.36).

Thiosegetalin B1(5)

Colourless powder, $[\alpha]_D$ −152.0° (c 0.10, MeOH); *m/z* 517 $(M+H)^+$ [Found: $(M+H)^+$, 517.2055. $C_{24}H_{33}N_6O_3S_2$ requires, 517.1953]; ν_{max} (KBr) cm^{-1} 3290, 1667; λ_{max} (MeOH) nm 221 (log ε 4.35) and 270 (log ε 4.27).

Thiosegetalin B2(6)

Colourless powder, $[\alpha]_D$ −30.0° (c 0.20, MeOH); *m/z* 517$(M+H)^+$ [Found: $(M+H)^+$, 517.2055. $C_{24}H_{33}N_6O_3S_2$ requires, 517.1953]; ν_{max} (KBr) cm^{-1} 3270, 1656; λ_{max} (MeOH) nm 219 (log ε 4.34), 270 (log ε 4.17).

Estrogen-like activity on uterine weight in ovariectomized rats

Four-week-old female rats of S.D. strain weighing 70–80 g, were used in groups of five animals. The day following ovarectomy (first day) each rat was subcutaneously administered daily with the test drug (2.5 mg kg^{-1} day^{-1} of segetalins A and B, thiosegetalins A1, A2 and B1) until the 14th day. On the 15th day, the uterus was excised and weighed to evaluate the activity. The activity of thiosegetalin B2 could not be evaluated because of its paucity.

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