Application of a new TLC chemical method for detection of cyclopeptides in plants

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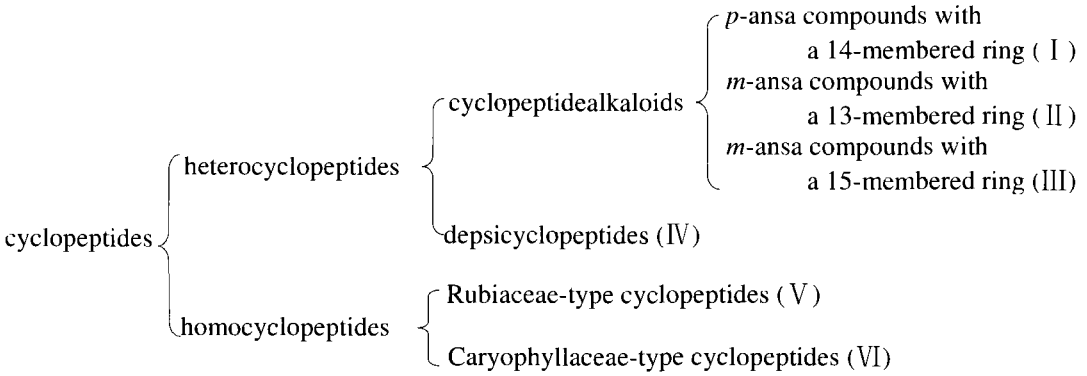
Abstract Cyclopeptides have been investigated phytochemically less often because until now there has not been a special chemical method to detect them. Since we found cyclopeptides in *Pseudostellaria heterophylla* (Caryophyllaceae) in 1991, we have gradually established a special chemical detection method for detecting cyclopeptides in plants, which induces a new thin layer chromatography (TLC) protosite reaction with ninhydrin reagent. With this method, our group isolated and determined 73 cyclopeptides from 17 plants which belong to 5 families and 14 genuses, they are from dicyclopeptides to undecacyclopeptides, including 68 new ones, and were determined based on spectral, chemical and enzymic methods, especially 2D NMR and FAB-MS. Meantime, with this method cyclopeptides can be distinguished from peptidic amides based on their behaviour in TLC.

Keywords: **TLC chemical detection method, cyclopeptides.**

We define cyclopeptides as cyclic compounds formed mainly with the peptide bonds of protein or non-protein amino acids. Since adouetines X, Y and Z were isolated from *Waltheria americana* in 1963 by Goutarel, more than 200 cyclopeptides have been obtained up to now and their structures have been determined. Cyclopeptides have mainly been found in plants of the following families: Annonaceae, Caryophyllaceae, Rhamnaceae, Rubiaceae; and also found in plants of Compositae, Hymenocardiaceae, Labiatae, Myrsinaceae, Pandaceae, Solanaceae, Sterculiaceae, Urticaceae, and Verbenaceae. The first systematic classification of cyclopeptides was proposed by us based on their skeletal structures. Cyclopeptides can be divided into two classes (heterocyclopeptides and homocyclopeptides), including six subclasses, as follows (fig. 1)[1]:



There is no special chemical method for detecting cyclopeptides in plants currently. Because there are no free amino groups (NH or NH_2) in structures of most cyclopeptides, they cannot react with ninhydrin reagent. In some phytochemical studies of types Ⅰ, Ⅱ and Ⅲ (cyclopeptide alkaloids), Dragendorff's reagent (a special reagent for detecting alkaloids) has been used to detect them, which is a non-specific and lower sensitive method. Since we found cyclopeptides heterophyllin A and B from *Pseudostellaria heterophylla* (Caryophyllaceae) in 1991[2], we have gradually established a special chemical method for detecting cyclopeptides in plants. This method is a new thin layer chromatography (TLC) protosite reaction with ninhydrin reagent. By this method, 73 cyclopeptides were isolated by our group and their structures were elucidated from 17 plants which belong to 5 families and 14 genuses, from dicyclopeptides to undecacyclopeptides, including 68 new ones (table 1). Peptidic amides , which are defined as amides with a peptide bond of the amino group (NH or NH_2) of one α-amino acid

combined with the carboxyl group (COOH) in the side chain of the α-amino acid (lactams) or other carboxyl groups of carboxylic acids, can be detected by this method, too. With this method we found that pyroglutamic acid with cardiovascular and nervous system activities is one of the main constituents in Chinese patent drug "Shengmaisan". We also found that pyroglutamic acid existed in some famous traditional Chinese medicines, such as *Panax ginseng*, *Schizandra chinensis*, *Ophiopogon japonicus*, and *Gastrodia elata*. In 2D-TLC, cyclopeptides can be distinguished from peptidic amides well based on their behaviour. In this note we describe this new method and list all the cyclopeptide structures and their sources obtained by our group with this method.

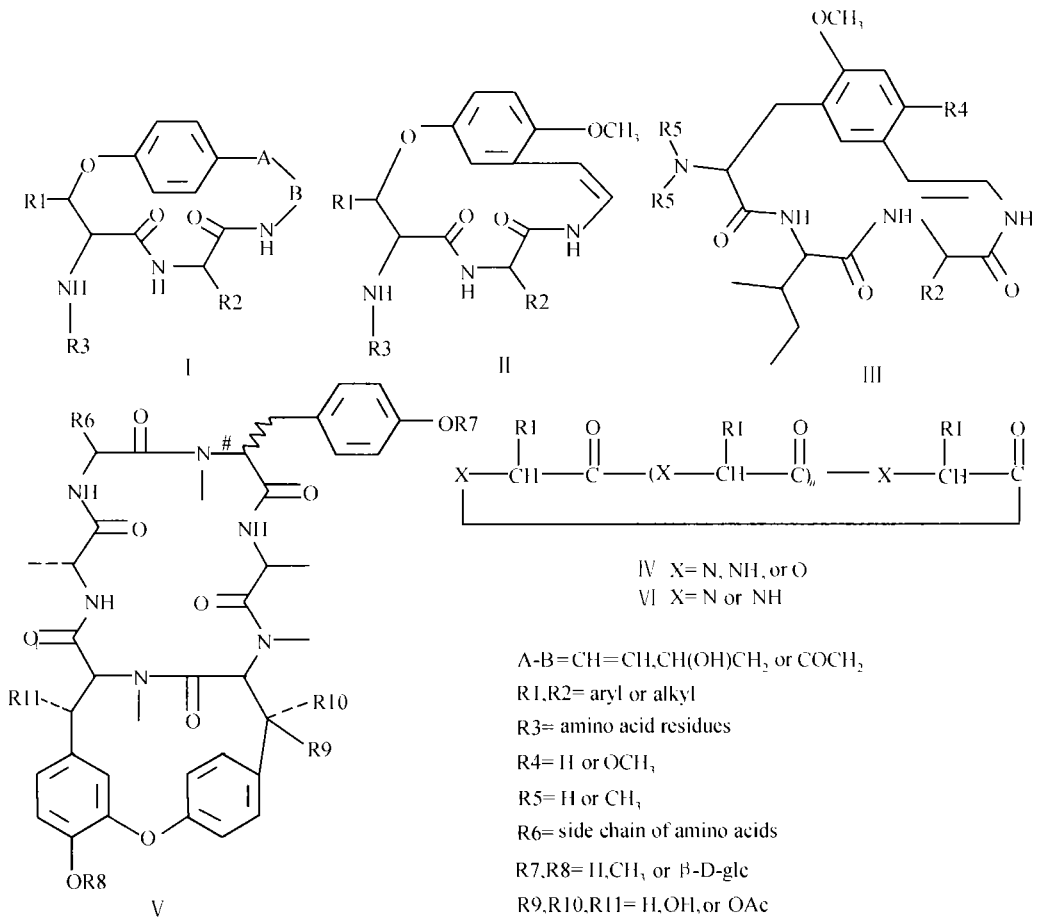


Fig. 1. Six types of cyclopeptides.

1 Experimental

(i) Materials. All solvents and reagents used were of chemical purity. Thin layer chromatographic (TLC) silica gel G plates and column chromatographic (CC) silica gel H were products of the Qindao Marine Chemical Factory, China. CC reverse phase-18 (RP-18) and MCI were supplied by Merck Company. Samples for detection were extracts and/or fractions from plants. The example plant *Pseudostellaria heterophylla* was cultivated in Jiangsu Province, China and was collected in 1993.

(ii) Sample preparation. We extracted 50—100 g of dried crushed plant materials with 95% EtOH by refluxing for 3 h, and the extracts were concentrated *in vacuo*. The EtOH extracts were partitioned with EtOAc or $CHCl_3$ and then BuOH. Removal of solvent furnished the sample fractions, in which EtOAc or $CHCl_3$ fraction was used to detect cyclopeptides and peptidic amides, and BuOH

fractions were used for detection of cyclopeptide glycosides. If the plant materials were the seeds of plants or contained more oil principles, the first process was the defatting with petroleum ether by refluxing for 3 h and then the preparation of the samples according to the above-mentioned method. If the prepared samples contain excessive pigments or water-soluble principles, we suggest that they should be separated at first by normal (silica gel H, $CHCl_3$-MeOH) or reverse (RP-18 or MCI, MeOH-H_2O) phase CC, respectively. For example, 100 g of dried crushed roots of *Pseudostellaria heterophylla* (Miq.) Pax ex Pax et Hoffm. were prepared according to the above-mentioned method, and the EtOAc fractions were detected according to the following method.

(iii) Cyclopeptide and peptidic amide detection. The sample was dotted at one corner of each of two identical 25 mm×50 mm silica gel G plates (plates 1 and 2) and these plates were developed with $CHCl_3$-MeOH (8.5:1.5 or 9:1) in 1D-TLC. After removal of the solvent, plate 2 was placed and hanged in a sealed glass vessel with about 1 mL concentrated HCl and hydrolyzed in a drying incubator (110℃) for 1—2 h. Cooled for a few minutes, plate 2 was taken out, and the HCl was volatilized with a ventilator. Then plates 1 (non-hydrolyzed plate) and 2 (hydrolyzed plate) were sprayed with 0.2% ninhydrin-acetone reagent and detected after heating with a drier for several minutes. Repeat the above-mentioned process once more. If there are some purplish red spots in most cases and/or yellow spots in a few cases in plate 2, but there is no spots at the same sites in plate 1, it is indicated that the detected samples contain cyclopeptides and peptidic amides.

(iv) Distinction between cyclopeptide and peptidic amide. The sample was dotted at one corner of a 50 mm×50 mm silica gel G plate (plate 3) according to the method of 2D-TLC, then the plate was developed with $CHCl_3$-MeOH (8.5:1.5 or 9:1) in the first dimension. After removal of the solvent, plate 3 was placed and hanged in a sealed glass vessel with about 1 mL concentrated HCl and hydrolyzed in a drying incubator (110℃) for 1—2 h. Cooled for a few minutes, plate 3 was taken out, and the HCl was volatilized with a ventilator. Then plate 3 was developed with $CHCl_3$-MeOH-HAc (8:2:2 drops) or $CHCl_3$-MeOH-H_2O-HAc (7:3:0.5:4 drops) in the second dimension. After removal of the solvent, plate 3 was sprayed with 0.2% ninhydrin-acetone reagent and detected after heating with a drier for several minutes. Repeat the above-mentioned process once more. If there is just one reddish purple spot in the same direction of the second dimension, it is indicated that the detected samples contain peptidic amides. If there are some purplish red or yellow spots in the same direction of the second dimension, it is indicated that the detected samples contain cyclopeptides.

2 Results and discussion

(i) Evaluation of results. Comparison of the spots of plate 1 (1D-TLC non-hydrolyzed plate) with those of plate 2 (1D-TLC hydrolyzed plate) showed a positive reaction only when plate 2 showed some purplish red or yellow spots, but plate 1 did not have such spots at the same sites, indicating that the detected samples contained cyclopeptides and peptidic amides. The spot numbers, color and color changes were analyzed after spraying in the second dimension of 2D-TLC of plate 3 (2D-TLC hydrolyzed plate). It contains peptidic amides only if there is only one reddish purple spot in the same direction of the second dimension, but it contains cyclopeptides if there are some purplish red or yellow spots (for details see subsect. (iv) of sect. 2).

(ii) Detection results of *Pseudostellaria heterophylla*. The TLC results of the EtOAc fractions prepared from the roots of *Pseudostellaria heterophylla* are shown in fig. 2. The results indicated that the plant contains at least three cyclopeptides. By the 1D-TLC method used here, eight cyclopeptide heterophyllins from A to H were isolated (table 1).

(iii) Applications in separation. With this 1D-TLC method, 73 cyclopeptides were isolated from 17 plants (table 1) and their structures were determined by various spectral (especially 2D NMR, FAB-MS) and chemical methods.

(iv) How to distinguish cyclopeptides from peptidic amides. In addition to the cyclopeptides found from plants by this method, peptidic amides, including pyroglutamic acid and its ester derivatives

and its analogues, and other amides which react positively with ninhydrin reagent, were isolated[3,4][1]. In order to distinguish between them, we compared their 1D- and 2D-TLC behavior in detail and found that there are differences in spot numbers of 2D-TLC, in color and color changes after spraying. Cyclopeptides have two or more spots in the same direction of the second dimension of 2D-TLC, and the color is commonly purplish red or yellow, which fades quickly. However, peptidic amides have one long spot in the same direction of the second dimension of 2D-TLC, and the color is commonly reddish purple, which fades slowly.

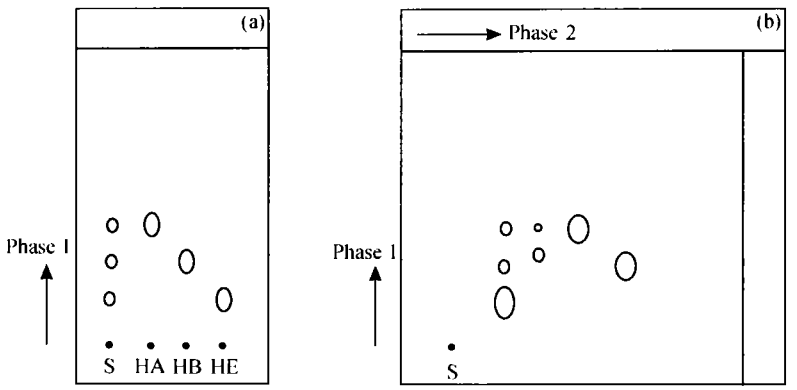


Fig. 2. TLC results of EtOAc fractions from *P. heterophylla* for detecting cyclopeptides. (a) 1D-TLC. Solid phase: silical gel G; mobile phase: $CHCl_3$-CH_3OH (85 : 15); detection reagent: 0.2% ninhydrin-acetone. (b) 2D-TLC. Solid phase: silical gel G; mobile phase: phase 1, $CHCl_3$-CH_3OH (85 : 15); phase 2, $CHCl_3$-CH_3OH-HAc (8 : 2 : 2 drops); detection reagent: 0.2% ninhydrin-acetone. S indicates the EtOAc fractions of *P. heterophylla*. HA, HB and HE are cyclopeptide heterophyllins A, B and E, respectively.

Table 1 Cyclopeptides (1—73) isolated and elucidated based on 2D NMR and FAB-MS[a)]

No.	Source	Cyclopeptide (No.)	Structure[b)]	Ref.
1	*Annona glabra* (Annonaceae, seeds)	glabrin A (1)	cyclo-(Pro-Gly-Leu-Val-Ile-Tyr)	[5]
		glabrin B (2)	cyclo-(Pro-OMet-Val^1-Ala-Val^2-Tyr-Gly-Thr)	[5]
		glabrin C (3)	cyclo-(Pro-Gly-Tyr-Val^1-Leu^1-Ala-Leu^2-Val^2)	[6]
		glabrin D (4)	cyclo-(Pro^1-Pro^2-Val-Tyr-Gly-Pro^3-Glu)	[6]
2	*A. muricata* (seeds)	annomuricatin A (5)	cyclo-(Pro-Phe-Val-Ser-Ala-Gly)	[7]
		annomuricatin B (6)	cyclo-(Pro-Asn-Ala-Trp-Leu-Gly-Thr)	[8]
3	*A. squamosa* (seeds)	annosquamosin A (7)	cyclo-(Pro-OMet-Thr-Ala-Ile-Val-Gly-Tyr)	[9]
4	*Arenaria juncea* (Caryophyllaceae, roots)	arenarin A (8)	cyclo-(Ser^2-Ser^1-Phe^2-Ile-Pro^2-Pro^1-Phe^1)	
5	*Brachystemma calycinum* (Caryophyllaceae, roots)	brachystemin A (9)	cyclo-(Pro^1-Phe-Leu-Ala^1-Thr-Pro^2-Ala^2-Gly)	1)
6	*Dianthus superbus* (Caryophyllaceae, whole plants)	dianthin A (10)	cyclo-(Ala-Tyr-Asn-Phe-Gly-Leu)	[10]
		dianthin B (11)	cyclo-(Ile-Phe^2-Phe^1-Pro^2-Gly-Pro^1)	[10]
7	*Drymaria diandra* (Caryophyllaceae, whole plants)	drymarin A (12)	cyclo-(Phe^1-Pro^1- Pro^2- Pro^3- Phe^2- Phe^3-Val-Ile-Ala)	

(*To be continued on page* 1829)

1) Tan, N. H., Wang, S. M., He, M. et al., Dicyclopeptides of *Panax notoginseng* and lactams of *Panax ginseng*, Chinese Chemical Letters, 2000, submitted.

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No.	Source	Cyclopeptide (No.)	Structure[b)]	Ref.
		drymarin B (13)	cyclo-(Pro^1-Phe-Tyr-Pro^2-Gly-Leu)	
		drymarin C (14)	cyclo-(Pro-Pro-Phe-Phe-Val-Ile-Ala-Phe-Leu)	
		drymarin D (15)	cyclo-(Tyr^1-Pro^1-Tyr^2-Phe-Val-Asp-Pro^2-Gly)	
8	*Panax ginseng* (Araliaceae, roots)	PNG-1 (16)	cyclo-(Leu-Thr)	2)
		PNG-2 (17)	cyclo-(Leu-Ile)	2)
		PNG-3 (18)	cyclo-(Leu-Val)	2)
		PNG-4 (19)	cyclo-(Ile-Val)	2)
		PNG-5 (20)	cyclo-(Leu-Ser)	2)
		PNG-6 (21)	cyclo-(Leu-Tyr)	2)
		PNG-7 (22)	cyclo-(Val-Pro)	2)
		PNG-8 (23)	cyclo-(Ala-Pro)	2)
		PNG-9 (24)	cyclo-(Phe-Tyr)	2)
		PNG-10 (25)	cyclo-(Phe-Ala)	2)
		PNG-11 (26)	cyclo-(Phe-Val)	2)
		PNG-12 (27)	cyclo-(Leu-Ala)	2)
		PNG-13 (28)	cyclo-(Ile-Ala)	2)
		PNG-14 (29)	cyclo-(Val-Ala)	2)
9	*Polycarpon prostratum* (Caryophyllaceae, whole plants)	polycarponin A (30)	cyclo-(Pro^1-Gly-Phe^1-Phe^2-Ala^1-Ile^1-Ala^2-Ile^2-Pro^2)	
		polycarponin B (31)	cyclo-(Gly^1-Ile-Val^1-Leu^1-Val^2-Gly^2-Leu^2-Pro)	
		polycarponin C (32)	cyclo-(Pro^1-Thr-Leu^1-Pro^2-Pro^3-Val-Leu^2-Phe)	
10	*Psammosilene tunicoides* (Caryophyllaceae, roots)	psammosilenin A (33)	cyclo-(Pro^1-Phe^1- Pro^2- Phe^2- Phe^3- Ala-Pro^3- Leu)	[11]
		psammosilenin B (34)	cyclo-(Pro^1-Gly- Phe^1- Val- Pro^2-Phe^2- Thr-Ile)	[11]
		(35)	cyclo-(Ala-Ala)	
		(36)	cyclo-(Val-Ala)	
		(37)	cyclo-(Ala-Leu)	
		(38)	cyclo-(Ala-Ile)	
11	*Pseudostellaria heterophylla* (Caryophyllaceae, roots)	heterophyllin A (39)	cyclo-(Pro-Val-Ile^1-Phe-Gly-Ile^2-Thr)	[2]
		heterophyllin B (40)	cyclo-(Pro^1- Pro^2- Pro^3-Ile-Phe-Gly^1-Gly^2-Leu)	[2]
		heterophyllin C (41)	cyclo-(Pro^1-Ile^3-Ile^1-Pro^2-Ile^2-Leu-Gly)	[12]
		heterophyllin D (42)	cyclo-(Phe^1-Ile-Thr-Val-Phe^2-Gly)	
		heterophyllin E (43)	cyclo-(Pro^1-Val-Tyr^1-Ala^1-Gly^1- Pro^2-Tyr^2-Leu-Ala^2-Gly^2)	
		heterophyllin F (44)	cyclo-(Gly-Ile^1-Ile^2-Leu^1-Leu^2-Leu^3)	
		heterophyllin G (45)	cyclo-(Pro-Val-Ile^1-Phe-Gly-Ile^2-n-penta Thr)	
		heterophyllin H (46)	cyclo-(Tyr-Pro)	
12	*Rubia yunnanensis* (Rubiaceae, roots)	RY-1 (47)	type V (see fig. 1)	[13]
			R_6 R_7 R_8 R_9 R_{10} R_{11} #	

(*To be continued on page* 1830)

2) Cheng, Y. X., Zhou, J., Tan, N. H. et al., A new cyclopeptide from *Brachystemma calycinum*, Chinese Chemical Letters, 2000, submitted.

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No.	Source	Cyclopeptide (No.)	Structure[b]							Ref.
			CH_3	CH_3	glc	H	H	H	L	
		RY-II (48)	type V (see fig. 1)							[14]
			R_6	R_7	R_8	R_9	R_{10}	R_{11}	#	
			CH_2OH	CH_3	glc	H	H	H	L	
		RY-III (49)	type V (see fig. 1)							[15]
			R_6	R_7	R_8	R_9	R_{10}	R_{11}	#	
			CH_3	glc	CH_3	H	H	H	L	
		RY-V (50)	type V (see fig. 1)							[13]
			R_6	R_7	R_8	R_9	R_{10}	R_{11}	#	
			CH_3	CH_3	OH	H	H	H	L	
13	*Schizandra chinensis* (Schizandraceae, fruits)	schizandrochin A (51)	cyclo-(Leu-Pro)							[16]
		schizandrochin B (52)	cyclo-(Phe-Pro)							[16]
		schizandrochin C (53)	cyclo-(Phe-Leu)							[16]
		schizandrochin D (54)	cyclo-(Phe-Val)							[16]
		schizandrochin E (55)	cyclo-(Phe-Ile)							[16]
		schizandrochin F (56)	cyclo-(Phe-Phe)							[16]
14	*Silene szechuensis* (Caryophyllaceae, roots)	silenin A (57)	cyclo-(Pro^1-Leu^1-Ser-Phe-Pro^2-Tyr-Leu^2-Val)							[17]
		silenin B (58)	cyclo-(Pro^3-Phe^1-Leu^1-Ala-Pro^1-Leu^2-Pro^2-Phe^2)							[17]
		silenin C (59)	cyclo-(Pro^1-Gly-Phe^2-Tyr^2-Pro^2-Tyr^1-Ala-Phe^1)							[17]
15	*Stellaria delavayi* (Caryophyllaceae, roots)	stelladelin A (60)	cyclo-(Gly^1-Pro-Pro-Pro-Leu^2-Leu^1-Gly^2-Pro-Pro-Tyr^1-Tyr^2)							[18]
		stelladelin B (61)	cyclo-(Gly-Ile-Pro^2-Pro^1-Ala-Tyr-Asp-Leu)							[18]
		stelladelin C (62)	cyclo-(Val-Pro^3-Tyr^2-Pro^2-Pro^1-Phe-Tyr^1-Ser)							[18]
		stelladelin D (63)	cyclo-(Gly-Val-Pro^1-Ser-Pro^3-Tyr-Phe-Pro^2-Ala^2-Ala^1-Ile)							[19]
		stelladelin E (64)	cyclo-(Tyr-Tyr-Pro-Pro-Ile-Thr-Ile-Ala)							
16	*S. yunnanensis* (roots)	stellarin A (65)	cyclo-(Gly^1-Pro^1-Phe-Pro^2-Gly^2-Tyr-Gly^3)							[20]
		stellarin B (66)	cyclo-(Gly-Ser-HO Ile-Phe^1-Phe^2-Ala)							[21]
		stellarin C (67)	cyclo-(Gly-Ser^2-HO Ile-Phe^1-Phe^2-Ser^1)							[21]
		stellarin D (68)	cyclo-(Gly-Tyr-Leu-Phe-Pro^2-Ile-Pro^1)							[22]
		stellarin E (69)	cyclo-(Gly-Ile^1-Pro-Tyr-Ile^2-Ala^2-Ala^1)							[22]
		stellarin F (70)	cyclo-(Gly^1-Ala-Gly^2-Ser-Pro^2-Trp-Phe-Pro^1)							[23]
		stellarin G (71)	cyclo-(Gly-Ala^2-Tyr-Leu-Ala^1)							[23]
		stellarin H (72)	cyclo-(Phe-Ser^1-Leu^2-Val-Leu^1-Pro^1-Pro^2-Tyr-Ser^2)							[24]
		stellarin I (73)	cyclo-(Gly-Pro-Tyr-Leu-Ala)							[24]
17	*Vaccaria segetalis* (Caryophyllaceae, seeds)	vaccarin A (74)	cyclo-(Trp-Ala^1-Gly-Val-Ala^2)							[25]
		vaccarin B (75)	cyclo-(Pro-Gly-Leu-Ser-Phe^1-Ala-Phe^2)							[25]
		vaccarin C (76)	cyclo-(Pro^1-Gly-Tyr-Val-Pro^2-Leu-Trp)							[25]
		vaccarin D (77)	cyclo-(Pro-Val^1-Trp-Ala-Gly-Val^2)							[25]

a) Dicyclopeptides 26, 27, 28 and 29 are identical with dicyclopeptides 54, 37, 38 and 36, respectively. Cyclopeptides 1—25, 30 — 49, 51—72, 74 are new compounds, but cyclopeptides 50, 73, 75—77 are known compounds. b) Ala, Asn, Asp, Glu, Gly, Ile, HO Ile, Leu, OMet, Phe, Pro, Ser, Thr, *n*-pentra Thr, Trp, Tyr and Val are the abreviations of the following amino acids: alanine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, δ-hydroxyl isoleucine, leucine, S-oxo methionine, phenylalanine, proline, serine, threonine, O-$CH_2CH_2CH_2CH_2CH_3$ threonine, tryptophan, tyrosine and valine, respectively.

3 Conclusion

Ninhydrin is a common reagent for detecting amino acids, linear peptides and proteins. In this note we combined ninhydrin reagent with the TLC protosite reaction and established this new TLC chemical method for detecting cyclopeptides and peptidic amides. After application of this method for past several years, we have found that it is a good specific and sensitive chemical method for detecting cyclopeptides and peptidic amides. It can be used effectively not only to detect plant extracts whether or not they contain cyclopeptides and peptidic amides by 2D-TLC, but also to guide cyclopeptide separation and purification by 1D-TLC. We have found that cyclopeptides are commonly found in the plants of some families, especially Annonaceae, Caryophyllaceae, Rubiaceae and Rhamnaceae based on our investigations and the literature.

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