Antitumor Cyclic Hexapeptides from *Rubia* Plants: History, Chemistry, and Mechanism (2005–2011)

Si Meng Zhao[ab], Bin Kuang[ab], Jun Ting Fan[a], He Yan[ab], Wen Yan Xu[a], and Ning Hua Tan*[a]

Abstract: Rubiaceae-type cyclopeptides (RAs), cyclic hexapeptides from *Rubia* plants, have shown potential antitumor activity *in vitro* and *in vivo*. Based on the review about plant cyclopeptides (*Chem. Rev*., **2006**, *106*: 840), this mini-review will highlight new progress on the discovery, synthesis, and mechanism of RAs isolated during 2005 to 2011, covering recent work in our group.

Keywords: Antitumor · Cyclic hexapeptides · *Rubia* · Rubiaceae-type cyclopeptides

Ning Hua Tan (born in 1963) was educated in medicinal chemistry at China Pharmaceutical University (Ph.D. mentored by Profs Shou-Xun Zhao and Jun Zhou), Kunming Institute of Botany, Chinese Academy of Sciences (post-doctoral research with Prof. Jun Zhou), National Cancer Institute of USA (Visiting Scholar), University of Wisconsin-Madison of USA (Visiting Professor). Since 1998, she has been a professor at Kunming Institute of Botany. She received a first-class prize from the Yunnan Province's Awards for Natural Sciences (2006) and the National Science Fund for Distinguished Young Scholars from the National Natural

Correspondence: Prof. Dr. N. H. Tan[a]
Tel.: +86 871 5223 800
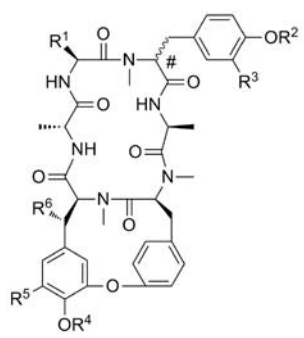Fax: +86 871 5223 800
E-mail: nhtan@mail.kib.ac.cn
[a]State Key Laboratory of Phytochemistry and
Plant Resources in West China
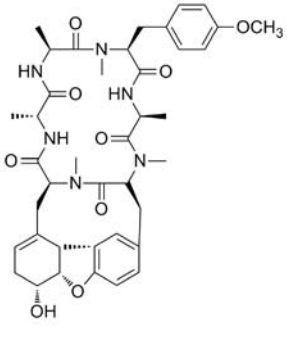Kunming Institute of Botany
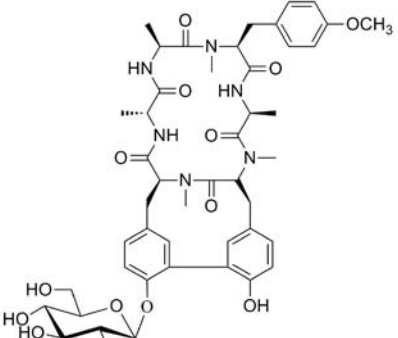Chinese Academy of Sciences
Kunming 650201, China
[b]Graduate University of Chinese Academy of Sciences
Beijing 100049, China

Science Foundation of China (2007). Her research interest focuses on drug discovery including natural product chemistry, drug screening and design, new drug R&D, especially chemistry and biology of plant cyclopeptides. She and her group have published over 200 papers and book chapters.

1. Introduction

Rubia plants, which belong to the family of Rubiaceae, fall into about 70 species distributed widely around the world. Among them, 36 species and 2 varieties exist in China. The roots of *Rubia cordifolia*, known as Rubiae Radix (Chinese name 'Qian-Cao'), are widely used as a traditional Chinese medicine for the treatment of tuberculosis, rheumatism, contusion, and menoxenia. Rubiaceae-type cyclopeptides (RAs), which are mainly isolated from *Rubia* plants, and formed with one α-D-alanine, one α-L-alanine, three *N*-methyl-α-L-tyrosines and one other proteinogenic α-L-amino acid, have shown potential antitumor activity *in vitro* and *in vivo*.[1] The skeleton structure of RAs is shown in Fig. 1. Bouvardin and Deoxybouvardin (RA-V) were the first two RAs isolated from *Bouvardia ternifolia* (Rubiaceae) in 1977 by Cole and coworkers.[2] In 1980s, Itokawa and coworkers in Japan found similar cyclic peptides from *R. akane* and *R. cordifolia*, and named this kind of compound as RAs.[3,4] In 2006, Tan and Zhou described the progress in the chemistry and biology of cyclopeptides from higher plants, in which 23 RAs discovered before 2005 were reviewed.[1] Morita and Takeya reviewed bioactive cyclic peptides from higher plants in 2010, including recently isolated RAs based on their own work.[5] This mini-review will highlight new progress in the discovery, synthesis, and mechanism of RAs isolated from 2005 to 2011, covering recent work in our group.

2. Discovery of New Cytotoxic RAs

With the distinctive bicyclic structural feature and significant antitumor activities, RAs have attracted great interest. Further studies on these chemically and pharmaceutically interesting cyclopeptides have led to the isolation of 15 new RAs from *Rubia* plants from 2005 to 2011. Takeya and coworkers reported seven RAs from the roots of *R. cordifolia* in 2008, *i.e.* RA-XVIII (**1**),[6] -XIX (**2**), -XX (**3**), -XXI (**4**), -XXII (**5**),[7] -XXIII (**6**), -XXIV (**7**).[8] The IC_{50} values of RAs **1**–**7** against P388 leukemia cells were in the range of 0.012–0.63 μg/mL. Two novel skeletons and six new RAs from *R. yunnanensis* were reported by Tan and coworkers in 2010, *i.e.* rubiyunnanins A and B (**8**,**9**),[9] rubiyunnanins C–H (**10**–**15**).[10] Compound **8** had one additional ring formed *via* a phenolic oxygen linkage and a new carbon–carbon bond between Tyr 5 and residue 6. **9** was discovered for the first time containing one carbon–carbon bond at the *ortho*-position of the hydroxyl groups of Tyr 5 and Tyr 6 instead of the typical phenolic oxygen linkage. The cytotoxicities against eleven cancer cell lines of compounds **8**–**15** were evaluated. Compound **10** showed significant cytotoxicity with IC_{50} valves in the range of 0.67–10.69 μM, whereas other compounds showed moderate or weak activities. RAs recently isolated are shown in Table 1.

In 2011, Takeya and coworkers established an immunoassay system for the quantitative estimation of minor antitumor cyclic hexapeptide RA-VII (**16**) (Fig. 1) from *R. cordifolia* and *R. akane*.

Table 1. RAs Discovered during 2005 to 2011 (glc = β-D-glucopyranosyl)

NO.	Name	Substituent							Species	Reference
		R¹	R²	R³	R⁴	R⁵	R⁶	#		
1	RA-XVIII	CH₃	CH₃	H	CH₃	OH	H	L	*R. cordifolia*	[6]
2	RA-XIX	CH₂CH(CH₃)₂	CH₃	H	CH₃	H	H	L	*R. cordifolia*	[7]
3	RA-XX	CH₂CH₃	CH₃	H	CH₃	H	H	L	*R. cordifolia*	[7]
4	RA-XXI	CH₂CH₃	CH₃	H	H	H	H	L	*R. cordifolia*	[7]
5	RA-XXII	CHOHCH₃	CH₃	H	H	H	H	L	*R. cordifolia*	[7]
6	RA-XXIII	CH₂CH₂CONH₂	CH₃	H	CH₃	H	H	L	*R. cordifolia*	[8]
7	RA-XXIV	CH₂CH₂CONH₂	CH₃	H	H	H	H	L	*R. cordifolia*	[8]
10	Rubiyunnanin C	CH₂CH₂COOCH₃	CH₃	H	H	H	H	L	*R.yunnanensis*	[10]
11	Rubiyunnanin D	CH₂CH₂COOH	H	H	H	H	H	L	*R.yunnanensis*	[10]
12	Rubiyunnanin E	CH₂CH₂COOH	CH₃	OH	H	H	H	L	*R.yunnanensis*	[10]
13	Rubiyunnanin F	CH₂CH₂CONH₂	CH₃	H	glc	H	H	L	*R.yunnanensis*	[10]
14	Rubiyunnanin G	CH₃	H	H	glc	H	H	L	*R.yunnanensis*	[10]
15	Rubiyunnanin H	CH₃	CH₃	OH	glc	H	OH	L	*R.yunnanensis*	[10]

Rubiyunnanin A (8)
From *R. yunnanensis* [9]

Rubiyunnanin B (9)
From *R. yunnanensis* [9]

RA-VII – glutarate – BSA conjugate was prepared as an antigen, and the clone of hybridoma which produces specific MAb IgG₁ 4A12 was produced and isolated. The IC₅₀ value of MAb 4A12 against RA-VII – glutarate – HSA was about 800 µg antigen/mL. The specificity against RAs was evaluated, and the result showed that the obtained MAb discerned differences in the sidechain structure of residue 2 but not of residue 6.[11]

3. Mechanism of RA Antitumor Effects

Due to their significant activities, RAs have attracted a lot of interests on the underlying mechanisms of their antitumor effects. In 2006, Sato and coworkers reported the anti-angiogenesis activity of RA-VII (16) on the proliferation, migration, stress fiber formation of bovine aortic endothelial cells (BAEC) and effects on mouse corneal angiogenesis. Compound 16 caused actin aggregation around the nuclei in BAEC, and inhibited

RA-VII (16) R¹=R²=R⁴=CH₃, R³=R⁵=R⁶=H

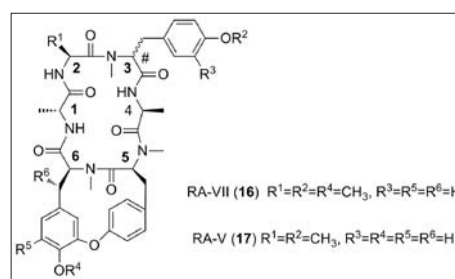RA-V (17) R¹=R²=CH₃, R³=R⁴=R⁵=R⁶=H

Fig. 1. Structural skeleton of RAs.

the formation of stress fibers by bFGF in BAEC. A pellet containing bFGF was inoculated into the mouse cornea, after 7 days' daily intraperitoneal injection of 16 (4mg/kg), the angiogenesis induced by bFGF was apparently inhibited. The effect of 16 on tumor vessels showed that it reduced the luminal tumor vessel area.[12] In 2011, Lau, Tan and coworkers described the anti-angiogenesis effects of RA-V (17) (Fig. 1) on human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMEC-1). Compound 17 was found to significantly inhibit the proliferation of HUVEC and HMEC-1 with IC₅₀ values of 1.42 and 4.00 nM. Compound 17 induced G₂/M phase cell cycle arrests in both cells. The tube formation, migration and adhesion of HUVEC and HMEC-1 could be inhibited significantly by 17. To investigate possible mechanisms, further relative proteins and their mRNA were detected. Results indicated that 17 decreased the levels of cyclin B1, cyclin D1, MMP-2, VEGF-R1, pVEGF-R2 and Tie2, and reduced the phosphorylations of extracellular signal-regulated kinase 1/2 (ERK) and IκB in both cell lines. Interestingly, the phosphorylations of mitogen-activated protein kinase (p38 MAPK) and phosphatidylinositol 3-kinase (PI3K) were decreased in 17-treated HMEC-1 only, while decreased c-jun-NH₂-kinase (JNK) phosphorylation was observed in 17-treated HUVEC only. The mRNA expression of Tie 2, VEGFR-2, MMP-2, MMP-9, Ang 1 was reduced in both cell lines, while Ang 2 was reduced in HMEC-1 only. Finally, a matrigel plug assay *in vivo* on mice to verify the effect of 17 on angiogenesis gives results consistent with that observed *in vitro*. Therefore the anti-angiogenic effects of 17 on HUVEC and HMEC-1 cells may be exerted through inhibition of ERK1/2 phosphorylation and MMP activation, leading to decreased cell proliferation and migration/invasion.[13]

When Tan and coworkers reported the discovery of new RAs (rubiyunnanins C–H (10–15)), a study on the effects of several RAs on NF-κB signal transduction were described. LPS, IFN-γ-induced RAW 264.7 macrophages and HEK-293-NF-κB luciferase stable cell lines were used to examine the effects of RAs on NO production and NF-κB signal pathway. RA-V (17) showed the best activities on NO production inhibition (IC₅₀ = 0.05 µM) and NF-κB signal pathway inhibition (IC₅₀ = 0.03 µM).[10]

Tan and coworkers reported the quantitative structure–activity relationship (QSAR) analysis of 54 RAs. Hologram quantitative structure–activity relationship (HQSAR), comparative molecular field analysis (CoMFA), and comparative

molecular similarity indices analysis (CoMSIA) methods were applied to develop 2D- and 3D-QSAR models. Results of HQSAR indicated that OMe groups at R^2 and R^6 are necessary to increase the activity. CoMFA and CoMSIA results showed that electropositive side chains at R^1 position and hydrophobic groups at R^3 and R^5 positions could increase the activity. Intra-molecular hydrogen bonds between residue 1 and 4 were necessary to keep the pharmacophoric conformation.[14]

4. Total Synthesis and Derivation of RAs

The significant biological activity and distinctive molecular architecture made RAs very attractive synthetic targets. Contributions made by Inoue, Boger and Zhu groups on the synthesis of RAs were reviewed by Tan and Zhou in 2006.[1] To confirm the absolute structures of the new cyclic peptides, RA-XVIII (**1**), -XIX (**2**), -XX (**3**) have recently been prepared by Takeya and coworkers starting from RA-V (**17**). Treatment of **17** with nitric acid and sulfuric acid gave nitro compound **18**, which was treated with (trimethylsilyl) diazomethane to afford the methyl ether **19**. Catalytic hydrogenation of **19** with Pd/C gave **20**, which after treatment with copper(II) nitrate and copper(I) oxide produced **1** (Scheme 1).[6]

N-protected dipeptide **21** was coupled with Boc-Leu-OH using PyBOP to afford tripeptide **22**; further treatment of **22** with Cbz-D-Ala-OH gave tetrapeptide **23**. Compound **23** was reacted with lithium hydroperoxide to afford the tetrapeptide **24**. Cycloisodityrosine **25**, prepared by degradation of natural **16**, was coupled with **24** to give the hexapeptide **26**. Deprotection and subsequent macrocyclization of **26** using DPPA gave compound **2** (Scheme 2). Compound **3** was semi-synthesized through a similar protocol from **2**.[7]

In 2008, Takeya and coworkers reported the design and synthesis of **27**, an analogue having two cycloisodityrosine units in one molecule in order to obtain information on the effect of the side-chain conformation of Tyr 3 upon the biological activity. The synthetic route for the preparation of **27** is shown in Scheme 3. The IC_{50} value of **27** against P-388 leukemia cells was evaluated, demonstrating that the side-chain conformation of Tyr 3 was not the major factor which determined the activity of RAs.[15]
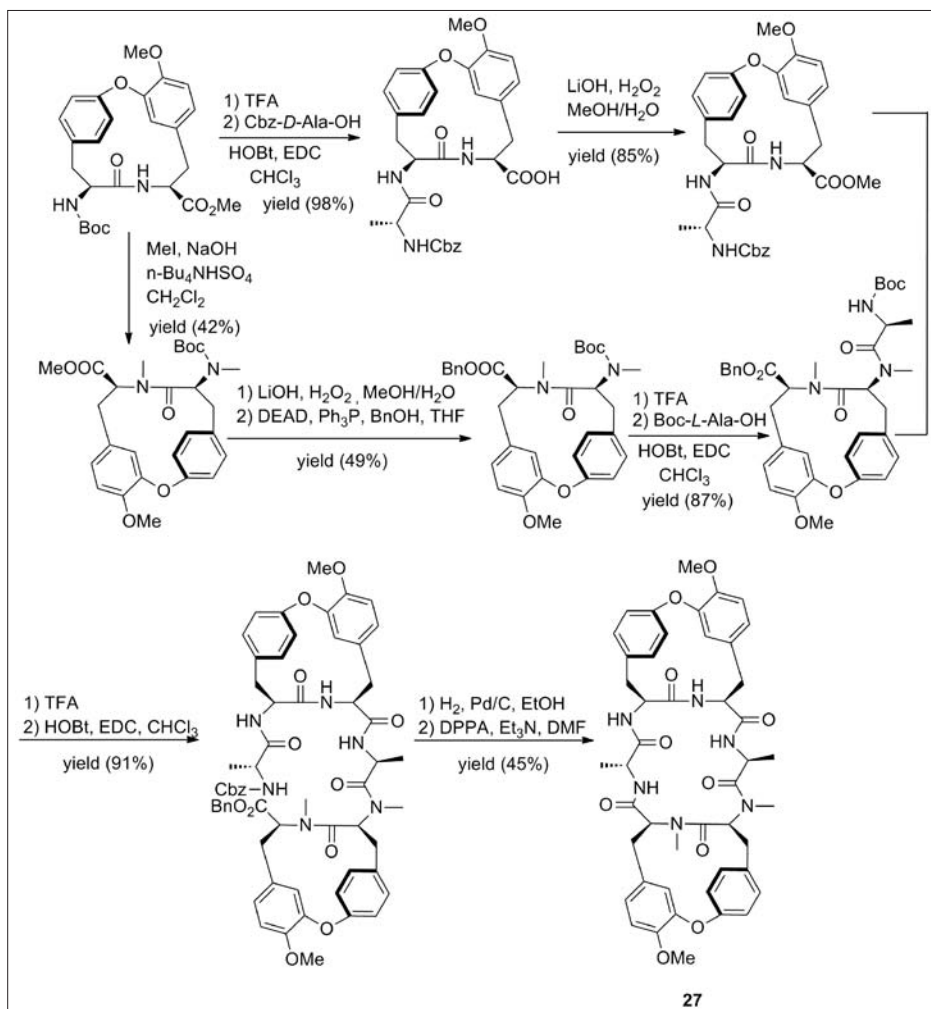
In 2011, Takeya and coworkers reported the synthesis of penta-*N*-methyl and hexa-*N*-methyl analogues of RA-VII (**28** and **29**) to study the effects of intramolecular hydrogen bonds in RAs (Scheme 4). Results showed that the intramolecular
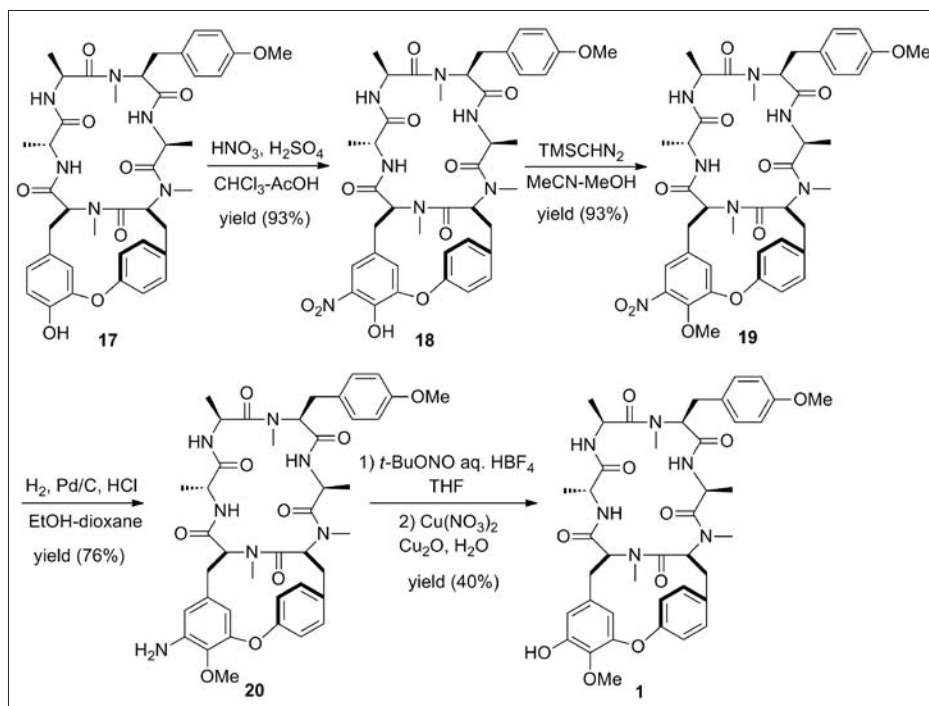
Scheme 1. Semi-synthesis of **1** from **17**.
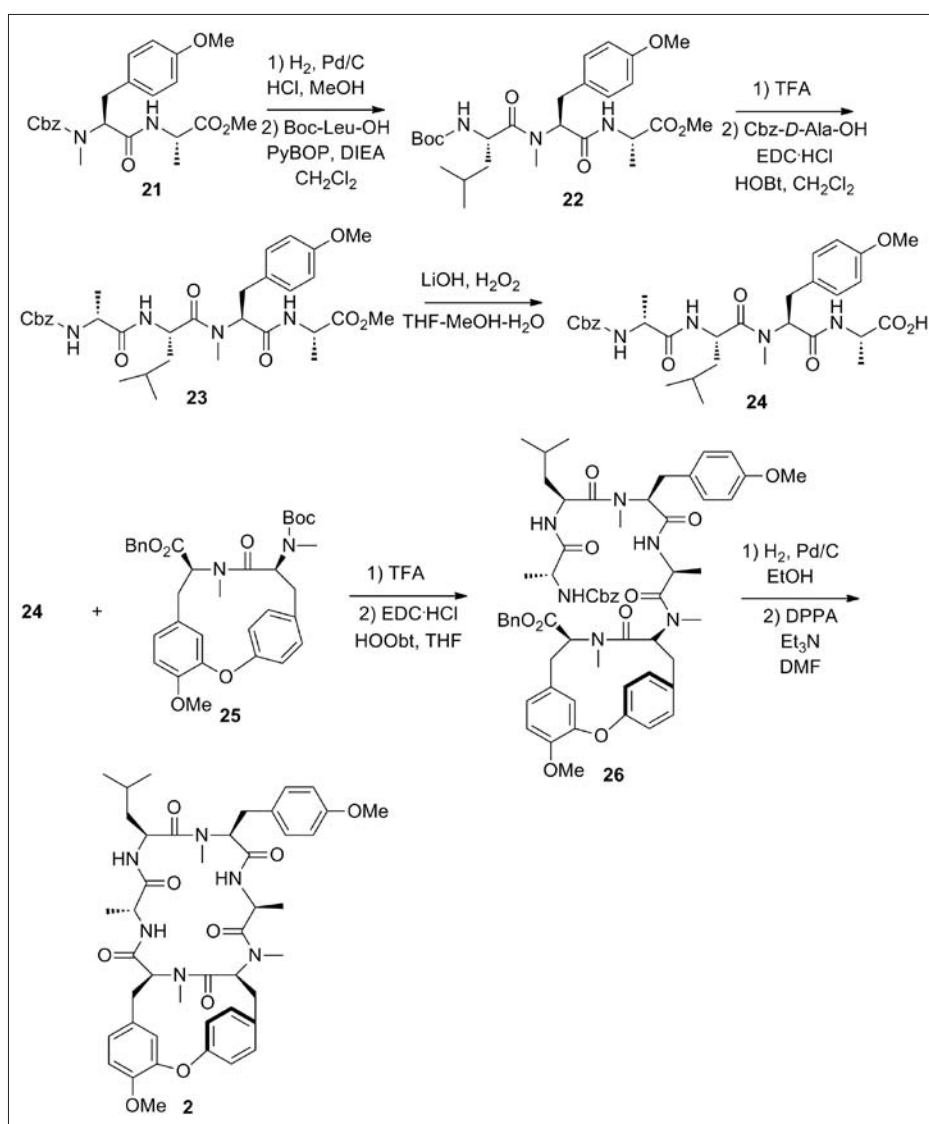
Scheme 2. Route of the semi-synthesis of **2**.

Scheme 3. Route of the synthesis of **27**.

6. Conclusion

The significant antitumor activities and distinctive structural features have made RAs an attractive research area. In this mini-review we have highlighted new progress on the discovery, synthesis, and mechanism of RAs isolated from 2005 to 2011. Fifteen new cytotoxic RAs including two novel skeleton cyclopeptides were isolated and six RAs were synthesized. The underlying mechanism for providing antitumor activity, particularly anti-angiogensis, was clarified and evidence was provided that RAs may act through inhibition of ERK1/2 phosphorylation and MMP activation, which then leads to decreased cell proliferation and migration/invasion. Most probably RAs represent also a new type of NF-κB natural inhibitor. Furthermore it has been proposed that RAs might be nonribosomal peptides (NRPs). We believe that cyclic hexapeptides from *Rubia* plants (RAs) will be isolated and their biological functions explored resulting in a series of novel natural products with interesting biological activity.
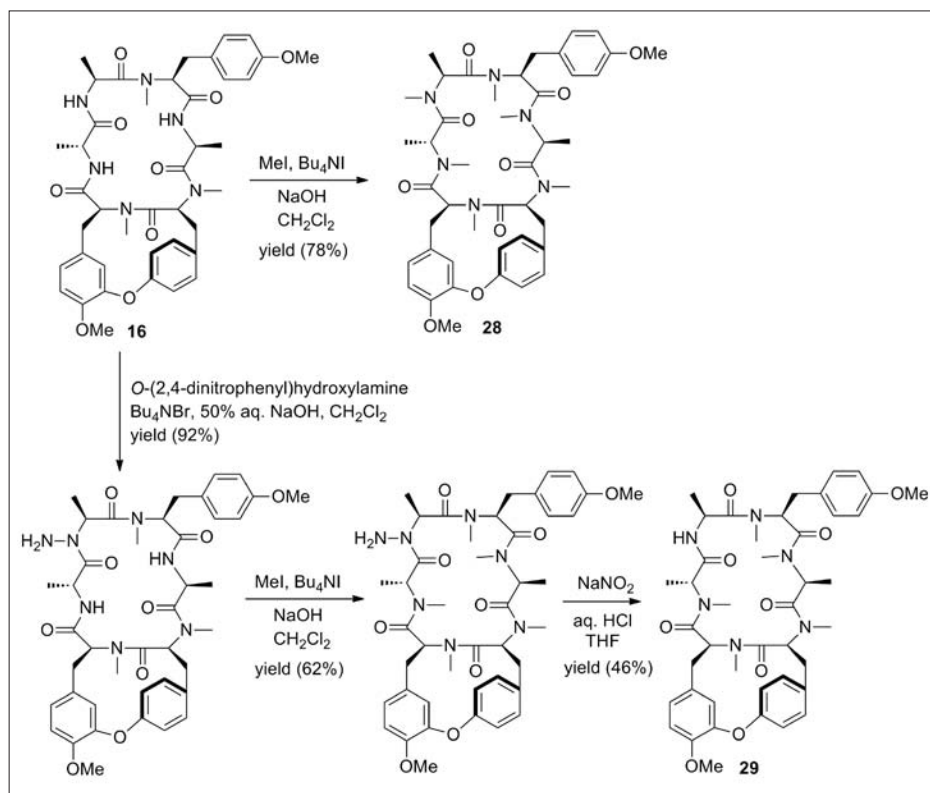
hydrogen bonds played important roles in retaining the conformation of RAs.[16]

5. Biosynthesis of RAs

Most cyclopeptides are products of the non-ribosomal peptide synthetase (NRPS) pathway and some cyclopeptides have been discovered as products of the ribosomal peptide synthetase (RPS) pathway, particularly isolated from microbes. Tan and Zhou reviewed biosynthesis of plant cyclopeptides in 2006 and proposed that cyclotides (large plant cyclopeptides) are gene products and small plant cyclopeptides may be of different origin.[1] Recently, segetalin A-H and heterophyllin B from plants were confirmed as gene-coded products through the RPS pathway.[17,18] In 2009, Du, Tan and coworkers proposed RAs as nonribosomal peptides (NRPs) which would be assembled by specific enzymes and proposed a possible biosynthetic pathway (Fig. 2).[19] In 2010, Tan and coworkers reported two novel skeletons of RAs (**8,9**) providing further support for the assumption of RAs as nonribosomal peptides (NRPs) (Fig. 2).[9]

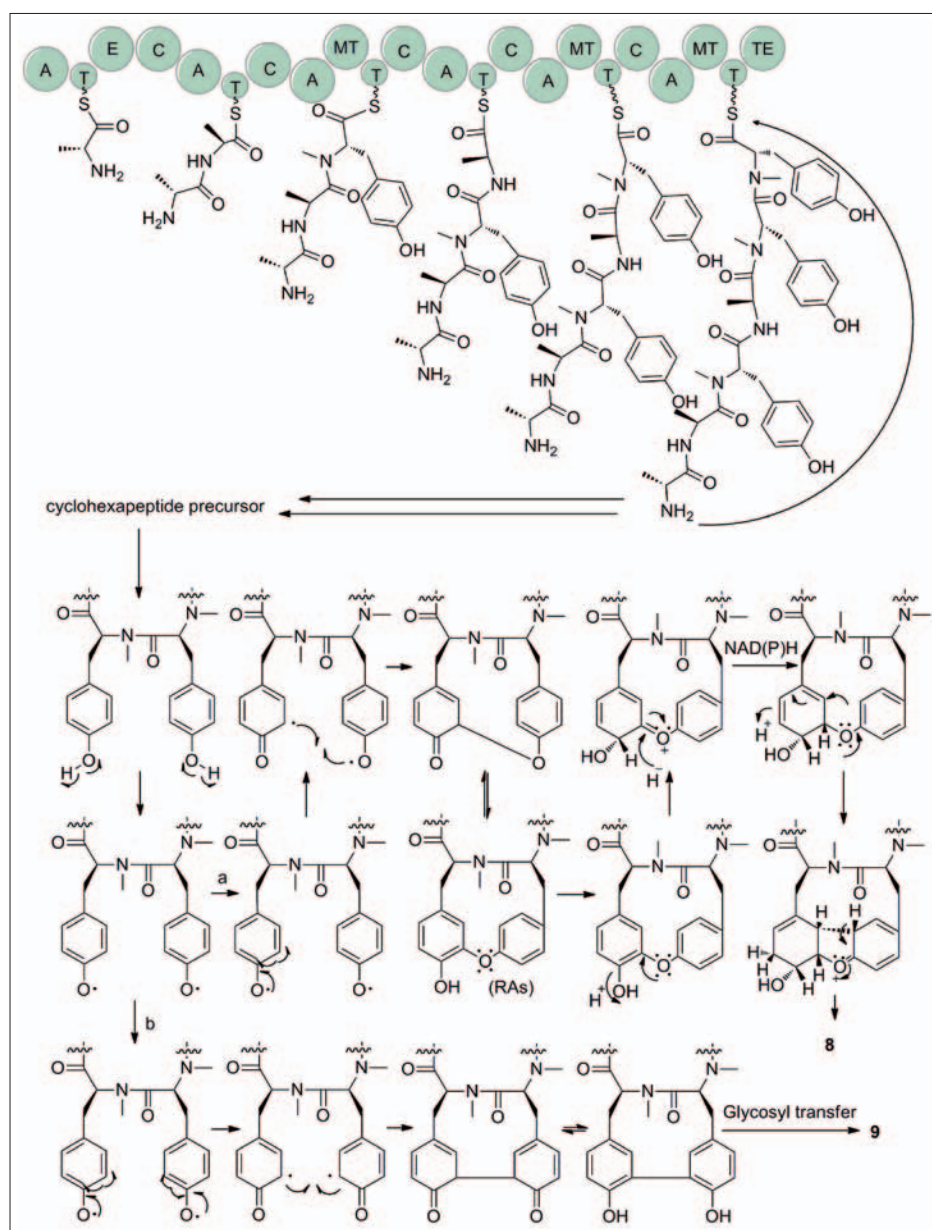Scheme 4. Route of the synthesis of *N*-methyl analogues of RA-VII (**28** and **29**).

[18] J. A. Condie, G. Nowak, D. W. Reed, J. J. Balsevich, M. J. Reaney, P. G. Arnison, P. S. Covello, *Plant Journal* **2011**, *67*, 682.
[19] L. C. Du, N. H. Tan, W. Y. Xu, L. L. Lou, *Acta Bot. Yunnanica* **2009**, *31*, 374.

Fig. 2. Possible biosynthetic pathway of RAs. The starter is assumed to be D-Ala-1. A, adenylation domain; T, thiolation domain; E, epimerase domain; C, condensation domain; MT, methyltransferase domain; TE, thioeaterase domain.

[1] N. H. Tan, J. Zhou, *Chem. Rev.* **2006**, *106*, 840.
[2] S. D. Jolad, J. J. Hoffmann, S. J. Torrance, R. M. Wiedhopf, J. R. Cole, S. K. Arora, R. B. Bates, R. L. Cargiulo, G. R. Kriek, *J. Am. Chem. Soc.* **1977**, *99*, 8040.
[3] H. Itokawa, K. Takeya, K. Mihara, N. Mori, T. Hamanaka, T. Sonobe, Y. Iitaka, *Chem. Pharm. Bull.* **1983**, *31*, 1424.
[4] H. Itokawa, K. Takeya, N. Mori, T. Sonobe, S. Mihashi, T. Hamanaka, *Chem. Pharm. Bull.* **1986**, *34*, 3762.
[5] H. Morita, K. Takeya, *Heterocycles* **2010**, *80*, 739.
[6] J. E. Lee, Y. Hitotsuyanagi, I. H. Kim, T. Hasuda, K. Takeya, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 808.
[7] J. E. Lee, Y. Hitotsuyanagi, K. Takeya, *Tetrahedron* **2008**, *64*, 4117.
[8] J. E. Lee, Y. Hitotsuyanagi, H. Fukaya, K. Kondo, K. Takeya, *Chem. Pharm. Bull.* **2008**, *56*, 730.
[9] J. T. Fan, Y. S. Chen, W. Y. Xu, L. C. Du, G. Z. Zeng, Y. M. Zhang, J. Su, Y. Li, N. H. Tan, *Tetrahedron Lett.* **2010**, *51*, 6810.
[10] J. T. Fan, J. Su, Y. M. Peng, Y. Li, J. Li, Y. B. Zhou, G. Z. Zeng, H. Yan, N. H. Tan, *Bioorg. Med. Chem.* **2010**, *18*, 8226.
[11] T. Hasuda, Y. Hitotsuyanagi, K. Takeya, *J. Nat. Med.* **2011**, *65*, 588.
[12] T. Koizumi, M. Abe, T. Yamakuni, Y. Ohizumi, Y. Hitotsuyanagi, K. Takeya, Y. Sato, *Cancer Sci.* **2006**, *97*, 665.
[13] G. G. L. Yue, J. T. Fan, J. K. M. Lee, G. Z. Zeng, T. W. F. Ho, K. P. Fung, P. C. Leung, N. H. Tan, C. B. S. Lau, *Brit. J. Pharmacol.* **2011**, DOI: 10.1111/j.1476-5381.2011.01458.x.
[14] H. Yan, X. L. Pan, N. H. Tan, J. T. Fan, G. Z. Zeng, H. J. Han, *Eur. J. Med. Chem.* **2009**, *44*, 3425.
[15] J. E. Lee, Y. Hitotsuyanagi, Y. Nakagawa, S. Kato, H. Fukaya, K. Takeya, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6458.
[16] Y. Hitotsuyanagi, J. E. Lee, S. Kato, I. H. Kim, H. Kohashi, H. Fukaya, K. Takeya, *Bioorg. Med. Chem.* **2011**, *19*, 2458.
[17] W. Y. Xu, H. T. Zhu, N. H. Tan, J. Tang, Y. J. Zhang, R. L. Cerny, L. C. Du, *Plant Cell Tiss. Organ Cult.* **2011**, DOI: 10.1007/s11240-011-0022-8.