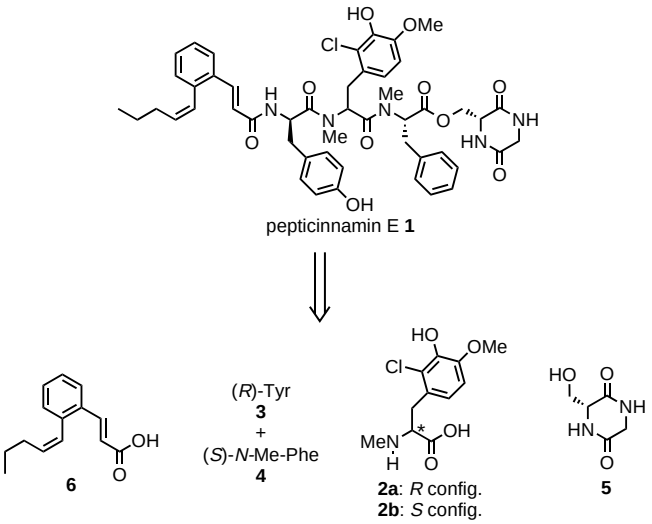
Synthesis and In Vitro Evaluation of the Ras Farnesyltransferase Inhibitor Pepticinnamin E**

Klaus Hinterding, Patrizia Hagenbuch, Janos Rétey,* and Herbert Waldmann*

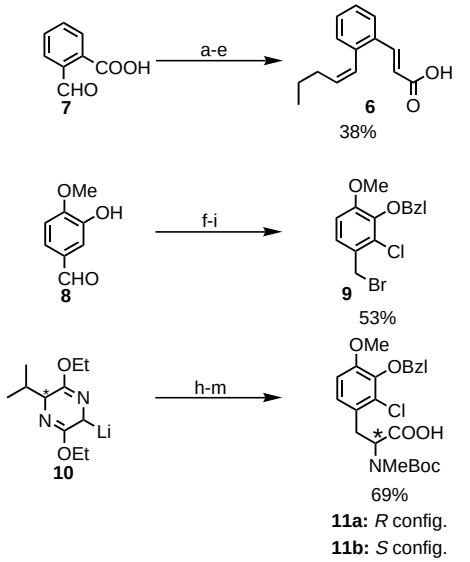
Ras proteins are S-farnesylated plasma membrane bound proteins which are decisively involved in the cellular transduction of mitogenic signals.[1] Point mutations in the *ras* genes are found in about 40% of all human tumors, and in more than 80% of some of the major malignancies such as colon, breast, and pancreas cancer.[2] To fulfill their signal-transducing function in the normal and transformed state, the Ras proteins must be farnesylated; non-lipidated Ras is cytosolic and inactive.[2, 3] Consequently, the targeted inhibition of Ras farnesylation was identified as a promising approach to the development of new antitumor agents.[2, 4, 5] Recent results[5] suggest, however, that inhibitors of protein farnesyltransferase (PFT) suppress the growth of transformed cells[6] also by blocking the farnesylation of other proteins,[7] but their respective intracellular target sites could not yet be identified. For the study of these biological processes and the development of new drugs, alternative farnesyltransferase inhibitors are of great relevance. Inhibitors with modular structure which can be varied rapidly by combinatorial techniques are of particular interest.[8] Furthermore, bisubstrate analogues which imitate both the farnesyl group and the peptide substrate are particularly promising; however, only a few inhibitors belonging to this class are available so far.[9] Pepticinnamin E (**1**, Scheme 1) is a natural PFT inhibitor from *Streptomyces* species[10] which could meet these demands. We now report on the synthesis of both diastereomers of this unusual peptidic natural product (the absolute configuration of the central non-proteinogenic amino acid was not known) and on the evaluation of their PFT-inhibiting properties employing a recombinant protein farnesyltransferase.

Retrosynthetic analysis of pepticinnamin E yielded, after cleavage of the amide and ester bonds, amino acids **2**–**4**, diketopiperazine **5**, and (pentenylphenyl)acrylic acid (**6**; Scheme 1). Acid **6**[11] was synthesized from the commercially available aldehyde **7** (Scheme 2). The methyl ester was first prepared and subjected to a *cis*-selective Wittig reaction to give the alkene. The ester was converted into the benzaldehyde by a two-step sequence; a subsequent completely *trans*-selective Knoevenagel condensation with malonic acid provided **6**.

The non-proteinogenic amino acids **11a** and **11b** were synthesized in enantiomerically pure form by the Schöllkopf



Scheme 1. Retrosynthetic analysis of pepticinnamin E (**1**).



Scheme 2. Synthesis of the pepticinnamin E fragments **6**, **9**, **11a**, and **11b**. a) MeI, K_2CO_3, acetone, 76%; b) $Ph_3P{=}C_4H_9$, THF, $-100\,^{\circ}C$, 84%, d.r. = 92:8; c) $LiAlH_4$, THF, 97%; d) PCC, CH_2Cl_2, 96%; e) $CH_2(COOH)_2$, pyridine, piperidine, 70%; f) Cl_2, CH_2Cl_2, 68%; g) BzlBr, K_2CO_3, DMF, 93%; h) $NaBH_4$, MeOH, then H_2O, 99%; CBr_4, PPh_3, Et_2O, 85%; j) **9**, THF, $-78\,^{\circ}C$, 85%, d.r. = 95:5; k) 0.5 N HCl, THF, then Boc_2O, NEt_3, MeOH, 88%; l) LiOH, THF, H_2O, 99%; m) NaH, MeI, THF, 96. Bzl = benzyl, PCC = pyridinium chlorochromate.

bis(lactim ether) method[12] (Scheme 2). The benzyl bromide **9** was made from the phenol **8**. First the chlorine substituent was introduced regioselectively at the *ortho* position by treatment with liquid chlorine in dichloromethane to yield the 1,2,3,4-tetrasubstituted arene. After protection of the phenol as benzyl ether the aldehyde could be reduced to the benzyl alcohol, which was converted into the benzyl bromide **9** with the Appel reagent. The alkylation of the lithiated bis(lactim ether) **10** proceeded in 85% yield and with a diastereomer ratio (d.r.) of 95:5. After cleavage of the bis(lactim ether)s and masking of the liberated amino termini as *tert*-butyloxycarbonyl (Boc) urethanes, the chiral auxiliary was separated by chromatography. Saponification of the ethyl ester with lithium

[*] Prof. Dr. J. Rétey, Prof. Dr. H. Waldmann, Dr. K. Hinterding, Dipl.-Chem. P. Hagenbuch
Institut für Organische Chemie der Universität
Richard-Willstätter-Allee 2, D-76128 Karlsruhe (Germany)
Fax: (+49) 721-608-4825
E-mail: waldmann@ochhades.chemie.uni-karlsruhe.de

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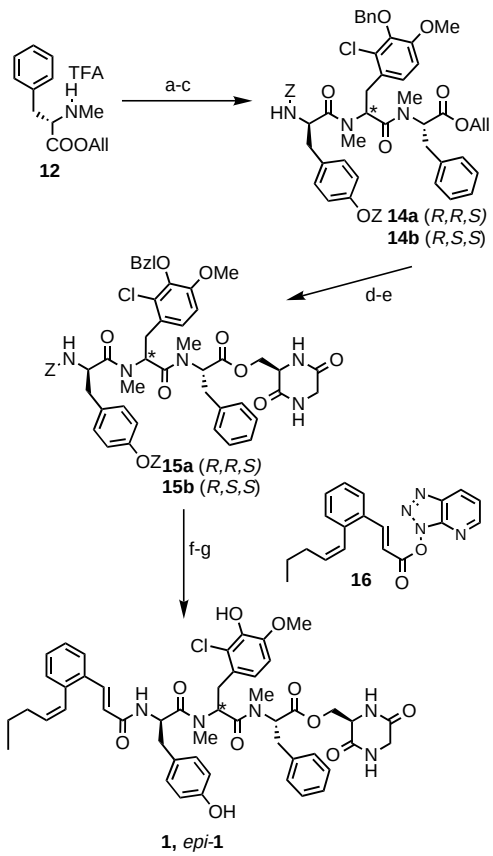
hydroxide and selective N-methylation of the Boc-urethane completed the synthesis of both enantiomers of the desired amino acid (**11a** and **11b**).

For the construction of both epimers of the central tripeptide (**14a** and **14b**), the respective (*R*)- or (*S*)-amino acid **11a** or **11b** was coupled with the phenylalanine derivative **12**, and after removal of the Boc group the amino acid chain was elongated with bis(benzyloxycarbonyl)-protected tyrosine **13** to yield **14a** or **14b** (Scheme 3). After Pd^0-mediated selective cleavage of the C-terminal allyl ester,



Scheme 3. Synthesis of pepticinnamin E (**1**). a) **11a** or **11b**, EDC, HOAt, DMF, 81% for *S*,*S* enantiomer, 77% for *R*,*S* enantiomer; b) HCl, Et_2O, quant.; c) Z-(*R*)-Tyr-(Z) (**13**), EDC, HOAt, DMF, 75% (77%); d) $[Pd(PPh_3)_4]$, morpholine, CH_2Cl_2, 89% (88%); e) DEAD, PPh_3, **5**, DMF, 53% (55%); f) H_2, Pd/C, HOAc, EtOH; g) **16**, DMF, 31% (33%) over two steps. DEAD = diethyl azodicarboxylate, EDC = *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide.

the required diketopiperazine **5** was introduced. Whereas several established methods for the formation of esters[13] gave unsatisfactory results, the activation of the alcohol **5** under Mitsunobu conditions[14] was the method of choice. The three benzyl protecting groups in esters **15a** and **15b** thus obtained were removed simultaneously by Pd-catalyzed hydrogenolysis. The liberated amino group was then selectively acylated with cinnamic acid derivative **16**, which was preactivated as the 7-aza-1-hydroxy-1*H*-benzotriazole (HOAt) ester, to give the natural product **1** or its diastereomer *epi*-**1**. Comparison of the retention times in HPLC with four different eluents, the ^1H NMR spectra (500 MHz), and the specific rotation of the synthetic compounds with reference values for an authentic sample of the natural product[15] proved that the central amino acid has the *S* configuration.

The inhibition of Ras farnesyltransferase was determined with an in vitro assay in which the enzyme-catalyzed farnesyl transfer from farnesylpyrophosphate (FPP) to a dansyl-labeled (dansyl = 5-(dimethylamino)naphthaline-1-sulfonyl) substrate peptide (Gly-Cys-Val-Leu-Ser, GCVLS) is followed fluorometrically.[16] The enzyme used was a recombinant Ras farnesyltransferase from *Saccharomyces cerevisiae*[18] which was cloned into the plasmid pT7-7 and overexpressed in the *Escherichia coli* BL21 system.[17] In this expression system the viral Φ10 promoter, which stems from bacteriophage T7, guarantees a high transcriptional efficiency. An effective translation is obtained by means of a ribosomal binding site (RBS, also called a Shine–Dalgarno sequence) in front of the start codon. Furthermore, the transcription does not depend on a bacterial but on a viral T7 polymerase, whose gene is integrated into the genome of the *E. coli* BL21 cells. To express the farnesyltransferase the genes *dpr1* and *ram2*, which encode the two subunits of the heterodimeric enzyme, were isolated from the yeast plasmids YEP13 and YEP24,[18] and an *Nde I* restriction site was introduced by site-directed mutagenesis with a polymerase chain reaction (PCR). This restriction site contains the start codon and guarantees a correct distance to the Shine–Dalgarno sequence, so that both genes inserted into the vectors are in the correct reading frame. Both genes were cloned into the pT7-7 plasmid through this restriction site. Since the vector contains only one resistance gene (the gene for ampicillin resistance, amp^r), only one recombinant plasmid can be used for the expression. Thus, from the pT7-7/*dpr1* plasmid the gene sequence and the RBS were cut out (→*dpr1* + RBS), the pT7-7/*ram2* plasmid was cleaved, and the fragment *dpr1* + RBS was inserted. The obtained plasmid (Figure 1) was introduced into *E. coli* BL21 cells, and the recombinant bacteria were used for the expression.

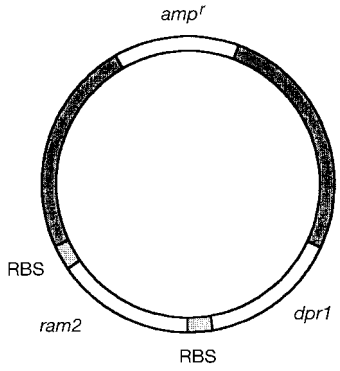


Figure 1. The pT7-7 plasmid used for the expression along with the two farnesyltransferase genes *dpr1* and *ram2*.

Analysis of the kinetic data by a Lineweaver–Burk plot (Figure 2) showed that the natural product is a competitive inhibitor with respect to both the peptide substrate and FPP. The determined K_I and K_M values are given in Table 1. In addition, the IC_{50} values for **1**, its diastereomer *epi*-**1**, and the

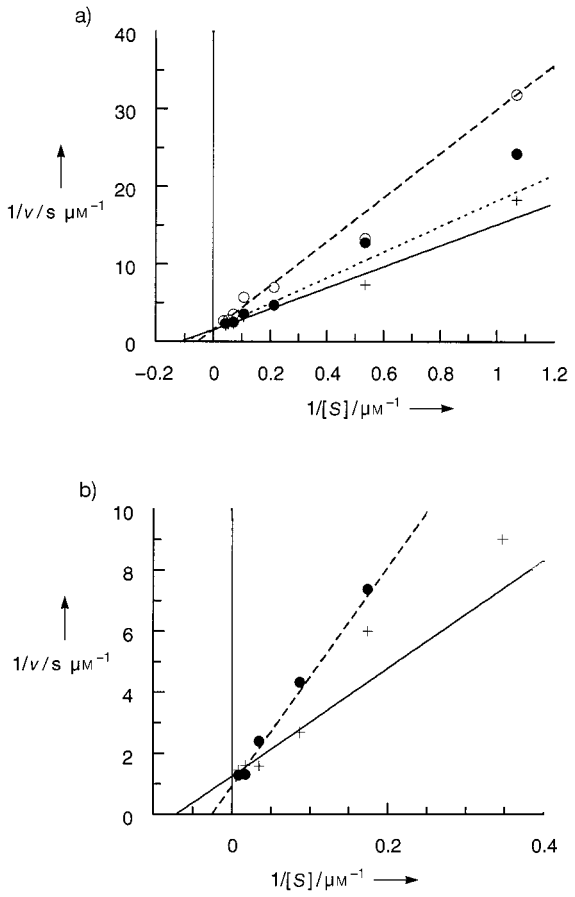


Figure 2. Lineweaver–Burk plots from which the K_I values for the inhibition of PFT by **1** were determined. For each measurement the reaction mixture (total volume 400 μL) contained 4 μg of enzyme (partially purified), 50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl (pH = 7.5), 5 mM dithiothreitol, 5 mM $MgCl_2$, and 10 μM $ZnCl_2$. a) Inhibition with respect to the peptide substrate at a constant concentration of FPP (75 μM) and different concentrations c of the inhibitor **1**: $c = 0$ (+, —), 5.5 (○, - - - - -), and 27.5 μM (○, – – – –). b) Inhibition with respect to FPP at a constant concentration of the peptide substrate (84.3 μM) and $c = 0$ (+, —) and 13.75 μM (○, – – – –). S = substrate, v = rate of reaction.

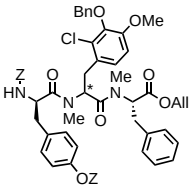
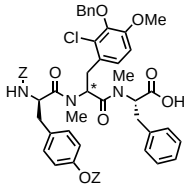
Table 1. K_M and K_I values determined from Michaelis–Menten and Lineweaver–Burk plots.

K_M [μM]		K_I [μM]	
dansyl-GCVLS	FPP	dansyl-GCVLS	FPP
9	14	30	8

tripeptide derivatives **14a** and **14b** as well as **17a** and **17b** were determined. These values, given in Table 2, demonstrate that the absolute configuration of the central amino acid is decisive for the inhibitory activity. On the one hand the natural product **1** is about six times stronger an inhibitor than its epimer *epi*-**1**, and, on the other hand, only acid **17b** of *R*,*S*,*S* configuration, but not the epimeric acid **17a** of *R*,*R*,*S* configuration, shows inhibitory activity under the conditions of the assay. In addition, the data prove that the C- and N-terminal modifications of the natural products are not important for inhibition of the farnesyl transfer (Table 2, entry 2).

In conclusion, we have synthesized the natural product pepticinnamin E for the first time and shown that it is a bisubstrate inhibitor for a recombinant farnesyltransferase. Furthermore, several structural parameters were identified which decisively influence the inhibition. With combinatorial synthesis further analogues of this modularly built farnesyltransferase inhibitor should be available rapidly and efficiently. These inhibitors should open up new opportunities for the study and suppression of the farnesylation of different proteins involved in cellular signal transduction cascades and the transformation of cells.

Table 2. IC_{50} values determined for pepticinnamin E (**1**) and several synthetic derivatives.

Entry	Compound		IC_{50} [μM]
1		**14a** (*R*,*R*,*S*) **14b** (*R*,*S*,*S*)	–[a] –[a]
2		**17a** (*R*,*R*,*S*) **17b** (*R*,*S*,*S*)	–[a] 67
3	pepticinnamin E	**1**	42
4	*epi*-pepticinnamin E	*epi*-**1**	237

[a] No inhibition.

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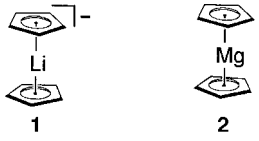
$[Cp_3Ba]^-$: The First Structurally Characterized Barate Complex**

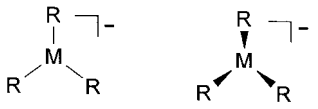
Sjoerd Harder*

In 1994 we reported that the in situ generation of "free" cyclopentadienyl (Cp) anions in the presence of CpLi resulted in the formation of the lithocene anion (**1**).[1] The structure of this complex is remarkably similar to that of its diagonally related neighbor magnesocene (**2**). Analogously, the treatment of Cp_2M (M = alkaline earth metal) with Cp anions could yield a $[Cp_3M]^-$ species. A thorough structural study on such compounds is justified for several reasons.



1 **2**

1. Although a few magnesium complexes of the type $[R_3Mg]^-$ have been structurally characterized,[2] at present no structural data are available for heavier alkaline earth metal complexes $[R_3Ca]^-$, $[R_3Sr]^-$, or $[R_3Ba]^-$.
2. How do the structures of $[Cp_3M]^-$ species (M = alkaline earth metal) compare to those of the isovalent Group 3 and Group 13 neighbors?
3. The divalent metallocenes of the heavier alkaline earth metal complexes have an unexpected preference for bent geometries, a structural feature that has attracted and still attracts much attention.[3] A similar trend may be found for the $[Cp_3M]^-$ complexes of the heavier alkaline earth metals. Will the metal center then prefer a planar (**3**) or a pyrimidal (**4**) coordination geometry?



3 **4**

First results of our studies on $[Cp_3M]^-$ complexes of the alkaline earth metals are presented here: the synthesis and structure elucidation of $[Cp_3Ba]^-$ in the presence of a weakly coordinating cation.

$[Cp_3Ba]^-$ can be synthesized simply by treating Cp_2Ba with a free Cp anion. Accordingly, several synthetic strategies can be employed. Free Cp anions can be generated in situ from CpNa and Ph_4PCl[4] and then treated with Cp_2Ba [Eq. (1)]. Another possibility is the direct reaction of Cp_2Ba with a stoichiometric quantity of Ph_4PCl [Eq. (2)].

$$CpNa + Ph_4PCl + Cp_2Ba \rightarrow [Cp_3Ba]^-[Ph_4P]^+ + NaCl \quad (1)$$

$$3\,Cp_2Ba + 2\,Ph_4PCl \rightarrow 2\,[Cp_3Ba]^-[Ph_4P]^+ + BaCl_2 \quad (2)$$

A drawback of both methods is the possible formation of side products such as $[Cp_2Na]^-$, $[Cp_2BaCl]^-$, $[CpBaCl_2]^-$, and other mixed compounds containing a variety of ions (Ba^{2+}, Na^+, Cp^-, and Cl^-). Therefore, a new method for the generation of Cp anions was developed: a Wittig reagent is mixed with cyclopentadiene to give $[Cp]^-[R_4P]^+$, which can be directly treated with Cp_2Ba [Eq. (3)].

$$Bu_3P{=}CHCH_2CH_2CH_3 + CpH + Cp_2Ba \rightarrow [Cp_3Ba]^-[Ph_4P]^+ \quad (3)$$

The crystals obtained from this reaction mixture[5] had the composition $[Cp_3Ba]^-[Ph_4P]^+[thf]$.[6] The $[Cp_3Ba]^-$ units form a linear coordination polymer (Figure 1) in which the

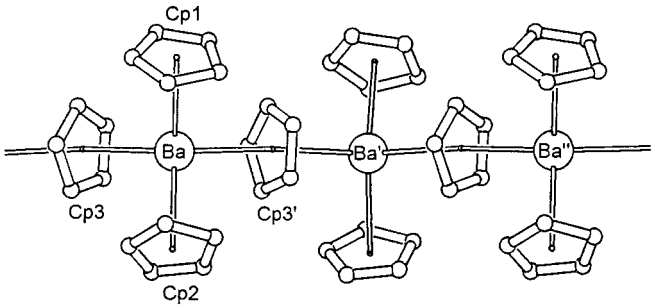


Figure 1. Structure of the linear $[Cp_3Ba]^-_\infty$ chain. All Cp rings are bound to Ba in an η^5 fashion. For the following Ba – C distances [Å] to the rings Cp1, Cp2, Cp3, and Cp3′, the range of Ba – C distances, the mean Ba – C distances, and the Ba – Cp_C distances are given. Cp1: 3.067(5) – 3.184(6), 3.123(6), 2.898(6); Cp2: 3.047(7) – 3.159(6), 3.096(6), 2.876(6); Cp3: 3.091(7) – 3.168(7), 3.129(6), 2.916(6); Cp3′: 3.123(7) – 3.220(7), 3.169(7), 2.969(6). The Cp_c-Ba-Cp_c' angles lie in the range 106.4(3) – 114.6(3)°.

Ba^{2+} cations are tetrahedrally surrounded by four Cp anions. Both the terminal and the bridging Cp anions are bound in an η^5 fashion to the barium centers. The Ba – C distances of the terminal and bridging Cp rings are remarkably similar. The barate $[Cp_3Ba]^-$ is the first structurally characterized unsubstituted Cp – Ba complex. The average Ba – Cp_c distance (Cp_c

[*] Dr. Sjoerd Harder
Universität Konstanz
Postfach 5560, M738, D-78457 Konstanz (Germany)
Fax: (+49) 7531-883137

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