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K-13 and OF4949: Evaluation of Key Partial Structures and Pharmacophore Delineation

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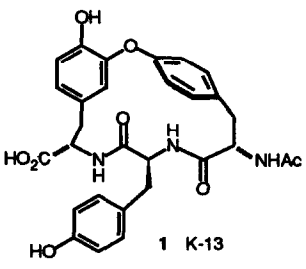
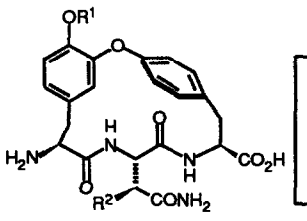
Abstract. The angiotensin I converting enzyme and aminopeptidase B inhibitory properties of key analogs of K-13 and OF4949, constituting key partial structures or substructures of the natural products, are detailed in studies to define the essential pharmacophores.

K-13 (**1**), an isodityrosine derived cyclic peptide isolated from *Micromonospora halophytica* subsp. *exilisia K-13* and identified in spectroscopic and chemical degradative studies,[1] has been shown to be a potent noncompetitive inhibitor of angiotensin I converting enzyme (I_{50} = 0.17 μg/mL, K_i = 0.35 μM) and a weak inhibitor of aminopeptidase B.[2] K-13 represents the newest member of a class of isodityrosine[3] derived cyclic peptides including OF4949-I - IV (**2-5**),[4] piperazinomycin,[5] bouvardin and related agents.[6,7] OF4949-I - IV were found to be potent inhibitors of aminopeptidase B, to lack conventional cytotoxic activity, and to possess confirmed antitumor activity.[8,9] Thus, the agents may constitute a new and potentially useful class of antitumor agents that act as immunopotentiators and which may not display host antigenicity or toxicity.[9] Consequently, in the course of synthetic studies on the isodityrosine derived agents,[10-15] we have prepared a range of key analogs of K-13 and OF4949 constituting key partial structures or substructures. Herein, we report the angiotensin I converting enzyme and aminopeptidase B inhibitory properties of a selected set of the agents (**7-29**)[10] and their *in vitro* cytotoxic activity in studies which further define the essential and potentiating structural features that contribute to their biological activity.

Aminopeptidase B (APB) Assay. The inhibitory activity on a dialyzed aminopeptidase B enzyme preparation[16,17] was assayed by the spectrophotometric determination at 405 nm for the rate of *p*-nitroaniline formation from the hydrolysis of L-arginine-*p*-nitroanilide. The enzymatic reaction was conducted for 1 h at 25°C and was initiated by the addition of a 20 μL aliquot of the enzyme preparation to a mixture of 200 μL of the L-arginine-*p*-nitroanilide solution and 780 μL of 0.1 M PIPES buffer containing 0.2 M NaCl, 1 mM DTT and increasing amounts of inhibitor (*i.e.*, 3, 6, 12, 25, 50, 100 μg)

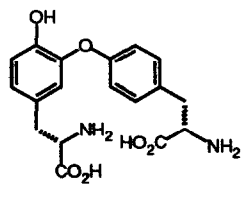


1 K-13



OF4949-I - IV

	R^1	R^2
2	CH_3	OH
3	H	OH
4	CH_3	H
5	H	H



6 isodityrosine

such that the total volume was 1 mL. Final absorbance in the absence of inhibitor was 0.36 and final absorbance due to background hydrolysis in the presence of excess APB enzyme inhibitor (OF4949-IV, 100 μg/mL) was 0.14. A control test of aminopeptidase enzyme activity due to the potential presence of leucine aminopeptidase (LAP) using a L-leucine-*p*-nitroanilide (200 μL) solution (absorbance = 0.12 at 100 μL of enzyme preparation, 5 x assay concentration) indicated no significant LAP activity. The results from this assay are presented in Table II.

Angiotensin Converting Enzyme (ACE) Assay. ACE inhibitory activity for the K-13 related samples was evaluated using rabbit lung acetone powder extract as the source of ACE and employing the spectrophotometric assay at 228 nm for the rate of hippuric acid formation from the substrate hippuryl-L-histidyl-L-leucine (HLL).[18] All samples were dissolved in DMSO. Each 250 μL assay mixture contained the following at the indicated final concentration: 100 mM phosphate buffer (pH 8.3), 100 mM NaCl, 5 mM HHL, 100 μL of rabbit lung acetone powder extract (1:20 dilution), and 50 μL of varied concentrations of compound solution or DMSO. Following 30 minutes of incubation at 37°C, the reaction was terminated by the addition of 250 μL of 1 N HCl followed by 1.5 mL of ethyl acetate. After mixing and centrifuging, 1.0 mL aliquots of the ethyl acetate layer were removed and evaporated to dryness. Hippuric acid was reconstituted in 1 mL of water and its concentration was determined spectrophotometrically at 288 nm. The results obtained from this inhibition assay are presented in Table III.

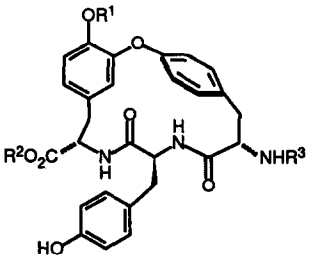
Fundamental Structural Features Responsible for Agents Properties. Select acyclic precursors and macrocyclic products constituting key substructures of isodityrosine, K-13 and OF4949-III and IV were subjected to comparative evaluation for *in vitro* cytotoxic activity[19] as well as comparative enzyme inhibitory activity in the two enzyme assays. The results expressed as the concentration necessary for 50% inhibition of cell growth relative to untreated controls (cytotoxic IC_{50}) and for 50% inhibition of enzyme activity (I_{50}) are detailed in Tables I-III. Consistent with past observations and important to the productive properties of the agents, K-13 (**1**), OF4949-III (**4**), OF4949-IV (**5**), and isodityrosine (**6**) lack inherent *in vitro* cytotoxic activity, Table I.

Table I. *In Vitro* Cytotoxic Activity, P388 (IC_{50}, μg/mL)

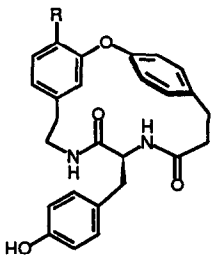
1	K-13	> 10
4	OF4949-III	> 10
5	OF4949-IV	> 10
6	isodityrosine	> 10

Table II. ACE Inhibition

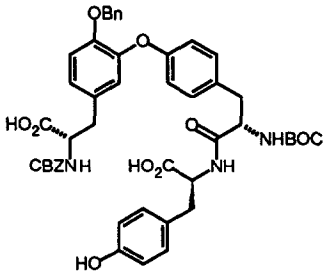
Agent	I_{50} (μg/mL)
1	0.1
7	0.5
8	0.6
9	3
10	5
11	10% at 100 μg/mL
12	inactive
13	inactive
4	70
5	11% at 100 μg/mL
6	13% at 200 μg/mL
captopril	0.005



	R^1	R^2	R^3
1	H	H	$COCH_3$
7	H	Me	$COCH_3$
8	H	Me	H
9	Me	H	$COCH_3$



10 R = OH
11 R = H



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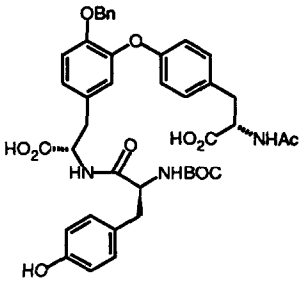

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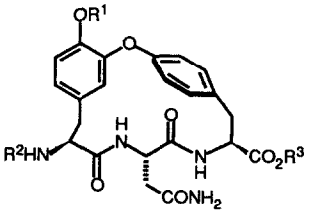
The evaluation of a range of agents structurally related to K-13 for ACE inhibitory activity revealed that the K-13 C-9 carboxylic acid potentiates the inhibitor activity but, unlike the classical ACE inhibitors, it is not essential for activity (**7** versus **1**), Table II. The comparable activity of **7** and **8** suggests that the C-15 acetamide contributes little to the productive ACE inhibition and the pronounced loss of activity with phenol *O*-methylation (**9** versus **1**) suggests a prominent role for the free phenol. Consistent with this observation and in contrast to initial expectations, removal of the C-9 carboxylate and C-15 acetamide provided an agent **10** that proved to be only 50x less effective than K-13 itself and further removal of the phenol OH from this structure provided the agent **11** that was essentially inactive. These results in conjunction with the lack of ACE inhibitory activity with **12-13** suggest that the macrocyclic structure and phenol OH constitute inherently important components of the ACE inhibitor pharmacophore.

Similar, although not identical, to the conclusions drawn from studies of the inhibition of aminopeptidase B isolated from Ehrlich ascites carcinoma employing derivatives of OF4949-I and -II,[9] the studies of the inhibition of rat liver aminopeptidase B detailed herein suggest an essential role for the OF4949 C-12 carboxamide and a strong potentiation role for the C-9 amine, Table III. The

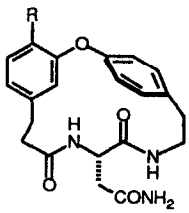
comparable activity of OF4949-IV and OF4949-IV methyl ester (**5** = **14**, also **15** ≡ **17**) underscores the lack of an important functional role for the C-15 carboxylate. The comparable activities of OF4949-IV and OF4949-III (**5** versus **4**, also **16** versus **17**) suggests little or no distinction between a C-4 hydroxy or methoxy substituent although significant perturbations at this site substantially reduced (OH ≡ OCH_3 >> OCH_2Ph, **16-18**) or abolishes activity (OH ≡ OCH_3 >> H, **19-21**). Assuming an unimportant functional role for the C-15 carboxylate, the relative importance of the C-9 amine versus the C-12 carboxamide may be assessed in the comparisons of **4-5** with **19-20**. This residual, albiet modest, activity of **19-20**, the surprisingly good activity of **15** (0.4 x **4**) and **16-17** bearing a CBZ protected amine, the inactivity of **24-29**, and the preceding observations suggest an essential role for the macrocyclic structure and the C-12 carboxamide, a strong but surprisingly nonessential role for the C-9 amine, a modest to strong potentiating role for the C-4 OH/OCH_3, and no role for the C-15 carboxylate.

Table III. APB Inhibition

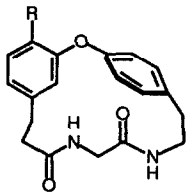
Agent	I_{50} (μg/mL)
5	20
4	40
14	20
15	110
16	120
17	130
18	14% at 220 μg/mL
19	210
20	220
21	inactive
22	inactive
23	inactive
24-29	inactive
1	13% at 230 μg/mL
6	inactive



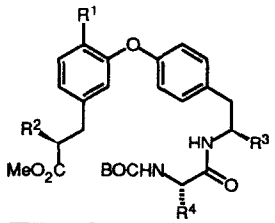
	R^1	R^2	R^3
4	Me	H	H
5	H	H	H
14	H	H	Me
15	Me	CBZ	H
16	H	CBZ	Me
17	Me	CBZ	Me
18	Bn	CBZ	Me



19 R = OH
20 R = OMe
21 R = H



22 R = OH
23 R = H



	R^1	R^2	R^3	R^4
24	OBn	NHCBZ	CO_2Me	CH_2CONH_2
25	OH	NHCBZ	CO_2Me	CH_2CONH_2
26	H	H	H	CH_2CONH_2
27	OH	H	H	CH_2CONH_2
28	OMe	H	H	CH_2CONH_2
29	H	H	H	H

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16. Preparation of rat liver aminopeptidase B (APB):[17] whole livers of male Sprague-Dawley rats (100 g, purchased from Harlan Sprague-Dawley, Indianapolis, IN) were homogenized employing an Elvehjem-Potter homogenizer in 200 mL ice-cold 0.1 M sodium phosphate (pH 7) buffer containing 0.25 M sucrose and 1 mM dithiothreitol (DTT). The homogenization vessel was kept over ice at all times. The homogenates were pooled and centrifuged in a Beckman J2-21 M/E

centrifuge for 45 minutes at 16,000 rpm. The pooled supernatants (190 mL, 0.0059 units/mg) were acidified by the gradual addition of cold 0.2 N HCl to pH 5.1. After standing 15 min, the suspension was centrifuged for 20 min at 20,000 rpm. The supernatants were pooled and brought to pH 7.0 (150 mL, 0.009 units/mg) by the slow addition of cold 0.2 N NaOH. Solid ammonium sulfate (36.4 g) was added over a period of 1.5 h to afford a cloudy suspension. After slow stirring for 1 h, the mixture was centrifuged for 20 min at 20,000 rpm. Solid ammonium sulfate (10.0 g, 52% total salt concentration) was added over 1 h. The suspension was centrifuged for 20 min at 20,000 rpm and the pellets were collected and dissolved in 15 mL 10 mM sodium phosphate (pH 7) buffer containing 5 mM sodium chloride and 1 mM dithiothreitol (DTT) and dialyzed against 10 volumes (150 mL) of the same buffer solution. The dialyzed solution was concentrated in a 10 mL Amicon pressure cell to a volume of 3 mL and stored at 4°C.

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