NOTES

Albocycline- and Carbomycin-type Macrolides, Inhibitors of Human Prolyl Endopeptidases

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Chemical screening[1,2] of microbial extracts using thin layer chromatography and various staining reagents results in a collection of pure natural products which advantageously can be examined in various target-directed biological screening attempts in order to identify new lead structures for commercial application. Our interest focused on enzymes involved in processing and degradation of biologically active peptides, for instance prolyl endopeptidases (PEP) [EC 3.4.21.26][3]. This paper deals with results from the screening towards inhibitors of prolyl endopeptidases pointing to macrocyclic lactones[4] like albocycline (**1**)[5,6], whose inhibition kinetics as well as its specificity towards different classes of proteases are reported.

A collection of structurally diverse pure secondary metabolites obtained from chemical screening strategies was assayed with prolyl endopeptidase derived from human placenta (hPEP). Besides the already reported fungal metabolite lipohexin[7,8] the 14-membered macrolide albocycline (**1**) with an IC_{50} of 9.0 μM shows a significant inhibitory effect on hPEP, whereas the structurally related 2,3-dihydroalbocycline (**2**) was found to be inactive up to concentrations of 200 μM. Based on these results we decided to investigate other structurally related secondary metabolites possessing a lactone moiety with a different ring size and/or a sugar residue. Whereas angolamycin, midecamycin, lankamycin, erythromycin, oleandomycin, spiramycin, tylosin, and the decarestricines B, D and M caused no inhibition up to a concentration of 200 μM in the assay, the 16-membered amino-sugar containing macrolides carbomycin A and B (**4** and **5**) were conspicuous due to their inhibitory effects on hPEP. The results of **4** and **5** as well as of further macrolides (Scheme 1) showing weaker inhibition of the enzyme tested are summarized in Table 1.

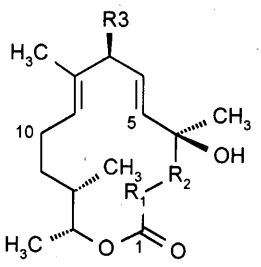
Albocycline (**1**) being found the most potent inhibitor towards hPEP among the active macrolides (Table 1) was selected for a more detailed characterization of its inhibitory effect, mode of action, as well as of its specifity. All studies of the interaction between enzyme and albocycline were performed in parallel with hPEP and bPEP from *Flavobacterium meningosepticum*[9]. Experimental details are described in previous papers[7,8].

Reversibility of albocycline (**1**)-PEP binding was investigated applying gel permeation chromatography. The mixture of albocycline (**1**) and enzyme, possessing 20% remaining activity after inhibitor addition, was completely separated after column passage corresponding to the differences in molecular weight. Using the PEP-assay, fractions 72~110 were shown to contain albocycline (**1**). Compared to a positive control up to 98% reactivated PEP was determined suggesting a reversible inhibition mode. This result was confirmed and further specified by kinetic measurements. During a time interval of 2 hours, no time dependence of the inhibitory effect of albocycline (**1**) against hPEP was observed. The Lineweaver-Burk plot (Fig. 1) characterized albocycline (**1**) as a competitive inhibitor of hPEP with a calculated *Ki* value of 14 μM. In contrast, a seven-fold weaker inhibitory effect on the bacterial enzyme was observed, reflected by a *Ki* value of 106 μM.

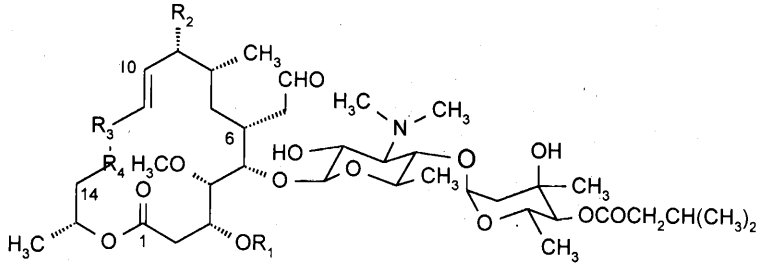
In order to determine the specificity of albocycline (**1**), the compound was tested in terms of inhibitory activity on representatives of 5 different classes of proteases. However, no effect of **1** on the activity of the cystein proteinase papain, the dipeptidase prolidase, the aminopeptidase prolyliminopeptidase, serine peptidase dipeptidyl peptidase IV, and of serine proteinases (α-chymotrypsin, trypsin, thrombin, proteinase K, pronase, subtilisin) was detected up to 100 μM.

Besides the number of synthetic peptide-like PEP inhibitors, as well as the tissue derived high molecular

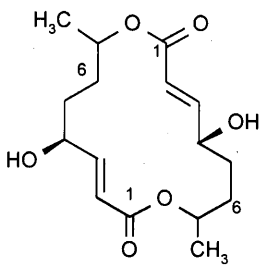
Scheme 1. Chemical structures of tested macrolides.



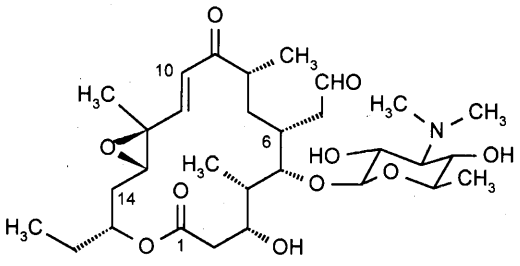
	R_1R_2	R_3
Albocycline (**1**)	HC=CH	OCH_3
2,3-Dihydroalbocycline (**2**)	CH_2-CH_2	OCH_3
Cineromycin B (**3**)	HC=CH	OH



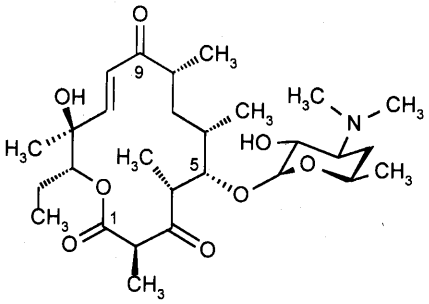
	R_1	R_2	R_3 R_4
Carbomycin A (**4**)	$COCH_3$	=O	O
Carbomycin B (**5**)	$COCH_3$	=O	C=C
Leucomycin A_1 (**6**)	H	OH	C=C



Helmidiol (**8**)



Cirramycin A_1 (**7**)



Pikromycin (**9**)

weight PEP inhibitors described so far[8] only a few examples have been reported from microbial sources. However, most of the known natural product inhibitors of PEP are peptides bearing an essential α-keto-β-amide functionality, *e.g.* poststatin[10] from *Streptomyces viridochromogenes*, and eurystatin A and B[11] isolated from *Streptomyces eurythermus*. In a previous paper we characterized a new member of this group, lipohexin, from *Moeszia lindtneri* and *Paecilomyces* sp. whose peptide backbone additionally contains a fatty acid side chain[8]. Recently, the first example of a natural product of a non-peptide inhibitor of PEP, the isotetracenone SNA-8073-B, has been reported[12]. With albocycline (**1**) an additional non-peptidic PEP inhibitor out of the group of macrocyclic lactones was discovered. With an IC_{50}-value of 9.0 μM albocyclin (**1**) shows an inhibitory effect in the same order of magnitude as other PEP inhibitors from natural sources like lipohexin (IC_{50}=3.5 μM)[8] and SNA-8073-B[12] (IC_{50}=8.0 μM). However, microbial metabolites like poststatin[10], eurystatin A and B[11] as well as synthetic pyrrolidine derivatives (*e.g.* JTP-4819[13]) have been reported to inhibit PEP from different sources in the lower nanomolar range.

Table 1. IC_{50} values of different macrolides tested as potential inhibitors of prolyl endopeptidase [EC 3.4.21.26].

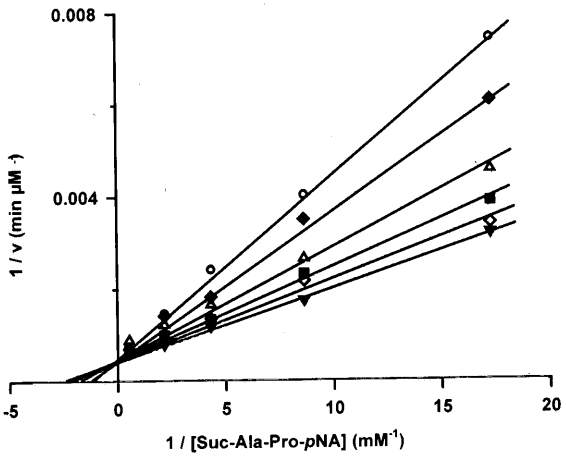
Macrolide	IC_{50} [μM] h PEP	IC_{50} [μM] b PEP
Albocycline (**1**)	9.0	96.0
2,3-Dihydroalbocycline (**2**)	—[a]	—[a]
Cineromycin B (**3**)	122.8	161.8
Carbomycin A (**4**)	26.3	23.1
Carbomycin B (**5**)	18.6	15.0
Leucomycin A_1 (**6**)	40.0	36.7
Cirramycin A_1 (**7**)	74.0	66.8
Helmidiol (**8**)	66.0	56.7
Pikromycin (**9**)	78.0	80.4

[a] No inhibitory effect observable up to concentrations of 200 μM.

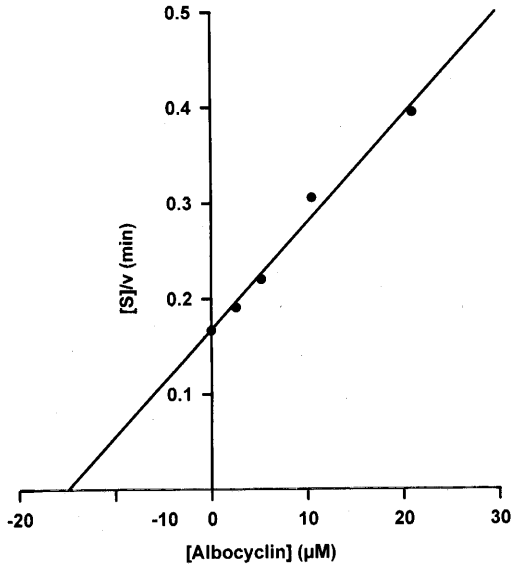
Specific PEP inhibitory testing of a number of struc-

Fig. 1. Inhibition kinetics of albocyclin (**1**) on the activity of prolyl endopeptidase from human placenta.

(A) Lineweaver-burk plot.
0 μM (▼), 1 μM (◇), 3 μM (■), 5 μM (△), 11 μM (◆) and 21 μM (○) of albocyclin (**1**).



(B) Determination of *Ki* for albocyclin (**1**).



turally related 10-, 14- and 16-membered macrolides with varying sugar moieties resulted in the discovery of further PEP-inhibitors in the macrolactone class, the carbomycins A and B (**4** and **5**) as well as the weaker active leucomycin A_1 (**6**), cirramycin A_1 (**7**), helmidiol (**8**) and pikromycin (**9**). Interestingly, all these metabolites comprise an unsaturated macrocyclic lactone ring system, but further unsaturated macrolides as spiramycin, tylosine, angolamycin and midecamycin proved to be inactive. Thus, an unsaturated character as well as a defined ring-size or the presence of an amino sugar moiety are not sufficient criteria for PEP inhibition. Macrolides without any double bond in the lactone ring like lankamycin, erythromycin and oleandomycin showed neither an effect on hPEP, nor on bPEP. Preliminary studies towards structure-activity relationships by testing albocycline derivatives suggest that the C-2/C-3 double bond seems to be required for the inhibitory effect on both, bacterial, and human PEP because 2,3-dihydroalbocycline (**2**) appeared to be inactive (Table 1). On the other hand, a drastic reduction of the inhibitory activity was also observed in case of cineromycin B (**3**) pointing to the substitution at C-7 also to be critical for PEP inhibition. At the present status, the inhibition effects in relation to structural features of the macrolides tested reveal no clear picture of structure-activity relationships.

In the course of specifitiy studies albocyclin (**1**) was characterized as a specific inhibitor of PEP isolated from human placenta because a seven-fold weaker inhibition of the enzyme isolated from *Flavobacterium meningosepticum* (bPEP) was observed. Comparable diffential inhibition effects were also seen with the other inhibitors of PEP with *Ki* values in the nanomolar to lower micromolar range for hPEP (*e.g.* lipohexin[8]). Surprisingly, carbomycin A and B (**4** and **5**) were not able to discriminate between hPEP and bPEP. Whether these specificity differences among members of the group of macrocyclic PEP inbibitors are based on different inhibition mechanisms is to be shown by further investigations.

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