METABOLIC PRODUCTS FROM MICROORGANISMS. 230 AMICLENOMYCIN-PEPTIDES, NEW ANTIMETABOLITES OF BIOTIN. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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Four new and two known peptide antibiotics containing amiclenomycin (Acm) have been isolated from a culture of *Streptomyces venezuelae* Tü 2460: L-MeIle-L-Acm (**1**), L-Ile-L-Acm (**2**), L-MeVal-L-Acm (**3**), L-MeIle-L-Acm-L-Gln (**4**), L-Ile-L-Acm-L-Gln (**5**) and L-Val-L-Acm-L-Gln (**6**). These di- and tripeptides exhibited antimicrobial activity on a minimal medium against Gram-negative bacteria, which could be reversed by biotin. It was shown that the antibiotics were decomposed by peptidases to provide amiclenomycin (**7**) after their uptake into cells of *Escherichia coli via* peptide-permeases. The antimicrobial activity of the amiclenomycin-peptides was the inhibition of DAPA-aminotransferase by the amiclenomycin-warhead, however, amiclenomycin itself was hardly transported into the cells. Since the amiclenomycin peptides misuse general transport systems, they are presented here as examples for the illicit transport concept.

HANKA[1] observed that 2~3% of the fermentation broths tested showed antimetabolite activity against *Escherichia coli* and *Bacillus subtilis*, and KOROBKOVA *et al.*[2] reported, that 25 out of 756 actinomycetes produced antimetabolites of leucine. Many anti-vitamins have been described, however, few antimetabolites structurally related to vitamins have been discovered with the exception of biotin analogues[3].

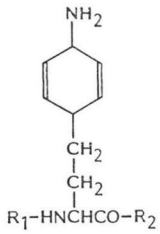
Searching for secondary metabolites with new structures, we screened a number of *Streptomyces* strains isolated from soil samples of various origins. Using a difference test as reported by HANKA[1], *Streptmyces venezuelae* Tü 2460 was found to exhibit antibacterial activity only on minimal media which could be reversed by biotin.

In this paper, we describe the optimization of the fermentation of *S. venezuelae* Tü 2460, which produces a group of new, closely related antibacterially active compounds: L-MeIle-L-Acm* (**1**), L-Ile-L-Acm (**2**), L-MeVal-L-Acm (**3**), L-MeIle-L-Acm-L-Gln (**4**), L-Ile-L-Acm-L-Gln (**5**) and L-Val-L-Acm-L-Gln (**6**) (Fig. 1). Furthermore, the mode of action of the new antimetabolites was investigated with respect to previous studies on the transport of inhibitors linked to peptides[4].

Isolation and structure elucidation of the six Acm-peptides are described in a separate paper[5]. Two compounds (**1** and **3**) were found to be identical with stravidin and MSD-235 S2[6].

* Abbreviations: Acm, amiclenomycin; KAPA, 7-keto-8-amino pelargonic acid; DAPA, 7,8-diamino pelargonic acid (prepared by the method of MELVILLE *et al.*[26]); DTB, desthiobiotin; SAM, *S*-adenosylmethionine.

Fig. 1. Structures of the Acm-peptides.



Acm-peptides		R_1	R_2
MeIle-Acm	(1)	MeIle	OH
Ile-Acm	(2)	Ile	OH
MeVal-Acm	(3)	MeVal	OH
MeIle-Acm-Gln	(4)	MeIle	Gln
Ile-Acm-Gln	(5)	Ile	Gln
Val-Acm-Gln	(6)	Val	Gln
Acm	(7)	H	OH

Materials and Methods

Organisms

S. venezuelae Tü 2460, the production organism, was isolated from a soil sample from Tel Jericho, Israel. Taxonomic studies were carried out following the 'Methods Manual' of SHIRLING and GOTTLIEB[7]. *E. coli* 12593/74, the standard test organism for the bioassay of Acm-peptides, was obtained from Prof. G. LEBEK, University of Bern, Switzerland. The other bacterial strains used were mutants of *E. coli* K12 and *Salmonella typhimurium* LT 2 and are listed in Table 1.

Media and Growth Conditions for The Test Organisms

Cells were grown in liquid culture, on agar slants or on agar plates containing either TY-medium (Tryptone 1%, yeast extract 0.5%, NaCl 0.5%) or DM-mineral salt medium[8], the latter supplemented with 0.4% glucose and 1 mM sodium citrate. If required, the media contained (per ml) 20 μg of amino acids, 40 μg of uracil, thymine, cytosine, and 1 μg of thiamine.

Bioassay of Acm-Peptides

The antibiotic content of the cultures was measured by a disk diffusion assay using *E. coli* 12593/74 on agar plates that contained 4 ml of BF seed agar with 3×10^8 cells per plate over 17.5 ml of BF basal agar (sodium lactate 0.5%, Na_2SO_4 0.5%, KH_2PO_4 0.05%, K_2HPO_4 0.15%, $MgCl_2 \cdot 2H_2O$ 0.02%, NH_4Cl 0.5%, glucose 1%, agar 1.5%).

A distinction and a quantitative determination of individual Acm-peptides in the fermentation broth was not possible by this method because of the heterogeneous mixture of closely related analogues. However, it was performed by preparative HPLC of partially purified samples[5]. Routinely, the total activity of antimetabolite content in the culture filtrate is given as units equivalent to one μg MeIle-Acm as a standard. Biological properties of the Acm-peptides were assayed by the cross test[10] on agar plates containing one of the other test organisms listed in Table 1.

Determination of MIC

Minimal inhibitory concentrations (MIC) were determined in microtiter plates that contained 0.2 ml of liquid BF-medium supplemented with (per ml) Ca-pantothenate 50 μg, thiamine 50 μg, folic acid 0.25 μg, niacin 50 μg, *p*-aminobenzoic acid 25 μg, pyridoxal hydrochloride 50 μg and riboflavine 25 μg, 0.01 ml of inoculum to a final concentration of 10^4 cells per ml and 0.01 ml of a dilution series of the Acm-peptides to final concentrations from 1,024 to 0.007 μg/ml.

Fermentation

Stock cultures were maintained as suspensions of spores in glycerol (30%) at −20°C. For inoculation, cultures were maintained on agar slants consisting of glucose 0.4%, yeast extract 0.4%, malt extract 1% and Bacto agar 2% (Difco). The microbial growth of a one-week slant was used to inoculate a 500-ml seed flask containing 100 ml seed medium, that consisted of yeast extract 2% and glycerol 2%. The production of the Acm-peptides was carried out in Erlenmeyer-flasks (500 ml) and fermentors (25 and 200 liters). The production medium consisted of glycerol 2%, $(NH_4)_2SO_4$ 1%, K_2HPO_4 0.17%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $FeSO_4 \cdot 7H_2O$ 0.002%, $ZnSO_4 \cdot 7H_2O$ 0.001% and $CaCl_2 \cdot 2H_2O$ 0.04%. 1% of $MgHPO_4 \cdot H_2O$ was added after 34 hours of incubation to reduce nitrogen repression. To prevent foaming, polyol antifoam was added. All media were sterilized at 121°C for 20 minutes. Fermentation conditions in shaker flasks with one intrusion: 120 rpm shaker (Typ 106 Infors AG), 40 hours at 27°C. Fermentation in tanks (25 and 200 liters) with intensor system: 0.5 v/v/minute, 1,000

Table 1. Bacterial strains used.

	Strain	Genotype and properties	Source or ref
Escherichia coli	MTM 22	*pyrD zeb*:: *Tn*10 *pepN*308	27
K 12	MTM 110	*pepN pepA pepB pepQ pepD*△ (*pro-lac*)	McCaman
	9200	F⁻ *pepN arg trp his thr leu thi gal lac mal xyl mtl rpsL*	28
	BR 158	*aroB malT tonB tsx*	29
Salmonella	TN 852	*leuBCD*485 *pepN*10 *pepA*1 *pepB*1 *pepD*3 *zfg*801:: *Tn*10	
typhimurium	TN 858	*leuBCD*485 *pepN*⁺ *pepA*1 *pepB*1 *pepD*3 *zfg*801:: *Tn*10	C. G. Miller
LT 2	TN 859	*leuBCD*485 *pepN*10 *pepA*⁺ *pepB*1 *pepD*3 *zfg*801:: *Tn*10	C. G. Miller
	TN 860	*leuBCD*485 *pepN*10 *pepA*1 *pepB*⁺ *pepD*3 *zfg*801:: *Tn*10	C. G. Miller
	TN 861	*leuBCD*485 *pepN*10 *pepA*1 *pepB*1 *pepD*⁺ *zfg*801:: *Tn*10	C. G. Miller

rpm, 3~4 days at 30°C. The cell mass of the seed flasks was harvested on a paper filter (Machery and Nagel, No. 615), washed with 3 volumes of sterile NaCl 0.9%, and resuspended in a small volume of the production medium to inoculate a fermentor.

Results

Taxonomic Studies

Among various strains of Streptomycetes that were isolated from soil samples, one strain, Tü 2460, exhibited antibacterial activity which was reversible by biotin.

Microscopic Characteristics

Branched substrate mycelium developed aerial mycelia with spores of cinnamomeus color in straight and flexus strains ('*Rectus Flexibilis*') on various media. Under an electron-microscope, these spores showed a smooth to warty surface without spiny or hairy structures. The spores were cylindrical and measured 0.6~0.7×0.8~1.0 μm.

Physiological Characteristics

The melanin reaction was positive on peptone iron agar (Difco) supplemented with yeast extract 0.1%. Utilization of carbohydrates was investigated by the method of Pridham and Gottlieb[11]. Only glucose and galactose were used for growth (Table 2).

The morphological and physiological characteristics of Tü 2460 best matched those of *Streptomyces xanthophaeus*[12,13]. According to Hütter[14], *S. xanthophaeus* is a synonym for *S. venezuelae*. The strain Tü 2460 differs from other strains of *S. venezuelae* in producing the antimetabolites of biotin described herein. Furthermore, strain Tü 2460 does not produce chloramphenicol as reported by Ehrlich *et al.*[15] under conditions used in these investigations.

Since the production of a secondary metabolite is typical for a strain, but not for a genus of a microorganism, this isolate was assigned the name *S. venezuelae* Tü 2460.

Fermentation

Acm-peptides were synthesized by *S. venezuelae* Tü 2460 after a short incubation time on different media consisting of various complex carbon and nitrogen sources like malt extract, oat meal or milk powder and meat extract, peptone from meat or caseine, soybean meal and cotton seed, for example. However, it was almost impossible to separate the biologically active substances from the large amounts of small peptides present in those complex broths. Therefore, a new peptide-free production medium consisting only of well defined components was composed (see Materials and Methods) and optimized

Fig. 2. Optimum temperature for growth (■) of strain Tü 2460 and production (●) of Acm-peptides.

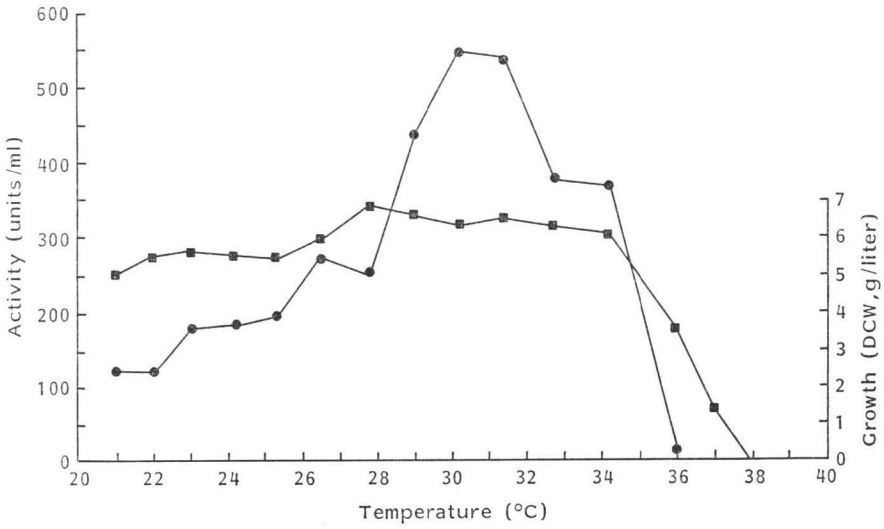
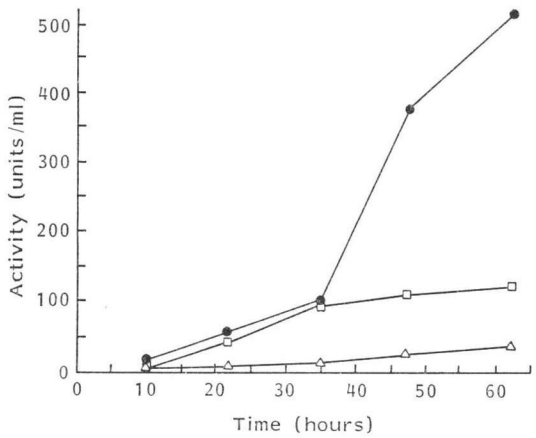


Table 2. Utilization of carbohydrates of *S. venezuelae* Tü 2460 on PRIDHAM-GOTTLIEB's basal medium.

Carbon source	Growth of Tü 2460
None	−
D-Arabinose	±
D-Xylose	−
D-Glucose	+
D-Galactose	+
D-Fructose	−
L-Rhamnose	±
Sucrose	−
Cellulose	−
Raffinose	−
myo-Inositol	−
D-Mannitol	−

+: Growth, ±: weak growth, −: no growth.

Fig. 3. Production of Acm-peptides at pH 5.5 (●), pH 6.5 (□) and pH 7.5 (△).



for maximal yields of the antibiotics.

The growth of Tü 2460 and biosynthesis of Acm-peptides was optimal at 30°C and pH 5.5 (see Figs. 2 and 3). Tü 2460 needs large amounts of oxygen for optimal growth. Limitation was prevented by vigorous agitation and high aeration rates (1,000 rpm, 0.5~1.0 v/v/minute).

Phosphate repression was observed at concentrations higher than 20 mM. The biosynthesis of the antibiotics was not affected by a glucose concentration up to 3% of the medium, but could be stimulated by replacing glucose by glycerol. Ammonium sulfate as nitrogen source in the mineral salt medium caused a significant nitrogen repression. This could be avoided by addition of $MgHPO_4 \cdot 3H_2O$ 1% after 34 hours of incubation (see Fig. 4).

Production reached amounts of 700 mg of Acm-peptides per liter of fermentation broth under these conditions. The content of the pure components was calculated by the composition of all Acm-peptides

Fig. 4. Nitrogen repression in the production of Acm-peptides.

Activity with (●) and without (○) addition of $MgHPO_4 \cdot 3H_2O$ after 34 hours of incubation.

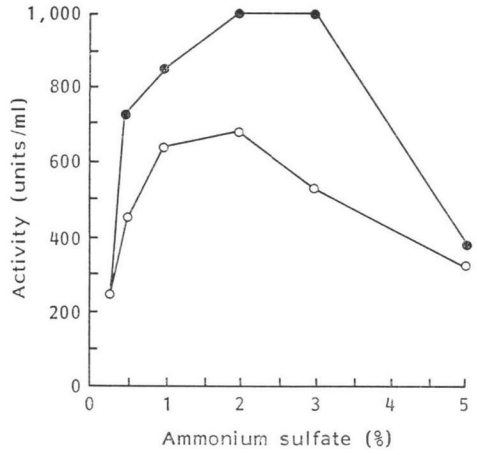


Table 3. Yield of Acm-peptides from Tü 2460.

	Acm-peptides	Yield % (w/w)
1	MeIle-Acm	24
2	Ile-Acm	3
3	MeVal-Acm	1
4	MeIle-Acm-Gln	24
5	Ile-Acm-Gln	47
6	Val-Acm-Gln	1

isolated up to now as shown in Table 3.

A kinetic of Acm-peptide production in a 25-liter reactor is shown in Fig. 5.

Biological Properties of Acm-peptides

The antibacterial spectra and the corresponding minimal inhibition concentrations were assayed in microtiter plates as described above. Acm-peptides showed broad spectrum activity against a number of clinically important organisms among Gram-negative bacteria (Table 4). They were inactive to most Gram-positive bacteria.

Moreover, Tü 2460 produced a polypeptide in addition to the Acm-di- and tripeptides. This polypeptide exhibited no antibacterial activity, but there was evidence for a synergistic activation when used in combination with for example the tripeptide **6**.

Transport

The test organism *E. coli* 12593/74 and two mutants of *E. coli* K12 with deficiencies in peptide uptake systems showed marked differences in their susceptibility to the various Acm-peptides (Table 5). *E. coli* K 12 D 448, which lacked only the oligopeptide permease, was susceptible to the dipeptides **1**, **2** and **3**, but resistant to the tripeptides **4**, **5** and **6**. *E. coli* K 12 D 449 was nearly resistant to all Acm-peptides produced by Tü 2460 because of deficiencies in both permeases. Furthermore, *E. coli* 12593/

Fig. 5. Fermentation kinetic of *S. venezuelae* Tü 2460.

Symbols: Glycerol (△), Pi (▲), total nitrogen (○), pO_2 (□), growth (■) and activity (●).

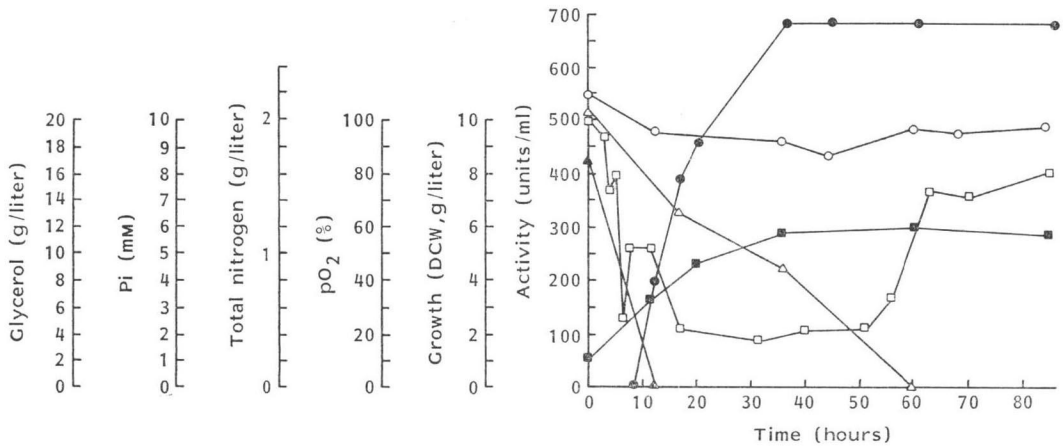


Table 4. Antibacterial activity of Acm-peptides.

Organism	MIC (μg/ml)					
	1	**2**	**3**	**4**	**5**	**6**
Escherichia coli ATCC 11775 TEM	0.125	0.03	0.06	0.06	0.5	0.01
E. coli ATCC 11775	0.25	0.007	0.125	0.06	0.125	0.01
E. coli BC 29 beta	0.125	0.03	0.03	0.06	0.25	0.08
Pseudomonas aeruginosa BC 19	>64	>64	>64	64	16	10.5
P. aeruginosa Hbg	>64	>64	>64	>64	>64	21.0
Klebsiella pneumoniae ATCC 10031	0.06	0.007	0.06	0.25	0.125	0.16
K. pneumoniae 1082	8	0.5	1	0.5	0.5	0.33
K. pneumoniae BC 6	8	16	1	2	0.5	2.62
Serratia marcescens ATCC 13880	4	1	0.5	0.5	2	1.3
S. marcescens BC 8	0.06	0.06	0.06	0.06	0.06	0.04
Enterobacter cloacae ATCC 13047	4	4	8	4	2	1.3
E. cloacae P 99	0.015	0.03	0.125	0.06	0.125	0.16
Citrobacter freundii ATCC 11601	0.015	0.015	0.015	0.125	0.06	0.04
Proteus inconstans ATCC 25825	0.25	0.007	0.06	0.125	1	0.16
Acinetobacter calcoaceticus ATCC 11959	64	>64	64	32	16	10.5
A. calcoaceticus ATCC 15473	64	>64	64	32	32	21.0

Table 5. Activity of the Acm-peptides against *E. coli* 12593/74 and two peptide permease mutants of *E. coli* K-12.

E. coli strain	Activity of Acm-peptides (10 μg/ml)*						
	1	**2**	**3**	**4**	**5**	**6**	**7**
12593/74	+	+	+	+	+	+	−
K-12 D 448 (*opp*)	+	+	+	−	−	−	−
K-12 D 449 (*opp dpp*)	−	−	(+)	−	−	−	−

* +: Growth inhibition, −: no activity, (+): weak activity.

74 was nearly resistant to Acm (**7**) prepared from **2** by enzymatic cleavage with Pronase P, but in the contrary to the Acm-peptides **1**~**6** this poor activity was shown to be reversible by the basic amino acids arginine, lysine and ornithine by the cross test (data not shown).

Mode of Action

A multiple peptidase deficient mutant of *E. coli* K 12 (MTM 110) was resistant to the 6 Acm-peptides in contrast to the controls (*E. coli* K 12 BR 158, peptidase positive; *E. coli* K 12 9200 and *E. coli* K 12 MTM 22, deficient only in peptidase N). In addition, the susceptibility of mutants of *S. typhimurium* LT 2 defective in all but one peptidase was assayed (Table 6). The Acm-peptides could only be activated, if peptidase N or A, and in addition peptidase D in the case of the dipeptides **1**~**3**, was present to release the antibiotically active compound into the cytoplasm.

The biological activity of the antibiotics produced by Tü 2460 has been found to be reversible by biotin as found for stravidin[8,18]. Therefore, the effects of biotin vitamers such as KAPA, DAPA and DTB, on the action of Acm-peptides were examined by the cross test[10]. In addition, the effects of pimelic acid, glutaric acid, L-alanine and SAM, which are also involved in the biotin biosynthesis of several organisms, were investigated in the same way. As shown in Table 7, the action of the Acm-peptides from Tü 2460 was reversed by biotin, DTB and DAPA, but not by KAPA or any other of the vitamers.

Acute toxicity of Acm-peptides was examined using NMRJ mice (18~22 g). No mouse died upon

Table 6. Activity of the Acm-peptides against peptidase deficient mutants of *S. typhimurium*.

Strain	Peptidase deficiency	Activity of Acm-peptides (10 μg/ml)*						
		1	2	3	4	5	6	7**
TN 852	*pepN pepA pepB pepD*	−	−	−	−	−	−	(+)
TN 858	*pepA pepB pepD*	+	+	+	+	+	+	(+)
TN 859	*pepN pepB pepD*	+	+	+	+	+	+	(+)
TN 860	*pepN pepA pepD*	−	−	−	−	−	−	(+)
TN 861	*pepN pepA pepB*	+	+	+	−	−	−	(+)

* +: Growth inhibition, −: no activity.
** Only weak activity at 1,000 μg/ml.

Table 7. Antagonistic effect of biotin and its vitamers.

Compound	Concentration (μg/ml)	Acm-peptides (10 μg/ml)	Acm* (50 μg/ml)	Actithiazic acid* (50 μg/ml)
None	0	+	+	+
Biotin	0.1	+	+	+
DTB	1.0	+	+	−
DAPA	10	+	+	−
KAPA	1,000	−	−	−
Pimelic acid	1,000	−	−	−
Glutaric acid	1,000	−	−	−
L-Alanine	1,000	−	−	−
SAM	1,000	−	−	−

−: No growth; +: growth after 2 days of incubation.
* Assayed with *Mycobacterium smegmatis* ATCC 607 (KITAHARA *et al.*[15]).

administering the antibiotics at a dose of 320 mg/kg subcutaneously.

Discussion

The Acm-peptides **2**, **4**~**6** isolated from the cultured broth of the soil isolate named *S. venezuelae* Tü 2460 are new antimetabolites competitive with biotin.

The production of these antibiotically active di- and tripeptides and of the two known antibiotics **1** and **3** could be stimulated up to 50-fold by optimization of the physico-chemical fermentation parameters (pH, temperature, aeration) and by development of an appropriate production medium which makes it feasible to avoid repression by glucose, phosphate and nitrogen.

From the above data, it could be concluded that after incorporation into the cells of Gram-negative bacteria the Acm-peptides **1**~**6** were converted to amiclenomycin (**7**), the essential biologically active part of these antibiotics. Acm is known to be a strong antimetabolite of biotin[9], which is inhibiting the DAPA-aminotransferase by binding probably to the KAPA-binding site[17,18]. BAGGALEY *et al.*[19] noted a structural correlation between KAPA and the Acm-moiety of stravidin.

The mode of action involves active transport misusing peptide permeases followed by intracellular peptidase cleavage to release the Acm-warhead. Although some Acm-peptides (**1**, **3** and **4**) are unusual in that their terminal amino group is *N*-methylated, they are transported adequately by bacterial peptide permeases, but may be cleaved more slowly[20,21]. The warhead itself is hardly antibacterially active because it is not transported into cells of *E. coli via* peptide permeases. Only at high concentrations it is taken up by the basic amino acid permease that is specific for arginine, lysine and ornithine. Hitherto, it has been reported to show activity against mycobacteria[9] only.

The long-chain peptide supports synergism to Acm-peptides, although it is not identical to streptavidin[22] or avidin[23]. The sequence of this polypeptide will be published elsewhere.

Unlike permeases involved in amino acid transport, peptide permeases are far less precise in molecular terms. Thus, it is possible for an amino acid mimetic, which is not transported by the specific amino acid permeases to be taken into the bacterial cell disguised as a peptide. This illicit transport concept[24,25] is increasingly being used to facilitate the rational design of novel peptides and could lead to further developments of microbial chemotherapy.

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