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Antibacterial Evaluations of Thiazomycin

—A Potent Thiazolyl Peptide Antibiotic from *Amycolatopsis fastidiosa*

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Abstract Thiazomycin is a novel thiazolyl peptide closely related to nocathiacin I. It was isolated from *Amycolatopsis fastidiosa* by chemical and biological screening. Thiazomycin showed highly potent bactericidal activity against Gram-positive pathogens (MIC range 0.002∼0.064 μg/ml) and did not show cross-resistance to clinically relevant antibiotic classes such as β-lactams, vancomycin, oxazolidinone and quinolones. It was highly efficacious against *Staphylococcus aureus* infection in mice exhibiting an ED_{99} value of 0.15 mg/kg by subcutaneous administration. It inhibited bacterial growth by selective inhibition of protein synthesis and it was thought to interact with L11 protein and 23S rRNA of the 50S ribosome. Structurally, it possesses an oxazolidine ring in the amino-sugar residue that provides further opportunities for selective chemical modifications that are not feasible with other thiazolyl peptides. More importantly such a modification can potentially lead to semi-synthetic compounds that overcome problems that have hampered clinical development of this class of compounds. Despite its positive attributes, emergence of an unacceptable frequency of resistance poses significant challenges for further development of thiazomycin and this class of molecules for therapeutic use.

Keywords antibiotics, thiazolyl peptide, protein synthesis inhibitors, natural products

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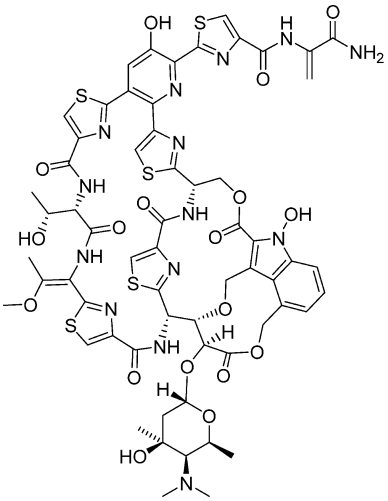
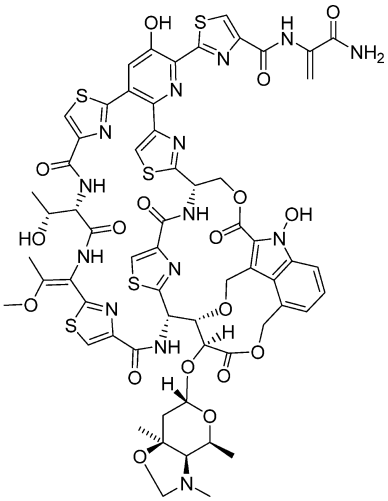
Introduction

The discovery of penicillin and other antibacterial agents to treat infections from pathogenic bacteria is arguably one of the greatest achievements of the science and medicine of mid-twentieth century [1, 2]. Incremental improvements to those discoveries have led to the development of new and improved antibiotics, which continue to serve humanity well. However, resistant bacteria are losing their susceptibility to these antibiotics rendering them ineffective. Fortunately the frequency of antibiotic resistance is variable and the bacteria that are resistant to one antibiotic are susceptible to others. The most alarming situation would be wherein bacterial strains become resistant to multiple antibiotics leading to limited or no treatment options. While these situations have started to emerge (*e.g.*, recalcitrant methicillin resistant *Staphylococcus aureus* and others), fortunately they also remain rare. In order to treat such infections and to avoid a potential epidemic, new treatment options must include discovery of new structural chemotypes that inhibit bacterial growth by new mode of action (*e.g.*, platensimycin [3, 4] and platencin [5, 6]).

Thiazolyl peptides are a class of naturally occurring antibiotics. The first member of this class of antibiotics was discovered in 1948 (*e.g.*, micrococcin) followed by the second well known member in 1954 (*e.g.*, thiostrepton) [7]. These compounds possess some of the most potent *in vitro* antibacterial activities known but could not be developed as clinical agents due to poor physicochemical properties,



Nocathiacin I



Thiazomycin

most notably their poor aqueous solubility. The lack of discoveries of new structural classes with antibiotic activities that could be further developed coupled with newer developments in chemistry and biology during this intervening period prompted us to re-examine the area of thiazolyl peptides. These efforts led to the discovery of a new member of thioglycohexide family, named thiazomycn in our laboratories [8]. Thiazomycin produced by *Amycolatopsis fastidiosa* was isolated as a congener of nocathiacin I and differs only in the amino-glycosidic structural unit. Nocathiacin I in the past was subjected to significant chemical and biological studies leading to the identification of improved semi-synthetic analogs and one such compound was selected for preclinical development [9～19]. Thiazomycin which possesses an oxazolidine ring instead of a dimethyl amino group present in nocathiacin I, provides further opportunities for chemical manipulations to increase its water solubility. Such a compound if successful would be an ideal choice for the development of an intravenous antibiotic. We report herein the biological characterization and *in vivo* evaluation of thiazomycin as an antibiotic. Its isolation, structure elucidation and large scale production is described in the accompanying paper [8].

Results

In Vitro Antibacterial Activities

Thiazomycin was a potent inhibitor of the growth of clinically relevant Gram-positive bacteria. The activity was measured as MIC and the data are shown in Table 1. The potency of thiazomycin was similar to that reported for most other thiazolyl peptides including nocathiacin I. It exhibited a low MIC (0.016 μg/ml) against the *Staphylococcus aureus* Smith strain and was equally potent against MRSA. The *in vitro* activity of this compound was even better against *Streptococcus pneumoniae*, whether the strains were penicillin sensitive or resistant, with MIC values of 0.004 and 0.002 μg/ml, respectively. Thiazomycin showed similar activity against *Streptococcus pyogenes* (MIC 0.002 μg/ml). The activities against *Enterococci* were good, but less potent than those against *Streptococcus pneumoniae*. It had an MIC value of 0.064 μg/ml against both vancomycin susceptible and vancomycin resistant phenotypes of *Enterococcus faecalis*. Thiazomycin also displayed better potency against vancomycin and linezolid resistant phenotypes of *Enterococcus faecium* and showed an MIC of 0.008 μg/ml. Since the mechanism of inhibition of thiazomycin was distinct and different from the clinically used drugs, it was not surprising that it did not exhibit cross resistance to the drug resistant organisms that were tested including *Staphylococcus aureus* that was resistant to protein synthesis inhibitors such as linezolid, macrolides, chloramphenicol, aminoglycosides, and tetracycline (Table 2). Thiazomycin did not inhibit growth of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* at 32 μg/ml. It also did not inhibit the growth of *Candida albicans*. These results clearly demonstrate that thiazomycin displayed preferential selectivity for Gram-positive bacteria over Gram-negative bacteria and eukaryotic microorganisms.

In Vivo Efficacy

The *in vivo* efficacy of thiazomycin was measured in a disseminated *Staphylococcus aureus* target organ assay

Table 1 *In vitro* antibacterial activity of thiazomycin (MIC in μg/ml)

Organism	Strain #	Nocathiacin I	Thiazomycin	Linezolid
Streptococcus pneumoniae (penicillin^S)	CL8002	0.004	0.004	1
S. pneumoniae (penicillin^R)	CL5771	<0.001	0.002	0.5
S. pyogenes	CL10440	0.006	0.002	4
Enterococcus faecalis (vancomycin^S)	CL8516	0.016	0.064	1
E. faecalis (vancomycin^R)	CL5246	0.050	0.064	1
E. faecium (vancomycin^R, linezolid^R)	CL5791	0.004	0.008	32
Staphylococcus aureus (MSSA)	MB2865	0.004	0.016	2
S. aureus (MRSA)	MB5393	0.008	0.032	2
S. aureus (vancomycin intermediate)	CL5706	NT	<0.03	NT
S. epidermidis	CL8040	0.016	0.064	1
Haemophilus influenzae	MSD 2261	>32	>32	>32
Escherichia coli	MB2884	>32	>32	>32
Pseudomonas aeruginosa	CL8222	>32	>32	>32
Candida albicans	MY1055	>32	>32	>32

NT=not tested.

Table 2 *In vitro* antibacterial activity of thiazomycin and comparator compounds against *S. aureus* resistant to protein synthesis inhibitors (MIC in μg/ml)[a]

Strain #	Resistance phenotype	Thiazomycin	Chloramphenicol	Gentamicin	Linezolid	Clarithromycin	Tetracycline
ATCC 29213	Control susceptible strain	0.008	0.12	16	2	0.25	0.5
CL8082	Tetracycline^R	0.008	0.25	16	2	0.25	16
CL5816	Linezolid^R	0.004	0.25	>64	64	0.5	32
CL8072	Macrolide^R (constitutive)	0.008	0.12	32	2	>64	0.5
CLB24148	Linezolid^R, Tetracycline^R, Gentamicin^R	0.004	64	>64	64	0.5	32
CLB24152	Linezolid^R, Tetracycline^R, Gentamicin^R, Clarithromycin^R	0.008	>64	>64	64	>64	32
CLB2408	Macrolide^R (Inducible), Gentamicin^R	0.008	64	>64	2	>64	0.5

[a] All media contained 2.5% lysed horse blood.

model in which CD-1 mice were infected with 2×10^3 CFU of *Staphylococcus aureus* MB2865 strain by IP injection and drug was administered within 20 minutes post-infection by specified route of administration and dosing regimens. Three to five mice were used in each group comprised of treatment and vehicle groups. Twenty four hours post infection the kidneys were removed, homogenized, plated and organisms recovered were counted. The activity was determined by comparing the recovered CFU of *Staphylococcus aureus* from the vehicle treated group with that from the compound treated groups. Thiazomycin was highly efficacious in this experiment. It exhibited greater than 5 log reduction of CFU at 0.78 mg/kg when dosed subcutaneously twice daily and the effect was dose dependent. The ED_{99} (dose of drug that reduces CFU load by 2 logs) was 0.15 mg/kg (Fig. 1A). In a

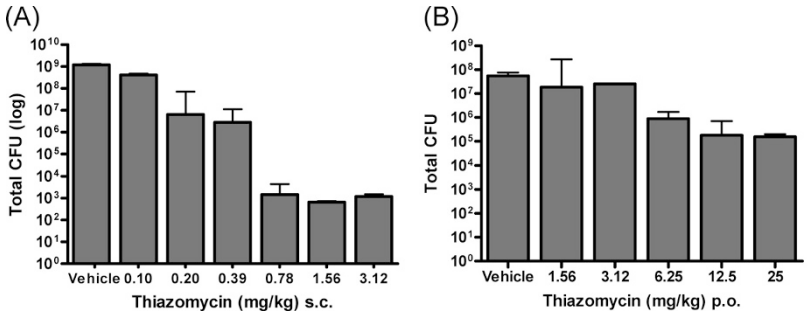


Fig. 1 *In vivo* efficacy of thiazomycin in *Staphylococcus aureus* mouse model.

Three mice each were infected with 2×10^3 *S. aureus* CFU by IP route. Twenty minutes post infection thiazomycin was dosed either subcutaneously or orally and again after 6 hours. Kidneys were removed after 24 hours, homogenized, plated and CFU were counted for treated and vehicle group. CFU counts and total of two doses are plotted. (A) Subcutaneous administration, (B) Oral administration.

similar experiment when thiazomycin was dosed orally twice daily, a 2.5 log reduction of *Staphylococcus aureus* CFU at 25 mg/kg was observed (Fig. 1B). These data were consistent with a suggestion that thiazomycin was highly efficacious by subcutaneous route of administration and weakly active by oral administration.

Inhibition of Macromolecular Synthesis and Determination of Mechanism of Action

Study of macromolecular synthesis by whole cell labeling provides an opportunity to measure the effect of inhibitors on the synthesis of macromolecules and allows determination of broad global mechanism of action. In this assay, syntheses of cell wall, DNA, RNA, protein and phospholipids in *Staphylococcus aureus* was measured in the presence of inhibitors. Thiazomycin inhibited protein synthesis with an IC_{50} value of 0.031 μg/ml. It did not inhibit synthesis of the other four macromolecules at 2.0 μg/ml indicating greater than 64 fold selectivity for the inhibition of protein synthesis (Fig. 2). This confirms that thiazomycin inhibits bacterial growth by inhibiting protein synthesis like other thiazolyl peptides. The similarities of the IC_{50} values of protein synthesis inhibition and MIC suggest that the termination of protein synthesis was the predominant mode of action of this compound for bacterial growth inhibition but distinct from clinical protein synthesis inhibitors such as linezolid, macrolides, chloramphenicol, aminoglycosides, and tetracycline.

***In vitro* Time-kill Curve Assay**

This assay allows the determination of whether the inhibitory effect of the antibiotic is bactericidal or bacteriostatic. These experiments were performed at 2×, 4×, 8× and 16× of MIC. At all concentrations, thiazomycin showed a 4~5 log reduction of colony

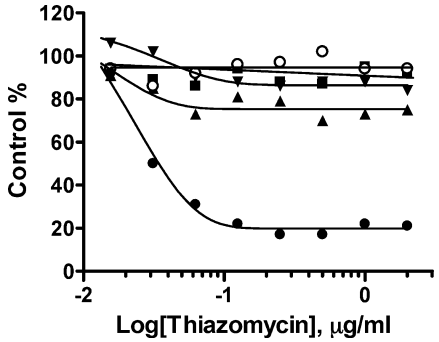


Fig. 2 Inhibition of macromolecular synthesis (*S. aureus*) by thiazomycin.

It inhibited protein synthesis (●, IC_{50}=0.031 μg/ml). It did not inhibit cell wall (■), DNA (▲), RNA (▼), phospholipids (○).

forming units (CFU) of *Staphylococcus aureus* in less than 3 hours suggesting that thiazomycin inhibits the growth of *Staphylococcus aureus* by a cidal mechanism, just as reported for nocathiacin I [17].

Mutation Analysis

Thiazomycin resistant mutants of *Staphylococcus aureus* MB2865 were obtained by selection on BHI agar medium at 0.064 μg/ml of thiazomycin. Sequence analysis revealed mutations in *rplK* gene encoding L11 protein. The mutations consisted of base pair changes, insertions and deletions as reported for nocathiacin I [17]. The deletion of base pairs 67~78, mapping to deletion of four amino acids (Pro^{23}-Val^{24}-Gly^{25}-Val^{26}) in L11 protein, resulted in a phenotype that showed significant resistance and the highest increase in MIC value (>32 μg/ml).

Discussion

Emergence of bacterial resistance to antibiotics is a significant problem and continues to increase every year both in nosocomial and community settings. To eliminate or to reduce the treatment failures and combat resistant organisms, particularly those that are resistant to multiple drugs, discovery and development of drugs with new chemotypes that inhibit bacterial growth by new mechanisms of action is urgently needed. Members of the thiazolyl peptide class of antibiotics are extremely potent but suffer from poor water solubility which renders them unsuitable for therapeutic uses. Newer members of this class have been subjected to significant chemical modifications leading to improvements in desired properties and selection of compounds for preclinical development.

Thiazomycin, one of the newer members of this class, was discovered by chemical screening designed specifically to discover new members of this class of compounds. Thiazomycin was highly potent *in vitro* against Gram-positive bacteria and was highly efficacious *in vivo* against *Staphylococcus aureus* in mice. Potency against *Streptococcus pneumoniae* was most remarkable. It showed activity against β-lactams-, vancomycin-, macrolides-, quinolones-, and other antibiotic-resistant bacterial phenotypes such as protein synthesis inhibitors (linezolid-, macrolide-, gentamicin-, chloramphenicol-, and tetracycline-) indicating no cross-resistance, which was consistent with its new chemical structure and new mechanism. Thiazomycin selectively inhibited protein synthesis and did not inhibit other macromolecules (*e.g.*, DNA, RNA, cell wall and phospholipids) suggesting its specific interaction at ribosomal level. A mutational mapping study suggested that all mutations occurred at proline rich region of L11 protein, the most predominant being deletion of four amino acids (Pro_{23}-Val_{24}-Gly_{25}-Pro_{26}) [17]. These results suggested a similar binding site as that of nocathiacin I and confirmed an identical molecular mode of action. This mechanism was different from that of the other protein synthesis inhibitors such as macrolides and oxazolidinones. Its bactericidal nature also differentiates this compound from many other protein synthesis inhibitors.

In summary, we have described the antibacterial activities of a new thiazolyl peptide, thiazomycin. It showed potent antibacterial activity against Gram-positive bacteria and has the same mode of action as nocathiacin I and is bactericidal [17]. Most importantly it has a chemical handle in the amino-sugar residue that would potentially allow for the synthesis of new and water soluble derivatives that are significantly different from other thiazolyl peptides analogs. Nevertheless, the emergence of an unacceptable frequency of resistance precludes further development of this important class of molecules for therapeutic uses (details of resistance will be published elsewhere by Overbye *et al.*).

Experimental

Reagents

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. Brain heart infusion (BHI) was obtained from from Difco and Trypticase soy broth (TSB) was purchased from Becton Dickinson.

Minimum Inhibitory Concentration

The MIC against each of the strains was determined as previously described [20]. The medium with a two-serial dilution of compounds in BHI broth was inoculated with 10^5 colony-forming units/ml, and was incubated at 37°C for 20 hours. MIC is defined as the lowest concentration of antibiotic which inhibited visible growth.

In Vivo Efficacy

A frozen stock of *Staphylococcus aureus* MB 2865 in a 10 ml tube of TSB was reconstituted and incubated at 35°C with shaking at 250 rpm for 8 hours. One ml of this 8 hours culture was used to seed 49 ml TSB in a 250-ml flask and incubated at 35°C with shaking at 250 rpm for 10 hours. The culture was centrifuged at 5000 rpm for 20 minutes and cell pellet was reconstituted in fresh TSB. This culture ($>10^9$ CFU/ml) was diluted to 0.3 : 10 ml in TSB and the absorbance was adjusted to 0.4 at 530 nm. This adjusted culture was then further diluted 1 : 5000 ($\sim 4\times10^4$ CFU/ml) in TSB and then further 1 : 10 in 5.0% hog gastric mucin. Three CD-1 mice (weighing about 20~22 g) per treatment group were infected (0.5 ml, 2×10^3/mouse, I.P.) with *Staphylococcus aureus* MB2865 inoculums described above. Therapy was initiated 20 minutes after infection and again 6 hours post infection challenge with the total amount delivered in two subcutaneous (s.c.) or oral (p.o.) doses of thiazomycin. Mice were observed for general health and mortality for 24 hours after challenge. After 24 hours, 3 mice per group were euthanized, both kidneys aseptically removed, placed in sterile Whirl Pak bags, weighed and then homogenized in 5 ml of sterile saline. Tissue homogenates were then serially diluted in 100-fold sterile saline and plated on mannitol salt agar. Plates were incubated at 35°C for 48 hours. Colony forming units (CFU) per gram of tissue were determined. The dosing

samples were prepared as follows. For s.c. dosing, 1.25 mg of thiazomycin was dissolved in 1.0 ml DMSO. It was adjusted to 10 ml by addition of 9.0 ml of Tween/water buffer making the concentration 0.125 mg/ml. This solution was two fold serially diluted 4.0 ml/4.0 ml five times in buffer (10% DMSO, 5.0% Tween, 85% water) to afford 0.062, 0.031, 0.016, 0.008 and 0.004 mg/ml concentration and 0.5 ml of these dilutions were administered s.c. twice a day (bid) affording final therapeutic doses of 3.12, 1.56, 0.78, 0.39. 0.2 and 0.1 mg/kg/dose. For oral administration, 4.0 mg of thiazomycin was dissolved in 0.8 ml DMSO and 7.2 ml of Tween/water buffer was added to make 0.5 mg/ml solution. Two fold serial dilutions with 4.0 ml/4.0 ml three times in buffer (10% DMSO, 5.0% Tween, 85% water) gave solutions with 0.25, 0.125 and 0.062 mg/ml concentration of thiazomycin. A 0.5 ml aliquot of these dilutions administered orally twice a day (bid) providing final therapeutic doses of 12.5, 6.25, 3.12 and 1.56 mg/kg/dose.

Whole Cell Labeling Assay

The assay was performed as previously described [4, 21]. Briefly, mid-log (A_{600}=0.5～0.6) *Staphylococcus aureus* growth were incubated at an increasing concentration of each inhibitor at 37°C for 20 minutes with 1.0 μCi/ml 2-[^{3}H]glycerol, 1.0 μCi/ml 6-[^{3}H]thymidine, 1.0 μCi/ml 5,6-[^{3}H]uracil, 5.0 μCi/ml 4,5-[^{3}H]leucine, or 5.0 μCi/ml 2,3-[^{3}H]alanine (or 2-[^{3}H]glycine) to measure phospholipids, DNA, RNA, protein, and cell wall synthesis respectively. Cell wall labeling with 2-[^{3}H]glycine (*Staphylococcus aureus*) was performed in the presence of 100 μg/ml chloramphenicol, which prevents protein synthesis. The reaction was stopped by addition of 10% TCA and the cells were harvested using a glass fiber filter (PerkinElmer Life Sciences, 1205-401). The filter was dried and counted with scintillation fluid.

***In Vitro* Time-kill Curve**

A 25 μl aliquot of an overnight culture (～10^9 CFU/ml) of *Staphylococcus aureus* MB2865 was diluted into six, 250-ml flasks, each containing 25 ml BHI broth. These were grown at 37°C for 2～3 generations until they reached an estimated 10^6 CFU/ml. After this time, a sample was removed for plating, and then to each flask was added either ciprofloxacin (0.24 μg/ml) or thiazomycin. Thiazomycin was added at 2×, 4×, 8× and 16× the MIC (0.032, 0.064, 0.128, 0.256 μg/ml) respectively. One flask without drug was used as a growth control. Aliquots were removed from each flask every 60 minutes, serially diluted and plated on BHI agar and counted.

Resistant Mutant Selection

Staphylococcus aureus MB2865 was grown overnight in BHI at 37°C. Mutants resistant to thiazomycin were selected by plating 100 μl of 10^9 CFU/ml on thiazomycin-containing plates. Selection was performed at 0.064 μg/ml with a final concentration 2.5% of DMSO in the agar. Individual colonies were streaked for isolation and MIC's were performed to determine the level of resistance to thiazomycin.

Cloning and Sequencing

Genomic DNA was isolated from selected strains by inoculating 5.0 ml BHI with a single colony, incubation overnight at 37°C with shaking. The pellet was re-suspended in 300 μl TE (10 mM Tris-Cl, pH 8.0 and 1.0 mM EDTA) and 25 mg/ml lysozyme. Next, 1.0 μl of RNAse (10 μg/ml) followed by 5.0 μl lysostaphin (2.0 μg/ml) was added. This was incubated for 90 minutes at 37°C. Proteinase K was added (final 25 μg/μl) along with 0.10 ml 10% sodium dodecyl sulfate (SDS) and incubated at 65°C for 15 minutes. Two solvent extractions were performed with 400 μl of phenol : chloroform : isoamyl alcohol (25 : 24 : 1). To the aqueous fraction 1/10 volume (=40 μl) of 7.5 M ammonium acetate was added along with two volumes of isopropyl alcohol. The DNA was centrifuged at 13,000 rpm for 10 minutes and the pellet was washed with 70% EtOH and subsequently dried. The DNA pellet was re-suspended in TE.

The *rplK* gene was amplified from genomic DNA with primers (Invitrogen) PrplK-1A (5′-ACCAGTAGAAGT-TGAATTCGATCA-3′) and PrplK-1B (5′-CGGGGGATC-CAATTTCCTCCCACATTACTTG-3′) using the Elongase amplifications system (Invitrogen) with the following cycle conditions: 94°C for 1 minute, then 35 cycles of denaturing (45 seconds at 95°C), annealing (45 seconds at 40°C) and extension (60 seconds at 68°C) using a Perkin-Elmer 397 thermal cycler. PCR fragments were viewed by agarose gel electrophoresis and visualized with ethidium bromide under ultra violet light. Selected fragments were purified with a PCR purification kit (Qiagen) and cloned into pCR-4TOPO (Invitrogen) for transformation into *Escherichia coli*. Colonies containing recombinant plasmids were grown in 5.0 ml LB supplemented with 100 μg/ml ampicillin and isolated by a plasmid isolation kit (Qiagen). Recombinant plasmids were screened by PCR and restriction digest and those containing the *rplK* insert were chosen for DNA sequencing. Sequencing was performed by using the Big Dye 3.1 sequencing kit and the ABI Genetic Analyzer 3100 (Perkin-Elmer). Sequence results were analyzed with the Vector NTI 8 (InfoMax).

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