Novel Tetrapeptide Inhibitors of Bacterial Protein Synthesis Produced by a *Streptomyces* sp.[†]

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ABSTRACT: In the course of a microbial product screening aimed at the discovery of novel antibiotics acting on bacterial protein synthesis, a complex of three structurally related tetrapeptides, namely, GE81112 factors A, B, and B1, was isolated from a *Streptomyces* sp. The screening was based on a cell-free assay of bacterial protein synthesis driven by a model mRNA containing natural initiation signals. In this study we report the production, isolation, and structure determination of these novel, potent and selective inhibitors of cell-free bacterial protein synthesis, which stably bind the 30S ribosomal subunit and inhibit the formation of fMet-puromycin. They did not inhibit translation by yeast ribosomes in vitro. Spectroscopic analyses revealed that they are tetrapeptides constituted by uncommon amino acids. While GE81112 factors A, B, and B1 were effective in inhibiting bacterial protein synthesis in vitro, they were less active against Gram-positive and Gram-negative bacterial cells. Cells grown in minimal medium were more susceptible to the compounds than those grown in rich medium, and this is most likely due to competition or regulation by medium components during peptide uptake. The novelty of the chemical structure and of the specific mode of action on the initiation phase of bacterial protein synthesis makes GE81112 a unique scaffold for designing new drugs.

The emergence of multidrug-resistant microbial pathogens is driving the search for novel and more effective antibiotics. In this context, a generalized approach to discovery involves target-based screening using assays based on validated and established vital targets. One of such targets is the translational machinery, whose structural and functional complexity offers many opportunities for identifying novel inhibitors capable of bypassing existing resistance mechanisms. Indeed, in addition to the ribosome, composed of three rRNAs and more than 50 proteins (*1*−*5*), the translational apparatus includes a set of proteins (the initiation, elongation, and termination factors) and scores of tRNAs and aminoacyl-tRNA synthetases (*6*−*8*). Furthermore, although many known antibiotics exert their action by inhibiting translation, several components and individual steps of the translational pathway represent unexploited antibiotic targets (*9*−*15*). This implies that novel inhibitors directed against these targets are likely to be effective against pathogens which have developed resistance to current inhibitors of protein synthesis. Finally, the selectivity of a significant number of antibiotics, which inhibits protein synthesis in bacterial but not in eukaryotic systems, is consistent with the large differences between the translation processes in bacterial and eukaryotic cells (*16*−*20*).

Early studies aimed at discovering inhibitors of protein synthesis in vitro have made use of cell-free systems programmed either with synthetic homopolymeric templates [e.g., poly(U) and poly(A)] to drive the synthesis of homopolypeptides (*21*) or with viral or total cellular RNAs (*22*, *23*). In recent years, coupled transcription/translation systems have been introduced for the identification of novel antibiotics acting on protein synthesis (*24*, *25*). In the present study, we devised and applied a screening program for inhibitors of bacterial translation driven by a model mRNA, designated 027 mRNA. This approach has led to the identification of new tetrapeptide antibiotics GE81112 factors A, B, and B1 (Figure 1) whose isolation and properties are described here below.

EXPERIMENTAL PROCEDURES

Bacterial Strains. *Bacillus subtilis* 566/1 (*thy A*, *thy B*) (*26*) and ATCC 6633, *Escherichia coli* K12, L47, JM109, and MG1655, *Staphylococcus aureus* ATCC 19636, *Streptococcus pyogenes* L49, *Streptococcus pneumoniae* L44, *Enterococcus faecalis* L560, *Moraxella catharralis* L3292, and *Candida albicans* L145 were from the Lepetit Culture Collection, Vicuron Pharmaceuticals, Gerenzano, Italy. *Strep-*

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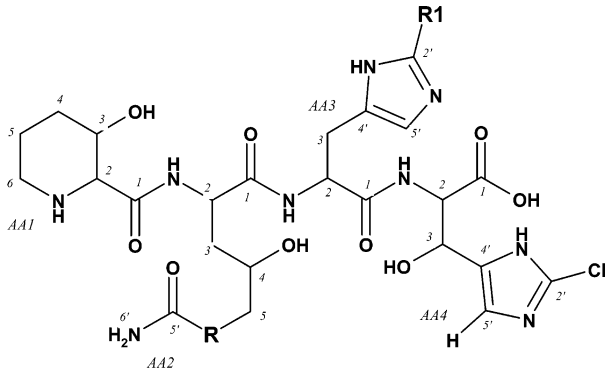

FIGURE 1: Structures of tetrapeptide factors A, B, and B1 of GE81112.

tomyces sp. utilized for tetrapeptide production was isolated in our laboratory and deposited in DSMZ as DSM 14386.

Protein Synthesis Assays. The cell-free screening assay applied to microbial extracts was performed using the *E. coli* translation system described below driven by 027 mRNA, a "natural-like" model mRNA. The microbial extracts which inhibited the natural-like translation were subjected to a secondary *E. coli* translation screening driven by poly(U) in order to discard known inhibitors of the elongation phase that are typically detectable by the poly(U) system.

Plasmid Constructions. Plasmid p024 was constructed by substituting the *Hga*I−*Pst*I fragment, excised from plasmid p022 and specifying 022 mRNA (*27*), with the fragment formed by the complementary oligonucleotides 5′-TTACG-TAATGCGTCTGCA-3′ and 5′-GACGCATTAC-3′. The resulting p024 construct maintains the 022 mRNA sequence with the addition of a *Sna*BI restriction site and an overlapping termination/initiation site. To obtain pTZ18-027, which contains the 022 coding sequence repeated 3.5 times, *Sna*BI-digested p024 was incubated with the partially complementary oligonucleotides 5′-GTAAAGAAGATCGTAGTAATC-3′ and 5′-GATCTTCTTTACGATTACTAC-3′, followed by fill-in reaction, ligation, and transformation of *E. coli* JM109. A clone containing the insert of desired length was identified by DNA sequence analysis.

Preparation of 027 mRNA. Plasmid pTZ18-027 (0.02 μM), linearized with *Hin*dIII, was incubated in 40 mM Tris-HCl, pH 8.1, buffer containing 20 mM $Mg(OAc)_2$,[1] 10 mM spermidine, 5 mM DTT, 1.6 units/mL T7 RNA polymerase, 0.1 mg/mL BSA, 0.05 unit/mL RNase inhibitor (Promega), 0.004 unit/mL PPase (Sigma), and 3.7 mM each ATP, GTP, CTP, and UTP. After 2.5 h incubation at 37 °C, an equal volume of 5 M LiCl was added, and the reaction mixture was kept at 0 °C for 30 min. The transcribed mRNA was then collected by centrifugation, resuspended in H_2O, and subjected to three cycles of ethanol precipitation. The final pellet, dissolved in H_2O, was stored in small aliquots at −80 °C in the presence of the RNase inhibitor.

Preparation of E. coli Cell-Free Extracts. E. coli K12 cells were grown at 37 °C in LB medium to mid-log. Ten gram aliquots of cells were resuspended in 7−10 mL of buffer A (10 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 20 mM NH_4Cl, 5 mM DTT) containing 5 mg/mL pepstatin, 0.25 mM PMSF, 1 mM benzamidine, and 5 mg/mL leupeptin and disrupted by three 3 min cycles in a KDL Dyno-mill (Bachofen AG, Basel, CH) with 2 min cooling intervals. The resulting mixture was centrifuged at 3000*g* for 20 min, and the clear supernatant was further centrifuged at 30000*g* for 30 min and then dialyzed twice against 25 volumes of buffer A lacking leupeptin for a total of 16 h. Aliquots (0.1−0.5 mL each) of the resulting S30 extract were stored in liquid nitrogen.

In Vitro Translation. For 027 mRNA-directed translation, each well of a 96-well microtiter plate contained, in a total volume of 50 μL, 7 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 10 mM Tris-HCl, pH 7.7, 2 mM DTT, 1 mM ATP, 2 mM GTP, 0.07 mM citrovorum factor (Serva), 40 μg/mL total *E. coli* tRNA (Boehringer), 200 μM each amino acid (4 μM Phe), 0.5 mM [^{3}H]Phe (2.5 mCi/mmol), 1 μL of the *E. coli* S30 extract, 5 pmol of 027 mRNA, and 5 μL of microbial library extract or known protein synthesis inhibitors in 10% DMSO. Under these conditions, the incorporation reaction was found to be linear for 75 min. After 60 min incubation at 37 °C, the reaction was stopped by the addition of 25 μL of 3 N NaOH and incubated for 30 min at room temperature before addition of 100 μL of 20% TCA. After 1 h incubation at 4 °C, the precipitate was recovered on a glass fiber filter (Unifilter-96, GF/B; Packard) using a filter microplate harvester (Filtermate 196; Packard). The acid-insoluble radioactivity present in each well was determined with a TopCount filtration unit (Packard) after addition of scintillation cocktail (Microscint 20; Packard). Poly(U)-directed poly(Phe) synthesis was carried out as described (*21*). The incubation mixture (50 μL) contained 10 mM Tris-HCl, pH 7.7, 14 mM $MgCl_2$, 100 mM NH_4Cl, 2 mM DTT, 2 mM GTP, 1 mM ATP, 40 μg of poly(U), 0.5−1 μL of *E. coli* S30 extract, 0.12 μg of *E. coli* tRNA, 0.14 μCi of [^{3}H]Phe (2.5 mCi/μmol), 4 μM nonradioactive Phe, and 5 μL of microbial library extract or known protein synthesis inhibitors in 10% DMSO. After 1 h incubation at 37 °C, the hot-TCA-insoluble radioactivity was determined as described above.

Ribosomal Binding of GE81112. The 30S (1.5 μM) or 50S (1.5 μM) *E. coli* ribosomal subunits in 500 μL of 20 mM Tris-HCl, pH 7.7, buffer, containing 10 mM $Mg(OAc)_2$, 60 mM NH_4Cl, and 0.1 mM DTT (buffer B) and supplemented with 10% sucrose, were incubated for 10 min at 20 °C in the presence of GE81112 at concentrations from 0.5 to 50 μM. To remove unbound antibiotic, the samples were layered over a cushion consisting of 1.5 mL of buffer B containing 20% sucrose and centrifuged 3 h at 4 °C and 40K rpm in a Beckman SW60 rotor. The pellets obtained were then resuspended in 50 μL of buffer B and tested in the 027 mRNA-directed translation assay.

f[^{35}S]Met-puromycin Formation. One micromolar 30S ribosomal subunits in 30 μL of 20 mM Tris-HCl, pH 7.7,

[1] Abbreviations: OAc, acetate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; DTT, 1,4-dithiothreitol; ESIMS, electrospray ionization mass spectrometry; FT-MS, Fourier transformed mass spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiquantum correlation; HTS, high-throughput screening; IC_{50}, inhibitor concentration at 50% of inhibition; MIC, minimal inhibitory concentration; MM, minimal medium; MS, mass spectrum; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; PMSF, phenylmethanesulfonyl fluoride; PK, pyruvate kinase; PPase, pyrophosphatase; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

buffer containing 7 mM $Mg(OAc)_2$, 80 mM NH_4Cl, and 0.1 mM DTT (buffer C) were incubated for 5 min at 20 °C in the presence of GE81112 at concentrations from 0.5 to 50 μM. Then 10 μL of buffer C containing 1.5 μM 027 mRNA, 1 μM IF1, 1 μM IF2, 1 μM IF3, 1.5 μM f[^{35}S]Met-tRNA, and 1 mM GTP was added, and the resulting mixtures were incubated for 15 min at 37 °C in buffer C. Finally, 20 μL of buffer containing 1 mM puromycin and 1 μM 50S was added. After 30 s of incubation at 37 °C, the reaction was quenched by the addition of 500 μL of NH_4HCO_3, pH 9.0. The f[^{35}S]Met-puromycin was extracted with ethyl acetate and quantified as described (*28*).

Preparation of Saccharomyces cerevisiae Cell-Free Extract. S. cerevisiae SKQ2M cells were grown at 30 °C in YPD medium to $A_{600} = 1$. The S30 extract was prepared essentially as described (*29*). Further details will be given elsewhere.

In Vitro Translation with S. cerevisiae Extract. 027 mRNA-directed translation was performed essentially as described (*29*). Further details will be given elsewhere.

Inhibition of Macromolecular Synthesis. B. subtilis 566/1 (*thyA*, *thyB*) was grown as reported (*26*). Briefly, after incubation overnight at 37 °C, 1 volume of this culture was diluted with 100 volumes of Davis−Mingioli medium containing 20 g/L glucose, 2.5 μg/mL Thy, and a mixture of all the natural amino acids, each at 0.2 mM concentration, with the exception of Phe which was 0.1 mM. At $A_{590} = 0.4$ macromolecular syntheses (DNA, RNA, protein, and cell wall peptidoglycan) were monitored by incorporation of the appropriate radioactive precursors according to the modified protocol from ref *26*: for DNA synthesis of [^{3}H]Thy (5 μCi/mL, 56.4 Ci/mmol), with 0.1 g/L unlabeled adenosine; for RNA synthesis of [5,6-^{3}H]Ura (2 μCi/mL, 50 Ci/mmol), with 2.5 μg/mL of unlabeled Ura; for protein synthesis, L-[2,6-^{3}H]Phe (1 μCi/mL, 48 Ci/mmol); for cell wall peptidoglycan, *N*-acetyl-D-[1-^{3}H]glucosamine (1 μCi/mL, 9.9 Ci/mmol). The antibiotic (200 μg/mL) was added after 15 min. Samples (0.1 mL) were taken at intervals up to 60 min of incubation and were precipitated with ice-cold 10% TCA for 30 min at 4 °C. TCA precipitate material was collected on glass fiber filters (Filtermat A; Wallac) using a 96-well cell harvester (Wallac), and radioactivity was determined in a β-plate scintillation counter (Wallac). Chloramphenicol (100 μg/mL) was tested as a control antibiotic.

Determination of in Vitro Antimicrobial Activity. In vitro antimicrobial activity was determined by broth microdilution assay as recommended by the National Committee for Clinical Laboratory Standards (*30*). The growth media utilized for MIC determination were cation-adjusted Difco Mueller−Hinton broth (MHB) for *E. coli*, *S. aureus*, *Moraxella catarrhalis*, and *E. faecalis* strains, Todd−Hewitt broth (THB) for *S. pyogenes* and *S. pneumoniae*, antibiotic medium no. 3 (AM3) and base medium Davis−Mingioli broth + 2% (w/v) glucose + 100 μg/mL asparagine (MMasp) for *B. subtilis*, Davis−Mingioli broth + 2% (w/v) glucose (MM) for *E. coli*, and RPMI 1640 medium (RPMI) and minimal 40 (MM40) for *C. albicans*. Typically, a 2-fold serial dilution of the test compound was performed in a sterile 96-well microplate inoculated with 10^4 CFU/mL test strain in appropriate medium. The microplate was then incubated for 18−24 h at 35 °C. The MIC was determined by visual examination of the microplate with the aid of a magnifying mirror apparatus. MIC is the lowest concentration of compound that showed no visible sign of microbial growth.

Fermentation. Streptomyces sp. DSM 14386 was grown in V6 medium (*31*). After 40 h, 100 mL of culture grown in 500 mL Erlenmeyer flasks at 28 °C and 200 rpm was inoculated into a 4 L fermenter, containing 3 L of the same seed medium. After 40 h at 28 °C and 700 rpm, 1.5 L was used to inoculate a 300 L fermenter containing 200 L of production medium having the following composition (g/L): glycerol, 30; soybean meal, 15; $CaCO_3$, 5; and NaCl, 2. Fermentation was run at 28 °C, 180 rpm stirring, with an air flow of 60 L/min for the first 18 h and then increased to 100 L/min (0.5 vvm) until the harvest time. Production was monitored by HPLC and with the bioassay on susceptible bacteria. Growth was measured as packed mycelium volume (PMV, %) by centrifuging 10 mL of culture in graduated vials.

Isolation of Tetrapeptides. The mycelium was removed from harvested broth by filtration with a Hyflo filter matrix. The filtrate was brought to pH 3.5 with HCl and was stirred overnight batchwise with 2.5 L of Dowex 50W-X2 cation-exchange resin (Dow Chemical Co.) in the acid form. The resin was then recovered, loaded on a column, and washed sequentially with 20 mM NaH_2PO_4 at pH 3.5, with 8:2 (v/v) 20 mM NaH_2PO_4−CH_3OH at pH 3.5, and then with 8:1:1 (v/v) CH_3COCH_3−H_2O−*n*-BuOH. Elution was performed with 85 mM $(NH_4)_3PO_4$, pH 10. The eluted fractions were immediately brought to pH 6 by addition of H_3PO_4. The antibiotic content of each fraction was evaluated by bioassay, i.e., inhibition of *E. coli* and *B. subtilis* cultivated in MM (see Determination of Antimicrobial Activity below), and by analytical HPLC. The pooled fractions containing GE81112 were stirred batchwise overnight with 1.2 L of polystyrene resin HP20 (Mitsubishi Chemicals Co.). The resin was then recovered by filtration and was eluted in a column at 40 mL/min flow rate with 1:9 (v/v) CH_3OH−H_2O (6 L) and then with 3:7 (v/v) CH_3OH−H_2O (4 L). The eluted fractions containing GE81112 factors were pooled, concentrated, and lyophilized to generate a solid preparation of the complex, hereafter mentioned as antibiotic GE81112. The individual pure factors were then obtained by repeated preparative HPLC over a Merck Hibar Lichrosorb RP8 column (25 × 250 mm, 7 μm, flow rate 3.5 mL/min, isocratic elution with 40 mM $HCOONH_4$ buffer at pH 4.5). The factors eluted in the order A, B1, and B, and the fractions showing homogeneous antibiotic content were pooled and concentrated under vacuum. The concentrated solutions were then lyophilized twice to yield purified GE81112 factors A, B1, and B.

Analytical Chromatography. The fermentation and purification process was monitored by HPLC on an Alltech Altima C18 column (250 × 4.6 mm, 5 μm, flow rate 1 mL/min, isocratic elution with 40 mM NH_4COOH at pH 4.5). Factor A, factor B1, and factor B eluted typically with 14, 18, and 21 min retention times, respectively. UV detection was at 230 nm.

NMR Study. NMR experiments were carried out on a Bruker AMX 600 spectrometer equipped with a pulse-field gradient module and a 5 mm Bruker TXI ^{13}C Z-grad probe operating at 600 MHz for ^{1}H and 150.9 MHz for ^{13}C. The spectra were recorded at 298 K using 8 mM samples in DMSO-d_6 (as such and acidified with TFA). Addition of TFA

improved the resolution and shape of the signals which otherwise were generally broad due to short T_2 relaxation. Proton and carbon chemical shifts were referenced to the residual solvent signal (DMSO-d_6) at 2.5 and 39.43 ppm, respectively. Two-dimensional experiments including TOCSY, DQF-COSY, HMQC, HMBC, and ROESY were performed using Bruker standard pulse sequences. Data were processed with WIN NMR software (Bruker).

MS Study. Accurate mass determinations were performed by FT-MS using a Bruker Daltonics APEX II, 4.7 T spectrometer fitted with an electrospray source. Experimental conditions were as follows: off-axis spray, 60 μL/h; drying gas, 200 °C; capillary voltage, 70 V; skimmer voltage, 10 V; accumulation, 40 scans. ESIMS fragmentation studies were performed in positive mode by using a Finnigan LCQDeca ion trap instrument. Experimental parameters were as follows: sheat gas, 40 arbitrary units; source voltage, 4.70 kV; capillary voltage, 25 V; tube lens offset, −10 V; capillary temperature, 250 °C. For fragmentation spectra the normalized collision energy was set at 25%. All of the spectra were acquired in the 100−700 amu mass range. The samples were dissolved in aqueous methanol.

Hydrolysis and Amino Acid Analysis. The GE81112 factors (100 μg) were hydrolyzed by treatment at 105 °C for 24 h in 6 N HCl containing 1% (w/v) phenol. The hydrolysate was analyzed by GC-MS as methyl esters and trifluoroacetyl derivatives. Briefly, the dried hydrolysis residue was incubated for 1 h at 100 °C with 3 N HCl in anhydrous methanol. It was then dried under a stream of nitrogen and treated at 60 °C for 15 min with trifluroacetic anhydride. The dried residue dissolved in dichloromethane was analyzed by GC-MS using a Finnigan TSQ700 triple stage quadrupole mass spectrometer interfaced with a Varian 3400 gas chromatograph. The GC separation was performed on a J&W Scientific DB-5, 30 m × 0.254 mm i.d. × 0.25 mm FT column. Conditions were as follows: carrier gas, helium; injection mode, splitless; injector temperature, 200 °C; transfer line temperature, 300 °C; temperature program, from 50 to 100 °C at 2.5 °C/min, to 250 °C at 10 °C/min, and 5 min at 250 °C. MS was performed by electron impact in positive ion mode with the following detection voltage setting: filament current, 400 mA; electron multiplier, 1400 V; electron energy, 70 eV. The hydrolysate was also analyzed by HPLC-MS. Derivatizations were performed with an *N*-hydroxysuccinimide-activated heterocyclic carbamate Waters AccQ-Fluor reagent kit (*32*, *33*), and the HPLC-MS was performed by splitting the eluate from the column between a diode array UV detector and a ThermoQuest Finigan LCQ Deca mass detector equipped with an ESI interface. The separation was performed at 37 °C on a C18 AccQ-Tag Waters column (3.9 × 150 mm) eluted at 1 mL/min flow rate with the multistep gradient: time (min) 0/5% phase B; time 5/5%; time 30/80%; time 35/95%; time 40/95%. Phase A was 140 mM NH_4OAc, pH 5, buffer and phase B was 0:40 (v/v) H_2O-CH_3CN. MS parameters were as follows: sheat gas, 40 arbitrary units; source voltage, 4.70 kV; capillary voltage, 10 V; tube lens offset, 40 V; capillary temperature, 200 °C. All of the spectra were acquired in the 100−700 amu mass range.

Table 1: Effect of Known Protein Synthesis Inhibitors on *E. coli* Translational Systems Programmed with 027 mRNA or Poly(U)[a]

inhibitor	IC_{50} (μg/mL) 027 mRNA	poly(U)
borrelidin	0.15	>100
mupirocin	0.12	>100
thermorubin	2	8
althiomycin	0.8	0.2
kasugamycin	8	>500
streptomycin	~1	150
gentamicin	30	400
neomycin	20	10
paromomycin	4	60
lincomycin	30	125
tetracyclin	12	6
nosiheptide	5	~2
GE2270	2	0.8
kirromycin	>1	0.16
pulvomycin	5	1.5
thiostrepton	0.8	0.7
micrococcin	0.3	0.5
carbomycin	0.25	0.25
erythromycin	≥1000	>1000
sparsomycin	<1	<1
pactamycin	1	>500
hygromycin A	0.25	1.5
puromycin	0.8	20
everninomycin	~100	~300
purpuromycin	7	7
amicetin	3	10
blasticidin S	0.5	1
chloramphenicol	20	≥1000
fusidic acid	200	80
fredericamycin	200	400
viridogrisein	4	400

[a] Serial dilutions of each antibiotic were added to the translation reaction mix as described in Experimental Procedures. The IC_{50} was the parameter chosen to compare the effect of different inhibitors on the two translational systems.

RESULTS

Screening of Microbial Products. A variety of known microbial products act by inhibiting protein synthesis. Many of them are of medical importance, but many are becoming progressively ineffective because of the spread of class-specific bacterial resistance. In this connection, to discover novel protein synthesis inhibitors, we screened broth extracts focusing on antibiotics that have likely escaped from screens using traditional assays. An assay based on the 027 mRNA construct was thus developed that showed improved detection capacity in comparison to the conventional poly(U)-dependent translation system, which has been used as a screening assay for many years. The 027 mRNA derives from 022 mRNA and contains an *E. coli* consensus translational initiation region, which includes a Shine−Dalgarno sequence and an AUG start codon (*27*). The latter is followed by an ORF consisting of 35 codons (specifying the amino acids Phe, Thr, and Ile), which is translated into an ≅4 kDa polypeptide in a process that is highly dependent on the presence of the translation initiation and elongation factors and cognate aminoacyl-tRNAs. Accordingly, a group of known antibiotics affecting protein synthesis were indeed found to be more active in the 027 mRNA than in the poly-(U)-dependent translation system (Table 1). In particular, the 027 mRNA-programmed system was sensitive to inhibitors of tRNA synthetases (e.g., borrelidin and mupirocin that inhibit threonyl-tRNA and isoleucyl-tRNA synthases, re-

Table 2: High-Resolution Electrospray Ionization Mass Spectral Data of Factors A, B, and B1

	factor A	factor B	factor B1
accurate mass measured	644.21858	659.22953	658.24590
mass calculated for the best hit	$C_{24}H_{35}N_9O_{10}Cl_1$: 644.218992	$C_{24}H_{36}N_{10}O_{10}Cl_1$: 659.2298915	$C_{24}H_{37}N_{11}O_9Cl_1$: 658.2458760
deviation	4.155e−04	3.665e−04	2.304e−05
elemental formula	$C_{24}H_{34}N_9O_{10}Cl_1$	$C_{24}H_{35}N_{10}O_{10}Cl_1$	$C_{24}H_{36}N_{11}O_9Cl_1$

spectively) (*34*, *35*) and more sensitive to some aminoglycosides (streptomycin, gentamicin, paromomycin, pactamycin) and to puromycin, chloramphenicol, and viridogrisein. However, it should be noticed that some antibiotics (e.g., erythromycin, everninomycin) were poor inhibitors of 027 mRNA-driven translation, possibly due to the largely hydrophobic nature of the peptide product of this mRNA; thus, some classes of protein synthesis inhibitors may still escape detection in an HTS program based on this translation system.

A screen based on 027 mRNA-driven assay was thus applied to the Vicuron Pharmaceuticals collection of broth extracts, and an extract with reproducible activity was selected from the first subset of 25000 extracts examined. The extract that contained the novel antibiotic GE81112 originated from a new soil isolate belonging to the *Streptomyces* genus (*36*).

Production and Purification of Tetrapeptides. Since LC-MS analysis of the crude broth extracts coupled with bioautography revealed that the active components in the complex matrix did not match the characteristics reported for any known microbial product, tank-scale fermentations were undertaken to fully characterize them. GE81112 was produced by *Streptomyces* sp. as a complex of three major factors, namely, A, B, and B1. The production of the antibiotic complex started after ca. 20 h from the time of inoculum and reached a maximum in the exponential phase of the microbial growth at ca. 10 mg/L after 50 h of fermentation (Figure 2). Multistep chromatographic purifications resulted in the isolation of three closely related tetrapeptides, **1**−**3**.

Structure Determination of Tetrapeptides. High-resolution FT-MS of GE81112 factors A, B, and B1 showed an isotope pattern of the protonated molecular ion consistent with the presence of one chlorine atom in a structure containing C, H, N, and O atoms. This information, along with the presence of 24 carbon atoms found in ^{13}C NMR spectra and the accurate mass determinations, indicated the elemental formulas reported in Table 2. The structures of the individual factors were assigned by NMR experiments and confirmed by MS studies and hydrolytic cleavage. The complete assignments of ^1H and ^{13}C NMR chemical shifts are presented in Table 3.

The structure of the first amino acid (AA1) was assigned as 3-hydroxypipecolic acid by 2D NMR experiments. Proton and carbon chemical shifts of CH β (δ 4.40 and 64.07, respectively) supported this interpretation although the OH signal could not be observed under the experimental conditions used. The connection of the second amino acid (AA2) to AA1 was indicated by long-range correlations (HMBC) between AA2 NH (δ 8.57) and carbonyl carbon of AA1 (δ 167.39). This AA2 amino acid corresponded to 2-amino-5-[(aminocarbonyl)oxy]-4-hydroxypentanoic acid. The [(aminocarbonyl)oxy] group assignment was consistent with the CH_2-5 proton (δ 3.83) and carbon (δ 67.75) chemical shifts,

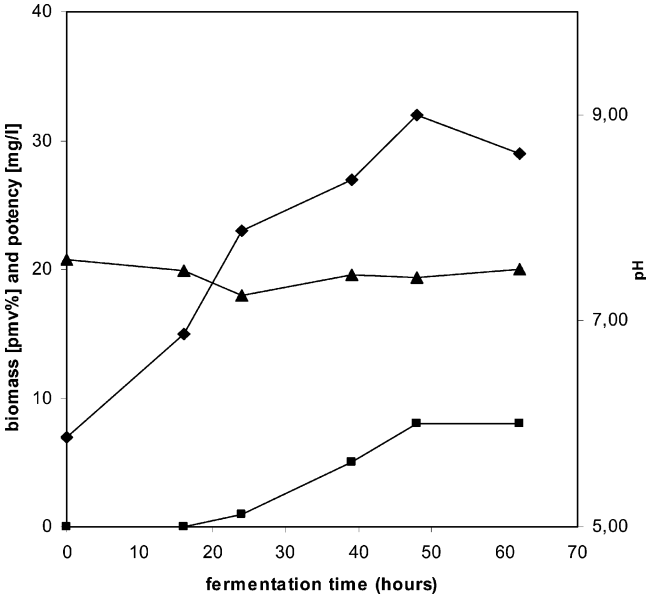

FIGURE 2: Time course of *Streptomyces* sp. DSM 14386 fermentation at 300 L scale. Antibiotic production (■) was monitored by HPLC, and growth (◆) was measured as packed mycelium volume (PMV, %). The pH values (▲) of the cultures are also reported.

with the ROESY cross-peak between H_2-5 and δ 6.46 (NH_2) and with the long-range coupling of H_2-5 with the quaternary carbon δ 156.7 (CO). The sequence of the remaining amino acids AA3 and AA4 was also determined from heteronuclear scalar couplings between the carbonyl carbon of each residue and the amide proton of the following amino acid. Amino acid AA3 was identified as histidine. In AA4, TOCSY correlations showed a spin system where CHβ (δ 5.06 and 66.88 chemical shifts) was connected to an OH group and to an aromatic ring. This aromatic ring was assigned as 2′-chloro-substituted imidazole, on the basis of COSY $^1H-^1H$ coupling of δ 5.06 with δ 6.94 protons, both on the sp^2 carbon, and of their HMBC correlations.

Comparison of ^1H NMR spectra of factor A to factors B and B1 revealed differences on the low-field imidazole NH broad signals which were at 14.07−14.7 in factor A and at 11.7−11.8 in factors B and B1. In both B factors these shifts were influenced by TFA addition. Furthermore, the H-2′ signal of the AA3 imidazole ring was absent in both B and B1 factors, and two exchangeable protons at 7.40 ppm were found, corresponding to a NH_2 group. These data indicated that AA3 is 2′-NH_2-substituted histidine. Peculiar signals were highlighted in the AA2 amino acid system of factor B1 that indicated the presence of an (aminocarbonyl)amino group in place of the (aminocarbonyl)oxy in position 5 in the A and B factors. A CH_2-5 system was indeed present with chemical shifts at δ 2.92/3.08 and 45.7, along with an exchangeable broad resonance at 6.16 ppm and weak COSY correlation of δ 6.16 with CH_2-5. The complete pattern of HMBC[3] and ROESY[4] correlations is detailed in Figures 3 and 4.

Table 3: ^{1}H and ^{13}C NMR Chemical Shift Assignments of GE81112 A, B, and B1 in DMSO-d_6 at 298 K[a]

			factor A		factor B		factor B1	
residue		group	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
AA1		NH_2^+	8.30, 9.07		8.34, 9.0		8.32, 9.01	
	1	CO		167.39		167.4		167.3
	2	CH	3.94	59.70	3.95	59.73	3.95	59.77
	3	CH	4.40	64.07	4.40	64.09	4.43	64.07
		OH	na		na		na	
	4	CH_2	1.62, 1.81	28.41	1.64, 1.83	28.4	1.64, 1.82	28.42
	5	CH_2	1.52, 1.88	15.90	1.52, 1.9	15.92	1.52, 1.87	15.91
	6	CH_2	2.90, 3.15	42.86	2.92, 3.13	42.87	2.90, 3.13	42.87
AA2		NH	8.57		8.54		8.60	
	1	CO		171.65		171.5		171.7
	2	CH	4.46	50.13	4.46	50.1	4.40	50.45
	3	CH_2	1.64	35.31	1.60, 1.70	35.47	1.57, 1.62	36.5
	4	CH	3.65	65.11	3.64	65.14	3.48	66.80
		OH	na		na		na	
	5	CH_2	3.83	67.75	3.83	67.77	2.92, 3.08	45.7
	Q	O or NH					6.16	
	5′	CO		156.7		156.7		159.3
	6′	NH_2	6.46		6.46		na	
AA3		NH	8.17		8.05		8.04	
	1	CO		170.16		170.5		170.5
	2	CH	4.70	51.30	4.60	51.2	4.60	51.16
	3	CH_2	2.87, 3.12	27.19	2.63, 2.86	27.30	2.63, 2.87	27.36
	1′	NH^+	14.07		11.72		11.73	
	2′	CH or Cq	8.95	133.65		146.7		146.7
	A	NH_2			7.40		7.43	
	3′	NH	14.27		11.75		11.80	
	4′	Cq		129.03		122.5		122.5
	5′	CH	7.29	116.83	6.48	110.3	6.49	110.4
AA4		NH	7.99		7.84		7.86	
	1	COOH		171.28		171.3		171.3
	2	CH	4.50	56.86	4.50	56.78	4.50	56.73
	3	CH	5.06	66.88	5.06	66.95	5.06	66.88
		OH	na		na		na	
	1′	NH	na		na		na	
	2′	Cq		128.4		128.4		128.4
	3′	N						
	4′	Cq		139.66		139.66		139.7
	5′	CH	6.94	117.8	6.94	117.85	6.93	117.7

[a] Chemical shifts were referenced with respect to the residual solvent signal at 2.5 ppm (^{1}H) and 39.43 ppm (^{13}C). The assignments were made by analysis of DQF-COSY, TOCSY, ROESY, HMQC, and HMBC spectra.

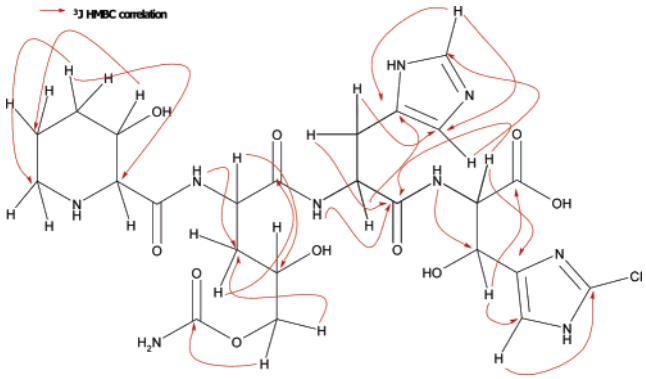

FIGURE 3: Long-range C−H coupling of GE81112 factor A. HMBC data were acquired as 4096 × 512 data points with 80 transients for the t_1 increment. Various delays ranging from 50 to 75 ms were taken for the development of long-range correlations.

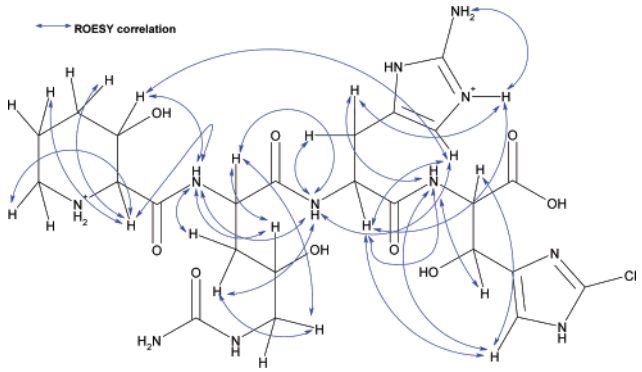

FIGURE 4: ROESY correlations of GE81112 factor B. ROESY spectra were recorded with 1024 increments in t_1 and 4096 complex data points in t_2, and 16−40 transients were averaged for each t_1 value. Various mixing times ranging from 150 to 300 ms were selected.

The NMR-derived structural assignments were consistent with the molecular formulas from FT-MS and with the calculated 12 double bond equivalents. MS^n studies provided additional support to the structural elucidation. The principal fragmentations were assigned as summarized in Figure 5. MS spectra showed losses of water and of hydrochloric acid, indicative of the presence of a hydroxyl group and of a chlorine atom, and loss of carbon dioxide, attributed to the carboxylic group of the C-terminal amino acid. Along the typical loss of substituents, the fragmentation of the peptidic backbone was observed. In MS^2 spectra, both factor A and factor B showed an ion at *m*/*z* 302, attributed to the N-terminal dipeptide AA1 + AA2. Its fragment at *m*/*z* 175 in MS^3 spectra was consistent with the AA2 moiety 2-amino-5-[(aminocarbonyl)oxy]-4-hydroxypentanoic acid. The ion at *m*/*z* 302 also lost water to give an ion at *m*/*z* 284 which in MS^4 spectra generated fragments at *m*/*z* 128. These fragments were consistent with the AA1 moiety 3-hydroxypipecolic

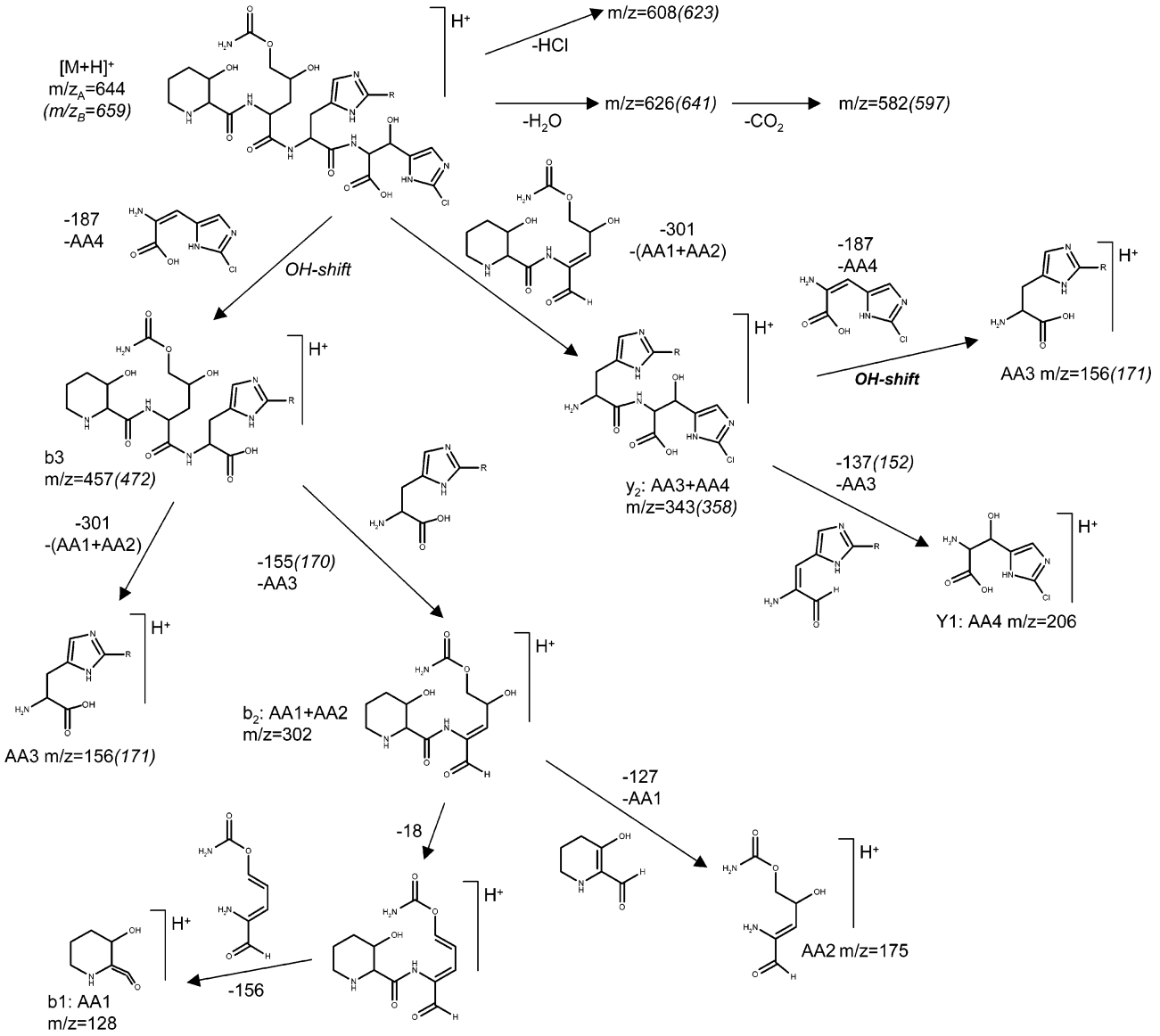

FIGURE 5: MS fragmentations of GE81112 factors A and B (values relative to factor B are reported in parentheses).

acid. In MS^2 spectra of factors B and A the most intense peaks at *m*/*z* 358 and 343 were attributed to the C-terminal dipeptide AA3 + AA4, differing by 15 atomic mass units. Additional evidence for the presence in factor B of an amino group arose from the loss of ammonia to give a *m*/*z* 154 ion. This was not observed in factor A. MS^3 of the ion at *m*/*z* 358 and 343 showed a fragment at *m*/*z* 206 which was common to factors B and A and was consistent with AA4, β-hydroxychlorohistidine, with transposition of the hydroxyl group of the AA4 to the carbon of the carboxylic group of the AA3 (*37*). The fragments at *m*/*z* 156 and *m*/*z* 171 were attributed to histidine and aminohistidine at AA3 positions in factors A and B, respectively. This attribution was also consistent with losses of neutral fragments corresponding to histidine (137 atomic mass unit loss) and to aminohistidine (152 atomic mass unit loss) in MS^2 spectra of factors A and B, respectively. The MS^n of factor B1 showed fragmentation patterns similar to the ones of factor B. The fragments found were consistent with the B1 structure determined by NMR studies (data not reported).

Additional evidence was derived from the acid hydrolysis of the antibiotics. The hydrolysate was analyzed with two alternative methods to maximize detection capacity. Histidine was found by the LC-MS method and was confirmed by comparison with an authentic sample. 3-Hydroxypipecolic and 2-amino-4,5-dihydroxypentanoic acids were detected by GC-MS. Their electron impact fragmentations confirmed the structural assignments. The β-hydroxychlorohistidine residue was not detectable by GC-MS, and a very low intensity peak with *m*/*z* 315 and isotopic pattern typical of chlorinated compounds was found by LC-MS analysis, probably because of chemical instability of this molecule under the hydrolysis/derivatization conditions applied.

Inhibition of in Vitro Protein Synthesis. GE81112 inhibited bacterial protein synthesis directed by 027 mRNA with an IC_{50} of approximately 0.07 μg/mL (Figure 6). Further characterization showed that factor A is less active than the complex of factors A, B, and B1 ($IC_{50} = 0.16$ μg/mL), while factors B and B1 are stronger inhibitors ($IC_{50} = 0.03$ μg/mL for each factor) (data not shown). The poly(U)-directed translation was affected at higher concentrations of GE81112, with IC_{50} of about 10 μg/mL (Figure 6). The possibility that a eukaryotic translation system might be inhibited was also investigated. Taking advantage of the fact that a translational

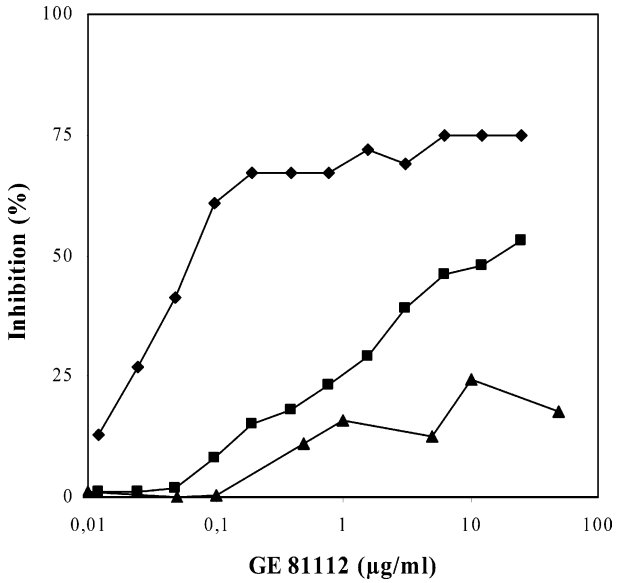

FIGURE 6: Effect of GE81112 on in vitro translation systems. *E. coli* protein synthesis was directed by 027 mRNA (◆) or poly(U) (■), and *S. cerevisiae* protein synthesis was directed by 027 mRNA (▲). The experiments were performed as described in Experimental Procedures in the presence of the indicated concentration of GE81112. *E. coli* and *S. cerevisiae* translation systems driven by 027 mRNA were performed under conditions that yield equivalent amounts of product in the absence of inhibitor.

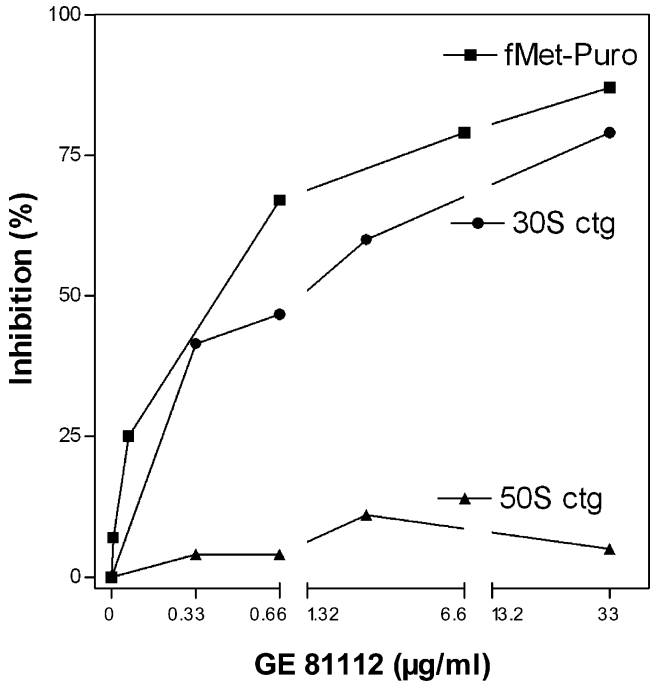

FIGURE 7: Mode of action of GE81112. The translational activity of 30S (▲) and 50S (●) ribosomal subunits preincubated in the presence of GE81112 concentrations indicated in the abscissa, centrifuged through a sucrose cushion (ctg), and supplemented with a stoichiometric amount of control 50S and 30S ribosomal subunits, respectively, was tested in a cell-free system programmed with 027 mRNA. The third curve (■) represents the activity in fMet-puromycin formation displayed by 70S monomers incubated in the presence of the indicated concentrations of GE81112. Additional experimental details are given in Experimental Procedures.

system derived from *S. cerevisiae* can be programmed with 027 mRNA, the inhibitory activity of GE81112 was tested in yeast translation. These experiments (Figure 6) demonstrated that the yeast system was substantially unaffected by GE81112 up to 100 μg/mL.

To identify the molecular target of GE81112, fixed amounts of either *E. coli* 30S or 50S ribosomal subunits were incubated with increasing concentrations of antibiotic and centrifuged in a swinging bucket rotor through a sucrose cushion. The pelleted subunits were then tested for their translational activity in the presence of a stoichiometric equivalent of the complementary control subunit. As seen in Figure 7, the translation activity of the GE81112-treated 30S subunits, combined with the control 50S subunits, was reduced by the preincubation with increasing antibiotic concentrations in a dose-dependent manner. On the other hand, GE81112-preincubated 50S subunits retained essentially full translational activity in the presence of the control 30S subunits. This finding clearly indicates that GE81112 targets the small ribosomal subunit, to which it remains stably bound even after centrifugation through sucrose while the 50S subunit either does not bind GE81112 or binds it with low affinity. To obtain further insight into the mechanism by which GE81112 inhibits translation, the formation of fMet-puromycin was studied as a function of increasing antibiotic concentrations. Figure 7 shows that GE81112 inhibits this reaction with a dose−response curve almost identical to that describing the inhibition of protein synthesis. Formation of fMet-puromycin requires the correct binding and positioning of the initiator tRNA in the ribosomal P site as well as the proper functioning of the peptidyl transferase center of the ribosome. In light of the fact that the latter is located on the 50S ribosomal subunit and that, as shown above, GE81112 does not bind or binds very weakly to the 50S subunit, this result indicates that the most likely functional target of this antibiotic is the initiation phase of translation.

Macromolecular Syntheses in Whole Cells. The protein synthesis target was confirmed in whole cell tests of macromolecular synthesis in *B. subtilis*. Figure 8 shows that protein synthesis was immediately blocked upon GE81112 addition, whereas DNA, RNA, and cell wall syntheses were substantially not affected, thus indicating that the antibiotic acted primarily on protein synthesis. Individual factors of the complex gave the same responses (data not shown).

Determination of Antimicrobial Activity. The antibiotic exhibited a restricted spectrum of antibacterial activities against a panel of major human pathogens (Table 4), with *M. catarrhalis* susceptible at 1−2 μg/mL concentrations, and *S. pneumoniae* and *E. faecalis* inhibited at higher concentrations. When the antibacterial activity of GE81112 factors was tested against *E. coli* and *B. subtilis* growing in MM, the MICs were found to be 4 orders of magnitude lower with respect to those determined in complete medium. Since the complete medium contains proteins, protein hydrolysates, and amino acids, we sought to investigate whether one or more of these components could be responsible for the loss of antibacterial activity. Addition of BSA, casein, or amino acids to the MM influenced only marginally the antibacterial activity of GE81112 (data not shown). On the other hand, protein hydrolysates significantly relieved the GE81112 inhibition. As seen in Figure 9, when the MICs for *E. coli* and *B. subtilis* were determined in MM in the presence of increasing amounts of casamino acids, the antibacterial activity of the antibiotic was progressively reduced in a

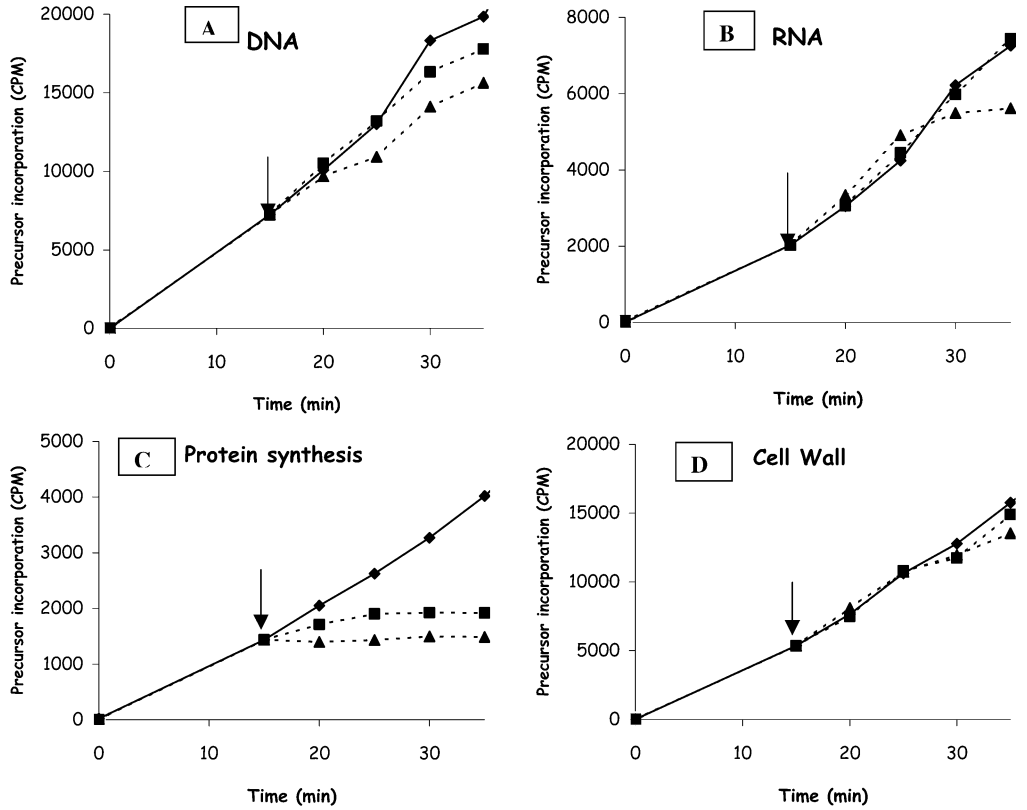

FIGURE 8: Inhibition tests of macromolecular syntheses in *B. subtilis*: DNA (A), RNA (B), protein (C), and cell wall (D). Key: control curve (◆); addition of GE81112 at 200 μg/L (■); addition of chloramphenicol at 100 μg/L (▲). The arrow indicates the time of antibiotic addition.

Table 4: Antimicrobial Activity of GE81112 Factors A, B, and B1

strain (medium)[b]	MIC[a] (μg/mL) factor A	factor B	factor B1
S. aureus ATCC 19636 (CAMHB)	>512	>512	>512
S. pyogenes L49 (THB)	>512	512	512
S. pneumoniae L44 (THB)	64	64	64
E. faecalis Van A L560 (CAMHB)	32	32	512
B. subtilis ATCC 6633 (MMasp)	0.13	0.008	0.06
B. subtilis ATCC 6633 (AM3)	>512	256	512
M. catarrhalis L3292 (CAMHB)	2	1	2
E. coli L47 (MM)	0.06	0.03	0.13
E. coli L47 (CAMHB)	>512	512	512
C. albicans L145 (MM40)	>512	>512	>512
C. albicans L145 (RPMI)	>512	>512	>512

[a] MICs were determined by broth microdilution assay. [b] Target microorganims were grown either in complete (CAMHB, THB, AM3) or in minimal media (MMasp, MM, MM40).

concentration-dependent manner in both organisms. GE81112 was devoid of any activity against *C. albicans* in both complete and minimal media (Table 4).

DISCUSSION

In the present study we describe a novel bacterial protein synthesis inhibitor of microbial origin, which was discovered through the screening of a collection of broth extracts by an in vitro translation system directed by the natural-like 027 mRNA. Extracts active both on poly(U) and on 027 mRNA-directed synthesis were not investigated. This approach led to the discovery of GE81112, an antibiotic which was found to target and stably bind the 30S ribosomal subunit and to inhibit the formation of fMet-puromycin. GE81112 did not bind or bound very weakly the 50S subunit. That translation

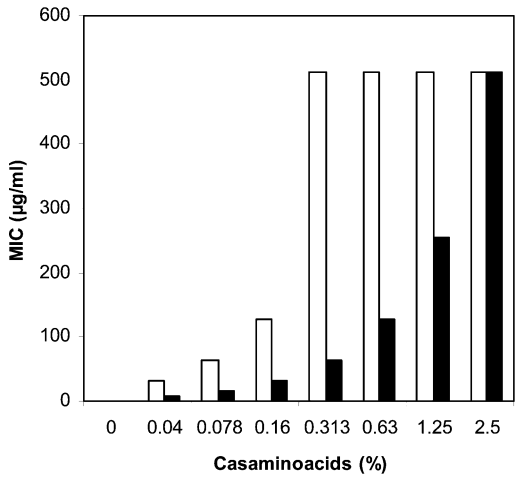

FIGURE 9: Effect of casamino acid addition on the MICs of GE81112. Approximately 10^4 CFU/mL *E. coli* MG1655 and *B. subtilis* ATCC 6633 was inoculated in Davis−Mingioli medium supplemented with the indicated amounts of casamino acids (w/v). MICs were determined after 24 h incubation at 37 °C. Key: (□) *B. subtilis*; (■) *E. coli*.

initiation was the inhibited step was confirmed by the fact that the overall translational activity and the capacity to form the first peptide bond, as measured from the formation of fMet-puromycin, were inhibited by GE81112 with almost superimposable dose−response curves.

Experiments with *B. subtilis* confirmed indeed that the antibiotic inhibited primarily protein synthesis in whole cells. Interestingly, GE81112 appeared selective in inhibiting the bacterial protein synthesis machinery, while the protein synthesis cell-free system derived from *S. cerevisiae* was substantially unaffected.

Since the screen by which GE81112 was identified was intentionally biased for inhibitors of translation initiation, these findings on its mechanism of action are consistent with the screening strategy. Further experiments will be carried out to determine the precise step of translation initiation inhibited by GE81112. It should be noted that, under the conditions employed for studying the inhibition of in vitro bacterial protein synthesis, GE81112 inhibition did not reach 100% but ranged from approximately 50% to 95% depending upon the Mg^{2+} concentration (data not shown). A similar Mg^{2+} effect was also reported for other protein synthesis inhibitors (*9*, *11*, *12*), and it may explain the variable potency in inhibiting protein synthesis in buffers having different composition.

When the antibacterial activity of the antibiotic was tested in a complete medium, the potency was markedly reduced compared to the minimal medium in both *E. coli* and *B. subtilis.* In both organisms we found that casamino acids antagonized the antibiotic in a concentration-dependent manner. Since the inhibition was not observed with a mixture of the common amino acids (data not shown), it was concluded that competition was due to the presence of incompletely hydrolyzated oligopeptides. This behavior may be correlated to the peptidic nature of the antibiotic and may be caused by competition with oligopeptides for transport into the cell. In fact, the hydrophilic nature of GE81112 surely hampers its simple diffusion through the hydrophobic membrane barrier, and its entry into the cell probably requires an oligopeptide transport system. The differential antibiotic activity in minimal vs complete medium may also account for the restricted spectrum of GE81112 antibacterial activity against pathogens that are typically tested in complex growth media. Further studies are needed to elucidate the systems involved in GE81112 transport.

GE81112 is constituted by uncommon amino acids and is substantially different from other known classes of protein synthesis inhibitors (*9*−*15*). Since complexes of ribosome with a variety of known antibiotics have recently been investigated by X-ray studies (e.g., see refs *14* and *15*), GE81112 may provide an additional tool to gain insights on ribosome−antibiotic interactions resulting in functional inhibition.

Cross-resistances between GE81112 and gentamicin, tetracyclin, and erythromycin have been investigated but never found, likely because GE81112 belongs to a novel class of inhibitors.

Thus, GE81112 seems to be a novel potent and selective inhibitor of bacterial translation in vitro with poor activity against pathogens, probably because of permeability problems into the target cells. However, considering its relatively low molecular weight (in comparison to many protein synthesis inhibitors of microbial origin) and its selective mechanism of inhibition, GE81112 could represent a unique scaffold for additional semisynthetic work aimed at improving its activity against whole cells.

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