Macromolecular Biosynthesis Assay for Evaluation of Influence of an Antimicrobial on the Synthesis of Macromolecules

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[Abstract] One of the most compelling approaches in the discovery of novel antimicrobials is screening of natural sources. In our publication we report on the activity of a compound 8-hydroxyserrulat-14-en-19-oic acid (EN4), a diterpene isolated from the Australian plant *Eremophila neglecta*. We evaluate its applicability for treatment of implant-associated infections. A comprehensive analysis of the mechanism of action of EN4 against Staphylococci revealed its membranolytic properties and a general inhibition of macromolecular biosynthesis, which was confirmed in a macromolecular biosynthesis assay and suggested a multitarget activity. The method used to investigate an influence of EN4 on the synthesis of peptidoglycan, RNA, DNA and proteins is based on precipitation of macromolecules with trichloroacetic acid. These macromolecules are synthesised from respective [^{3}H]-labelled precursors. The incorporated radioactivity with and without an antimicrobial is measured and it reflects the mode of action of the tested compound. Antibiotics with known mechanisms of action are used as controls.

Materials and Reagents

1. [^{3}H] N-acetylglucosamine (Hartmann Analytic, catalog number: ART 0101)
2. [^{3}H] Uridine (Hartmann Analytic, catalog number: MT-602)
3. [^{3}H] Thymidine (PerkinElmer, catalog number: NET35500)
4. [^{3}H] Leucine (Hartmann Analytic, catalog number: MT-672)
5. Triptic soy broth (TSB) (Becton Dickinson, catalog number: 211825)
6. Control antimicrobials for the influence on synthesis of:
    a. Peptidoglycan: vancomycin (Vancocin 500 mg) (Teva Pharma)
    b. RNA: actinomycin D (Sigma-Aldrich, catalog number: A1410)
    c. DNA: ciprofloxacin (Ciproxin Infusion 0.2 g) (Bayer)
    d. Proteins: chloramphenicol (Applichem, catalog number: A1806)
    e. All macromolecules (antiseptic activity): chlorhexidine dihydrochloride (Sigma-Aldrich,

catalog number: C8527)

Note: All substances prepared according to the manufacturer's instructions; minimal inhibitory concentration (MIC) of each antimicrobial can be determined according to Clinical and Laboratory Standards Institute guidelines (see Reference 1) (the following MICs were determined for S. aureus WSPPA: $MIC_{vancomycin}$ = 2 µg/ml, $MIC_{actinomycin\ D}$ = 6.25 µg/ml, $MIC_{ciprofloxacin}$ = 2 µg/ml, $MIC_{chloramphenicol}$ = 8 µg/ml, $MIC_{chlorhexidine}$ = 0.78 µg/ml)

7. Scintillation cocktail (Ultima Gold, catalog number: 6013321).
8. Phosphate-buffered saline (PBS) (Reagens, catalog number: 9007695).
9. Na_2HPO_4 (Sigma-Aldrich, catalog number: S3264)
10. KH_2PO_4 (Sigma-Aldrich, catalog number: P8416)
11. $MgSO_4 \cdot 7H_2O$ (Fluka, catalog number: 63138)
12. NH_4Cl (Sigma-Aldrich, catalog number: A9434)
13. NaCl (Sigma-Aldrich, catalog number: S3014)
14. Sodium citrate tribasic dihydrate (Sigma-Aldrich, catalog number: S4641)
15. Glucose (B. Braun Medical, catalog number: 395176)
16. Various amino acids (*i.e.*, L-alanine (Sigma-Aldrich, catalog number: A7469)
17. L-Valine (Sigma-Aldrich, catalog number: V0513)
18. L-Isoleucine (Sigma-Aldrich, catalog number: I7403)
19. L-Aspartic acid (Sigma-Aldrich, catalog number: A7219)
20. L-Glutamic acid (Sigma-Aldrich, catalog number: G8415)
21. L-Serine (Sigma-Aldrich, catalog number: S4311)
22. L-Threonine (Sigma-Aldrich, catalog number: T8441)
23. L-Cysteine hydrochloride (Sigma-Aldrich, catalog number: C6852)
24. L-Arginine (Sigma-Aldrich, catalog number: A8094)
25. L-Leucine (Sigma-Aldrich, catalog number: L8912)
26. L-Lysine (Sigma-Aldrich, catalog number: L9037)
27. L-Proline (Sigma-Aldrich, catalog number: P5607)
28. L-Phenylalanine (Sigma-Aldrich, catalog number: P5482)
29. L-Tryptophan (Sigma-Aldrich, catalog number: T8941)
30. L-Histidine monohydrochloride (Sigma-Aldrich, catalog number: H5659
31. Glycine (Sigma-Aldrich, catalog number: G7126)
32. Cyanocobalamine (Sigma-Aldrich, catalog number: V2876)
33. p-Aminobenzoate (Fluka, catalog number: 06940)
34. Biotin (Sigma-Aldrich, catalog number: B3399)
35. Nicotinic acid (Sigma-Aldrich, catalog number: N0761)
36. D-pantothenic acid hemicalcium salt (Sigma-Aldrich, catalog number: P5155)

37. Pyridoxine hydrochloride (Sigma-Aldrich, catalog number: P6280)
38. Thiamine hydrochloride (Sigma-Aldrich, catalog number: T1270)
39. Riboflavin (Sigma-Aldrich, catalog number: R9504)
40. $ZnCl_2$ (Sigma-Aldrich, catalog number: 208086
41. $MnCl_2 \cdot 4H_2O$ (Sigma-Aldrich, catalog number: M5005)
42. BH_3O_3 (Fluka, catalog number: 15665)
43. $CoCl_2 \cdot 6H_2O$ (Sigma-Aldrich, catalog number: C8661)
44. $CuCl_2 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: C3279)
45. $NiCl_2 \cdot 6H_2O$ (Sigma-Aldrich, catalog number: 223387)
46. $Na_2MoO_4 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: M1003)
47. $FeCl_2 \cdot 4H_2O$ (Fluka, catalog number: 44939)
48. NaOH (Sigma-Aldrich, catalog number: S5881)
49. Uracil (Sigma-Aldrich, catalog number: U0750)
50. Cytosine (Sigma-Aldrich, catalog number: C3506)
51. Adenine (Sigma-Aldrich, catalog number: A2786)
52. Guanine (Sigma-Aldrich, catalog number: G11950)
53. Sodium dodecyl sulphate (SDS) (Sigma-Aldrich, catalog number: L4390)
54. Trichloroacetic acid (TCA) (Sigma-Aldrich, catalog number: T9159)
55. 15 ml TPP centrifuge tubes (TPP 91015)
56. 10% TCA, 5% TCA, 5% TCA/1.5 M NaCl (see Recipes)
57. Completely defined medium (CDM) (see Recipes)
    Note: This medium has been optimised for Staphylococcus (S.) aureus and may require further optimisation if other bacteria are investigated.

Equipment

1. 37 °C bacterial culture incubator
2. Sterile capped glass tubes (to reduce bacterial adherence) (GlasKeller, catalog number: 2613111)
3. Ultracentrifuge tubes (2 ml) (Eppendorf, catalog number: 003120.094)
4. Scintillation tubes (PerkinElmer, catalog number: 6000288)
5. Benchtop centrifuge
6. Vacuum pump
7. Liquid scintillation analyser (Tri-CARB, catalog number: 1900TR)

Procedure

Note: The glass tubes for incubation must be pre-warmed and the reagents and tubes for precipitation must be pre-cooled; to assess the interference with biosynthesis of one macromolecule prepare 14 pre-warmed glass tube (untreated control, investigated antimicrobial and five control antimicrobials, in duplicates).

1. Overnight bacterial culture (for *S. aureus* WSPPA approximately 1.5 x 10^8 CFU/ml) prepared in TSB is diluted 1:100 in 10 ml CDM and, for [^{3}H] Leucine incorporation, in 10 ml CDM-Leu.
   Note: For S. aureus WSPPA the overnight and logarithmic-phase cultures are prepared in 15 ml TPP centrifuge tube, without shaking.
2. Incubation: 5 h, 37 °C.
   Note: The incubation time must be adjusted respectively to the strain in order to reach the logarithmic growth phase.
3. The log-phase culture (0.9 ml for *S. aureus* WSPPA, which corresponds to approximately 2 x 10^7 CFU) is transferred in duplicates to pre-warmed glass tubes and all antimicrobials at concentration of 4x MIC are added; untreated controls are incubated with an adequate volume of a solvent used to prepare solution of investigated antimicrobial.
   Note: Untreated control must be prepared in exactly the same solvent as used for the antimicrobial of interest to control for the effect of solvent on bacterial biosynthesis. It is therefore recommended to dilute all antimicrobials to the concentrations of 4x MIC in the same solvent, which additionally does not interfere with bacterial viability (e.g. PBS). If due to its chemical properties any substance needs to be diluted in a harsh solvent then a concentrated stock solution is prepared in the harsh solvent followed by diluting in a mild solvent to the 4x MIC to reduce the concentration of the first solvent below a level affecting bacteria. The untreated controls are prepared in exactly the same way for incorporation of each of the precursors.
4. The samples are mixed thoroughly and [^{3}H]-labelled precursors are immediately transferred to separate tubes:
   [^{3}H] N-acetylglucosamine up to a concentration of 0.1 µCi per ml (for peptidoglycan synthesis)
   [^{3}H] Uridine up to 1 µCi per ml (for RNA synthesis)
   [^{3}H] Thymidine up to 1 µCi per ml (for DNA synthesis)
   [^{3}H] Leucine up to 3 µCi per ml (for protein synthesis)
5. Incubation: 37 °C.

At time points of interest 0.5 ml aliquots are transferred into 2 ml ultracentrifuge tubes containing 1 ml of ice-cold 10% TCA, mixed thoroughly and placed on ice for at least 1.5 h to facilitate the precipitation.

Note: Time points of interest can be determined in the time-killing study according to Clinical and Laboratory Standards Institute guidelines (see Reference 1). For S. aureus WSPPA time point of 1 h was chosen.

6. The precipitates are washed one time with 0.5 ml of 5% TCA/1.5 M NaCl followed by one-time washing with 0.5 ml of 5% TCA (16,100 *x g*, 10 min, 20 °C), supernatants are removed using vacuum pump.
7. After the second wash samples are solubilised with 0.5 ml of 0.1% SDS/0.1 M NaOH by vortexing at room temperature.
8. The solubilised precipitates are transferred into scintillation tubes and thoroughly mixed with 2 ml of scintillation cocktail.
9. The incorporated radioactivity is measured in counts per minute using liquid scintillation analyser and the results are expressed as percentage of untreated control (Figure 1A - D).

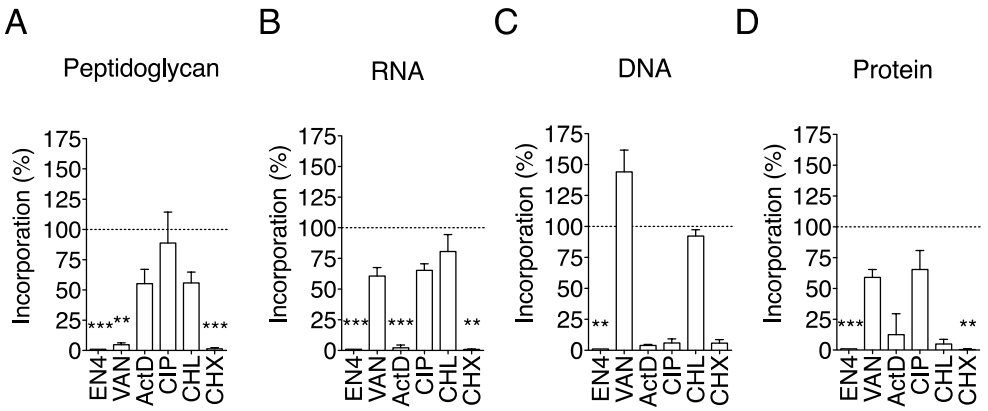

Figure 1. Inhibition of biosynthesis of macromolecules by EN4. Incorporation of [^{3}H] N-acetylglucosamine (A), [^{3}H] Uridine (B), [^{3}H] Thymidine (C) and [^{3}H] Leucine (D) by WSPPA treated for 1 h with EN4, vancomycin (VAN), actinomycin D (ActD), ciprofloxacin (CIP), chloramphenicol (CHL) or chlorhexidine (CHX) at 4x MIC, was expressed as percentage of untreated control (for peptidoglycan = 3444 ± 1212 cpm, RNA = 115538 ± 19533 cpm, DNA = 13862 ± 762 cpm, protein = 7065 ± 323 cpm); values shown are the means of at least two independent experiments prepared in duplicates ± SDs; dotted lines represent 100% incorporation. Significant reduction of biosynthesis as compared to

results for the untreated control is indicated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA (Kruskal-Wallis test) with a Dunns post test (Nowakowska *et al.,* 2013).

Recipes

1. CDM (1,000 ml)
   Mix 1.77 g of Na_2HPO_4, 1.36 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NH_4Cl, 0.5 g of NaCl, 294.1 g of sodium citrate tribasic dihydrate, 3.75 ml of 40% glucose, 160 mg each of various amino acids, L-Valine, L-Isoleucine, L-Aspartic acid, L-Glutamic acid, L-Serine, L-Threonine, L-Cysteine hydrochloride, L-Arginine, L-Leucine, L-Lysine, L-Proline, L-Phenylalanine, L-Tryptophan, L-Histidine, monohydrochloride, and 1.6 g of glycine, 0.05 mg of cyanocobalamine, 0.04 mg of p-aminobenzoate, 0.01 mg of biotin, 0.1 mg of nicotinic acid, 0.1 mg of D-pantothenic acid hemicalcium salt, 0.15 mg of pyridoxine hydrochloride, 0.1 mg of thiamine hydrochloride, 0.1 mg of riboflavin, 69.5 µg of $ZnCl_2$, 0.1 µg of $MnCl_2 \cdot 4H_2O$, 6 µg of BH_3O_3, 0.347 mg of $CoCl_2 \cdot 6H_2O$, 2.6 µg of $CuCl_2 \cdot 2H_2O$, 24 µg of $NiCl_2 \cdot 6H_2O$, 36 µg of $Na_2MoO_4 \cdot 2H_2O$, 0.15 mg of $FeCl_2 \cdot 4H_2O$, 120 mg of NaOH, 5 mg of uracil, 5 mg of cytosine, 5 mg of adenine, and 5 mg of guanine with 1,000 ml sterile dH_2O;
   The concentration of L-Leucine in CDM-Leu was 22.5 mg/L instead of 160 mg/L;
   Filter-sterilise (0.22 µm)
   Store at 4 °C.
2. 5% (10%) TCA (1,000 ml)
   Mix 50 g (100 g) of TCA with 1,000 ml dH_2O
   Store at 4 °C.
3. 5% TCA/1.5 M NaCl (100 ml)
   Mix 8.8 g NaCl with 100 ml of 5% TCA
   Store at 4°C.
4. 0.1% SDS/0.1M NaOH (100 ml)
   Gently mix by rotating 0.1 g sodium dodecyl sulphate (SDS) (w/v) and 0.6 g NaOH (w/v) with 100 ml dH_2O
   Store at room temperature.

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