# Supplementary Materials: Anti-Inflammatory Activity of Cyanobacterial Serine Protease Inhibitors Aeruginosin 828A and Cyanopeptolin 1020 in Human Hepatoma Cell Line Huh7 and Effects in Zebrafish (*Danio rerio*)

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## Transcriptional Analysis in Huh7 Cells after Exposure to Different Concentrations of AG 828A in HBSS Buffer (Figures S1–S3)

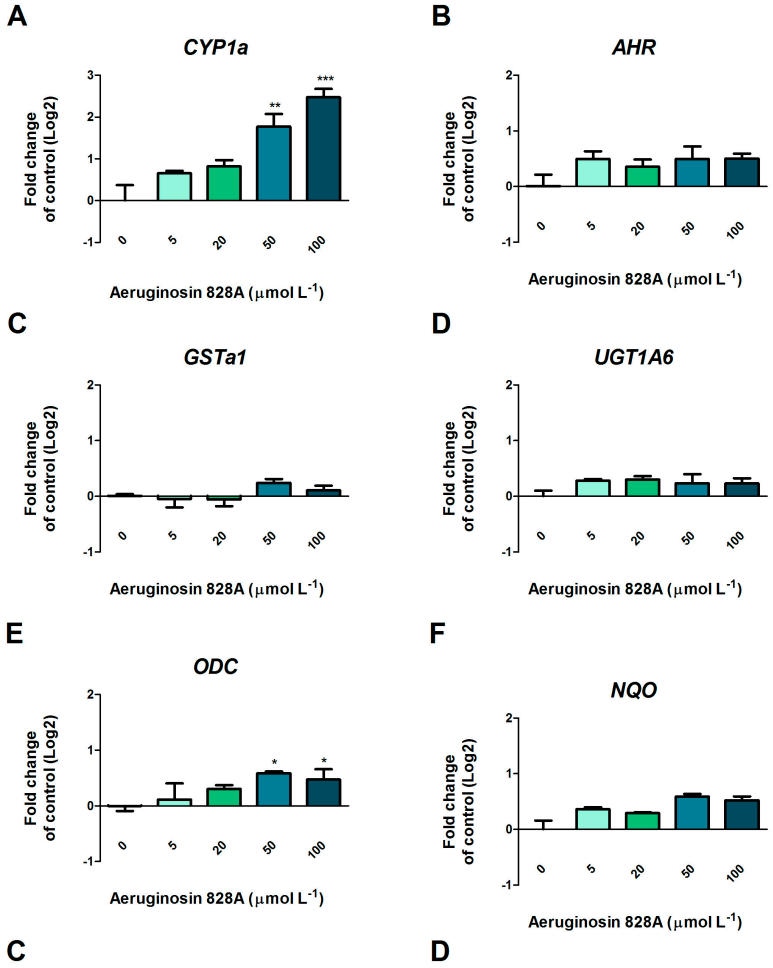


**Figure S1.** Transcription expression of aryl hydrocarbon receptor (AHR) regulated genes in Huh7 cells exposed to AG 828A in HBSS buffer compared to cells. (**A**) *Cytochrome P450 1A* (*CYP1A*); (**B**) *Aryl hydrocarbon receptor* (*AHR*); (**C**) *Glutathione S-Transferase a1* (*GSTa1*); (**D**) *Uridine diphospho-glucuronosyltransferase 1A6 (UGT1A6)*; (**E**) *Ornithin-decarboxylase* (*ODC*); (**F**) *nicotinamid adenindinucleotide phosphate* (*NADPH*) *chinon oxidoreductase* (*NQO*). Significant changes compared to control are indicated by asterisks (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$).

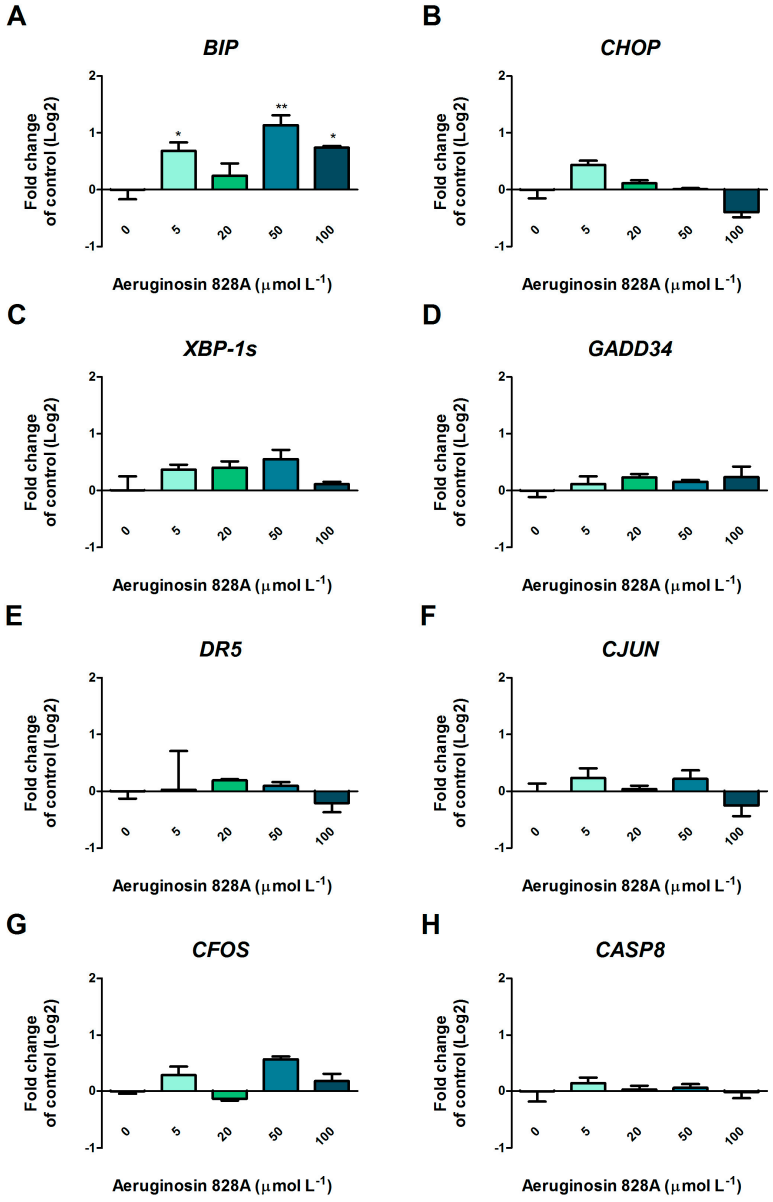


**Figure S2.** Transcriptional expression of genes that are known to be influenced by microcystin exposure in Huh7 cells exposed to AG 828A in Hank's Balanced Salt Solution (HBSS) buffer compared to control cells. (**A**) *Binding immunoglobulin protein* (*BIP*); (**B**) *CCAAT/enhancer binding protein (C/EBP) homologous protein* (*CHOP*); (**C**) *Spliced X-box binding protein 1* (*XBP1-*); (**D**) *growth arrest and DNA damage-inducible protein 34* (*GADD34*); (**E**) *Death receptor 5* (*DR5*); (**F**) *jun proto-oncogene* (*CJUN*); (**G**) *fos proto-oncogene* (*CFOS*); (**H**) *caspase 8* (*CASP8*). Significant changes compared to control are indicated by asterisks (* $p < 0.05$; ** $p < 0.001$).

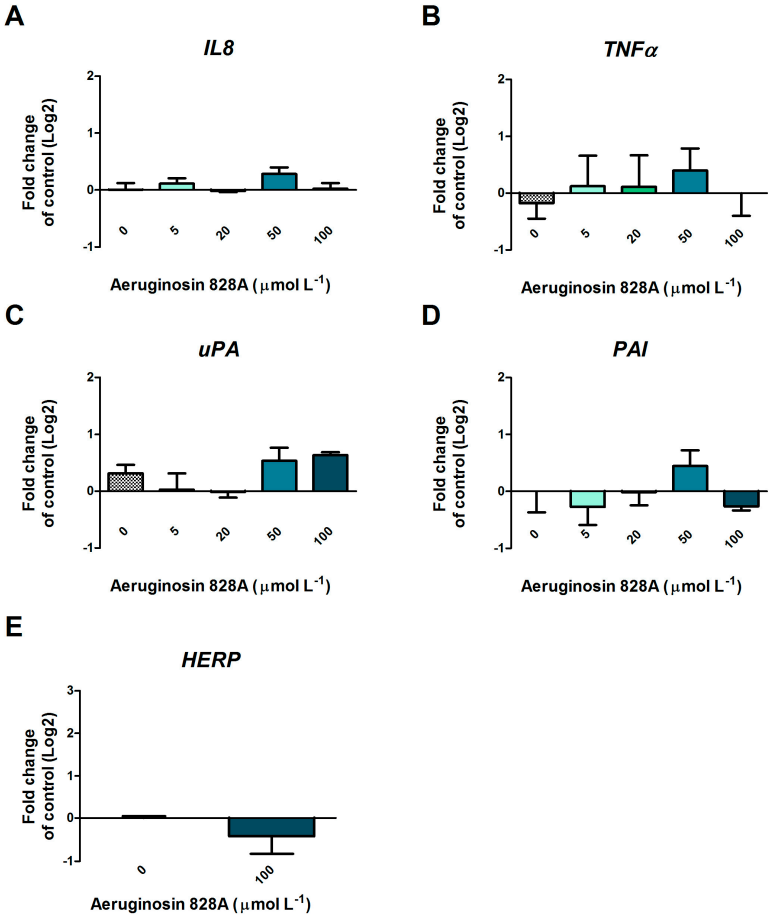


**Figure S3.** Transcriptional expression of genes involved in inflammation (**A**,**B**); the urokinase activation system (**C**,**D**) and endoplasmic reticulum (ER) stress (**E**) in Huh7 cells exposed to AG 828A in HBSS buffer compared to control cells. (**A**) *Interleukin 8* (*IL8*); (**B**) *Tumor necrosis factor α* (*TNF α*); (**C**) *Urokinase plasminogen activator* (*uPA*); (**D**) *Plasminogen activator inhibitor type 1* (*PAI*); (**E**) *Homocysteine inducible ER protein with ubiquitin like domain 1* (*HERP*).

## Transcriptional Analysis in Zebrafish Embryos after Exposure to Different Concentrations of AG 828A (Figures S4–S6)

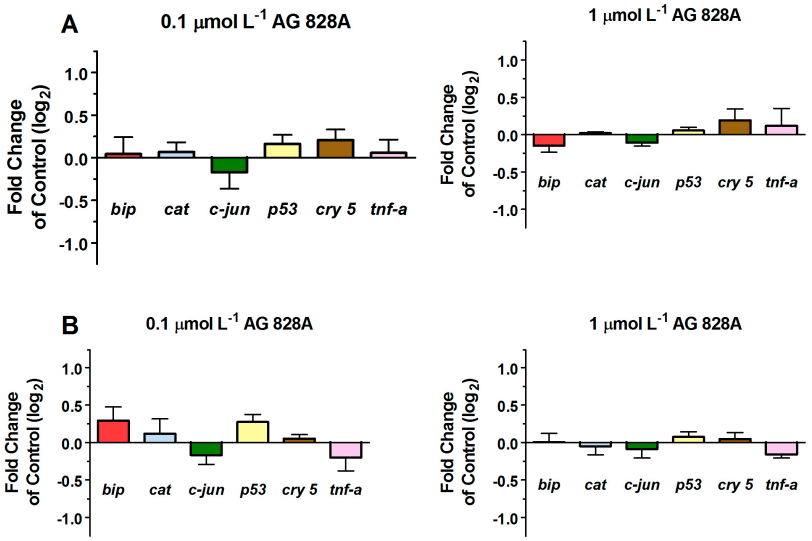

**Figure S4.** Transcription of genes involved in cellular stress responses (*bip*, *catalase* (*cat*), *c-jun*, *tumor suppressor protein 53* (*p53*), *cryptochrome 5* (*cry5*), *tnfα*) in zebrafish eleuthero-embryos exposed to AG 828A compared to control eleuthero-embryos. (**A**) 48 h exposure; (**B**) 96 h exposure.

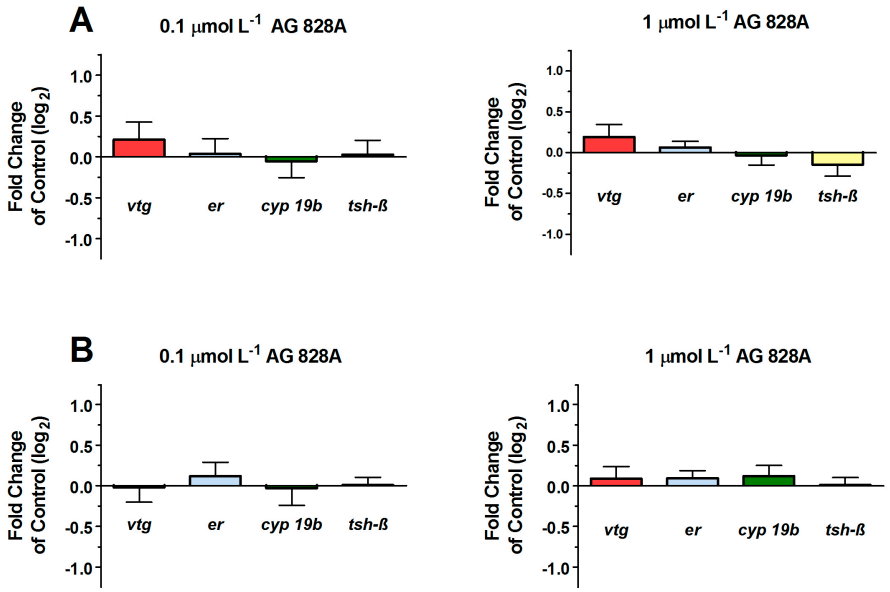


**Figure S5.** Transcription of genes involved in hormonal pathways (*vitellogenin* (*vtg*), *estrogen receptor* (*er*), *cytochrome P450 19b* (*cyp 19b*), *thyroid-stimulating hormone* (*tshβ*)) in zebrafish eleuthero-embryos exposed to AG 828A compared to control eleuthero-embryos. (**A**) 48 h exposure; (**B**) 96 h exposure.

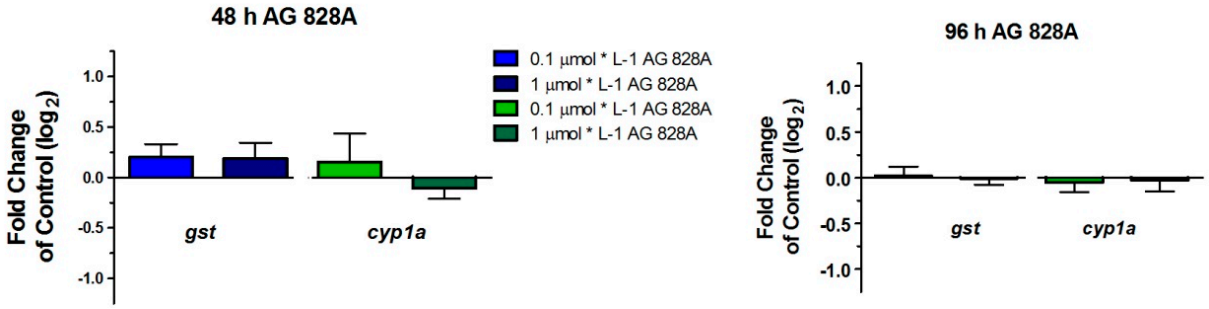


**Figure S6.** Transcription of genes involved in detoxification (*gst*, *cyp1a*) in zebrafish eleuthero-embryos exposed to AG 828A compared to control eleuthero-embryos for 48 h and 96 h exposure.

## Transcriptional Analysis in Zebrafish Liver Organ Culture after Exposure to Different Concentrations of AG 828A and the ER Stress Inducer Tunicamycin (Figures S7–S11)

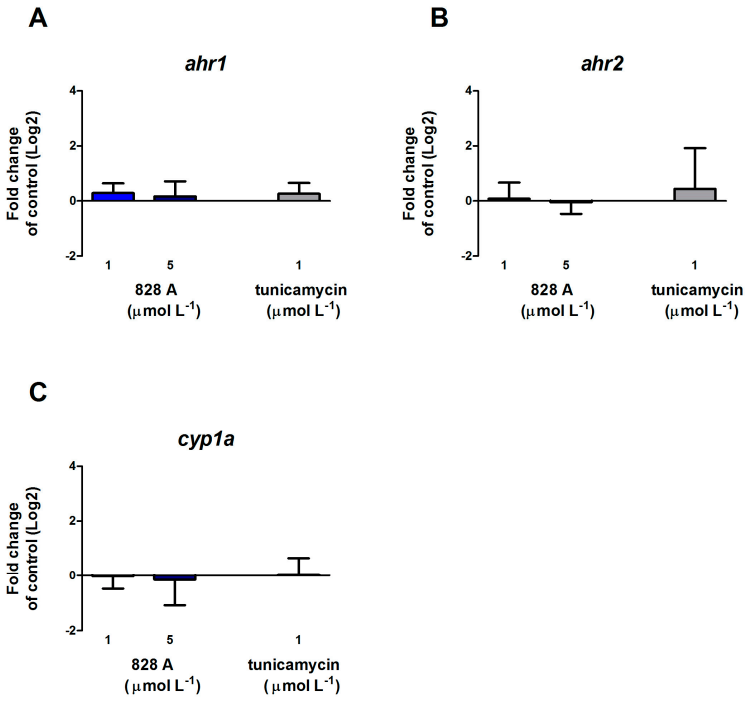


**Figure S7.** Transcriptional expression of AHR regulated genes in zebrafish liver organ cultures exposed to AG 828A for 5 h compared to control. (**A**) *Aryl hydrocarbon receptor 1*; (**B**) *Aryl hydrocarbon receptor 2*; (**C**) *Cytochrome P450 1A*.

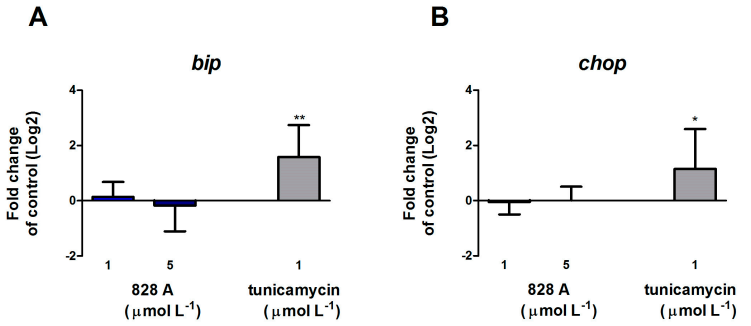


**Figure S8.** Transcriptional expression of genes involved in ER stress in zebrafish liver organ cultures exposed to AG 828A for 5 h compared to control. (**A**) *Binding immunoglobulin protein*; (**B**) *C/EBP homologous protein*. Significant changes compared to control are indicated by asterisks (* $p < 0.05$; ** $p < 0.001$).

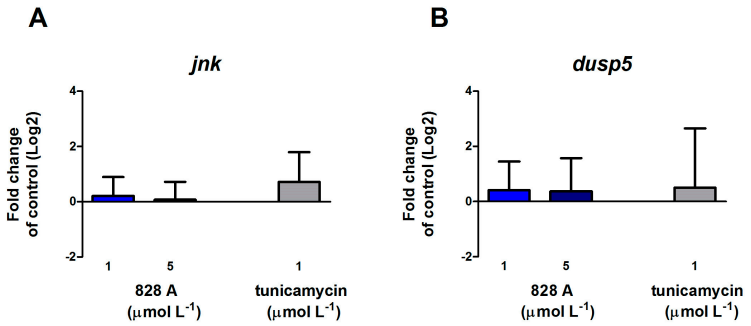

**Figure S9.** Transcriptional expression of genes involved in mitogen-activated protein kinase (MAPK) pathways in zebrafish liver organ cultures exposed to AG 828A for 5 h compared to control. (**A**) C-*Jun N-terminal kina (jnk)s*; (**B**) *Dual specificity phosphatase 5* (*dusp5*).

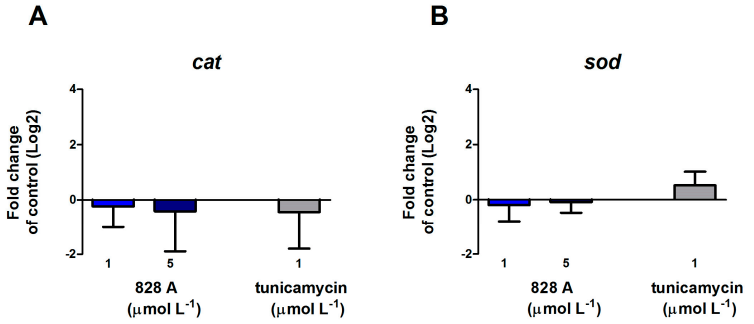


**Figure S10.** Transcriptional expression of genes involved in oxidative stress in zebrafish liver organ cultures exposed to AG 828A for 5 h compared to control. (**A**) *Catalase*; (**B**) *Superoxid dismutase* (*sod*).

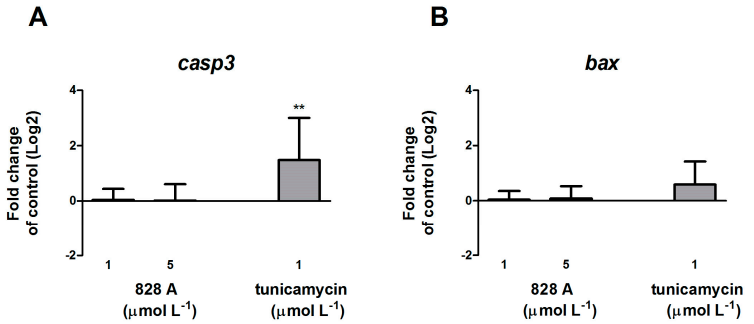


**Figure S11.** Transcriptional expression of genes involved in apoptosis in zebrafish liver organ culture exposed to AG 828A for 5 h compared to control. (**A**) *Caspase 3* (*casp3*); (**B**) *Bcl-2-like protein (bax*). Significant changes compared to control are indicated by asterisks (** $p < 0.001$).

## Analysis of the Purified Aeruginosin 828A and CP 1020 is Described in the Following, and Results are Presented in Figures S12–S17 and Table S1

Chromatography separation was accomplished on a Scherzo SM $C_{18}$ (2.1 × 100 mm, 3 micron, Imtakt USA, Portland, OR, USA) using an ultra high performance liquid chromatography stack arrangement consisting of a degasser, binary pump, auto sampler, thermostat and column oven (Agilent Series 1290, Agilent Technologies, Waldbronn, BW, Germany). A 1 µL aliquot of the purified sample was injected into the column and eluted with a linear gradient of 5%–95% solvent B over 4.5 min and kept at 95% B for 3 min and re-equilibration was performed in 3 min. Mobile phase A was water and mobile phase B was methanol plus each containing of 5 mM ammonium formate. The column was maintained at 40 °C and the column flow was set to 0.4 mL/min. Mass spectrometry spectra were assimilated on Q-TOF-MS system (Agilent Series 6540 Q-TOF, Agilent Technologies, Santa Clara, CA, USA) with a jet stream electrospray ion source (ESI). The ESI source was operated in positive mode with following parameter settings: nebulizer pressure 35 psig, nozzle voltage 0 V, sheath gas flow 11 L/min, sheath gas temperature 375 °C, drying gas flow 8 L/min, drying gas temperature 250 °C, capillary voltage 3000 V and fragmentor voltage 175 V respectively. Accurate mass spectra were acquired over an *m*/*z* of 100–1500 range by 8127 transitions per spectrum (1Hz). The acquired spectra were automatically recalibrated on-line by reference ions with exact masses 121.0509 and 922.0098 *m*/*z*. The system was running under the software MassHunter Acquisition and Qualitative Analysis version B.06.00 (Agilent Technologies, Santa Clara, CA, USA, 2012).

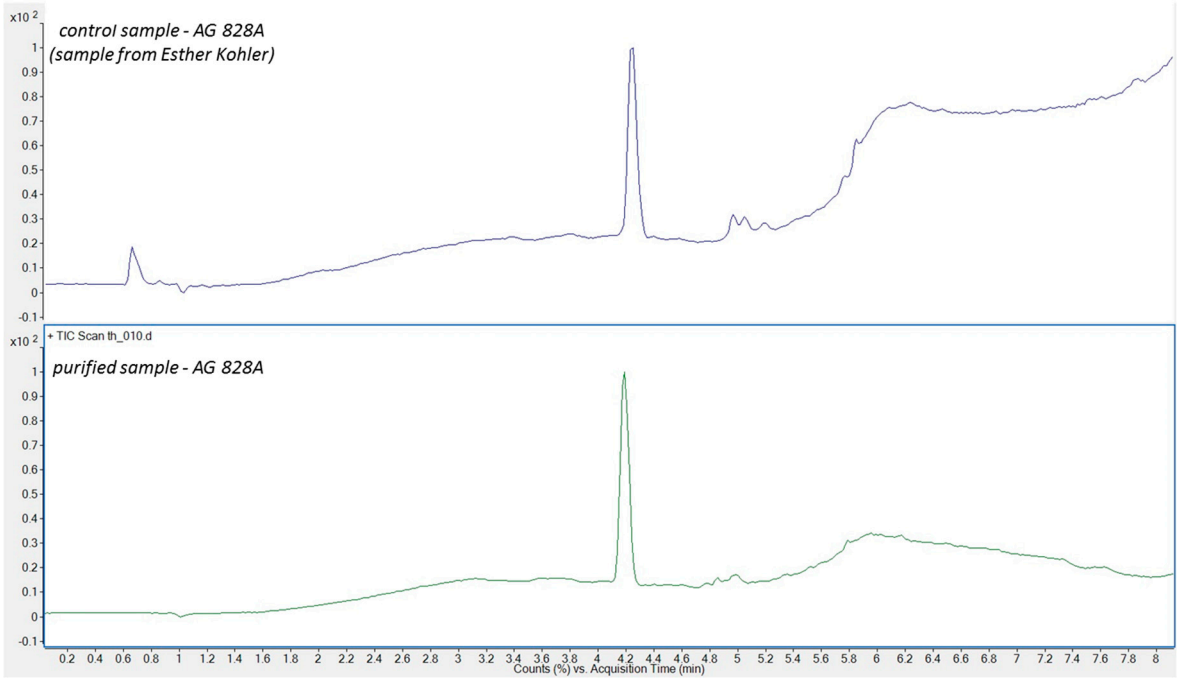


**Figure S12.** Total Ion Chromatogram of control sample AG 828A (provided from [14] as standard) and purified AG 828A of *Planktothrix rubescens* extract after the second fractionation step.

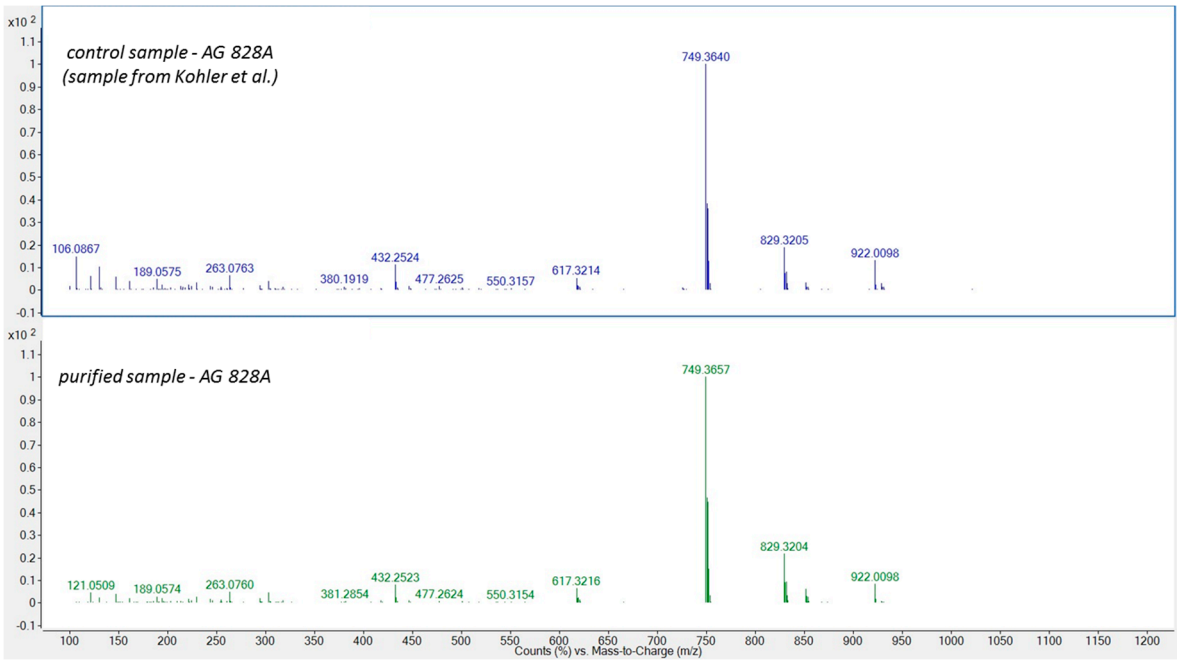


**Figure S13.** MS spectrum of AG 828A of the standard control sample (blue) and purified sample (green). Mass *m*/*z* 829 is the [M + H] signal and the base peak *m*/*z* 749 signify $[M + H - SO_3]^+$ of the targeted compound Ag 828A.


**Figure S14.** MS spectrum with charge carrier, neutral loss annotation and predicted isotope distribution (red bars) of AG 828A of the standard control sample and sample.

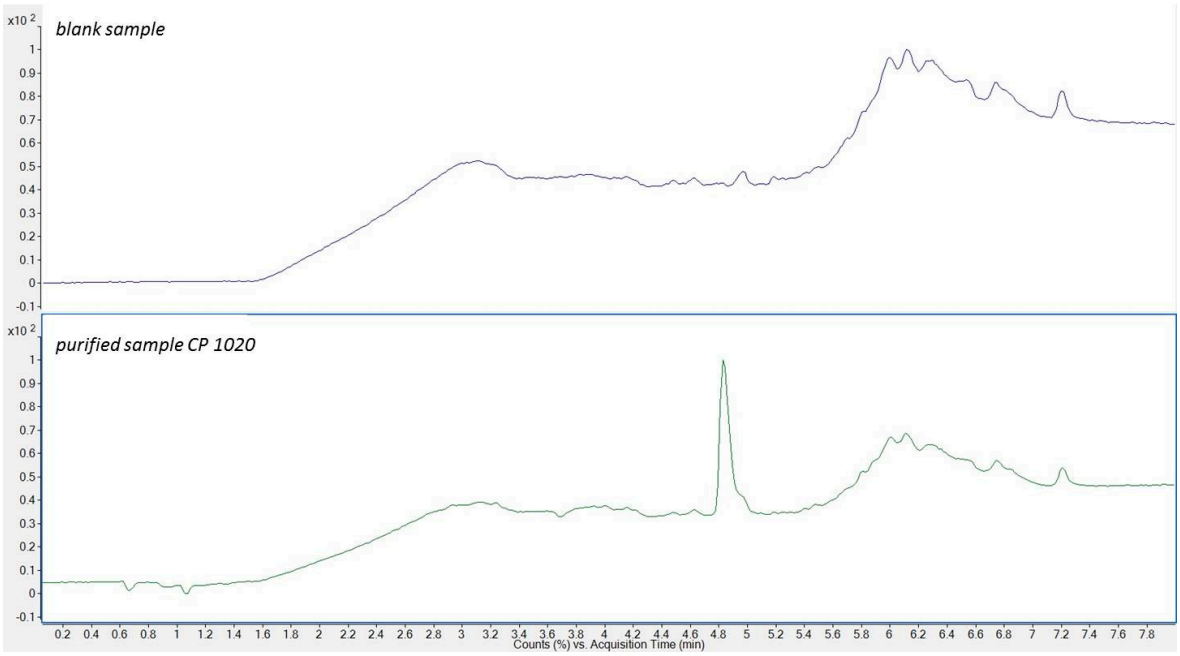


**Figure S15**. Total Ion Chromatogram of blank sample and purified sample CP 1020 of *Microcystis* extract after the fractionation step.

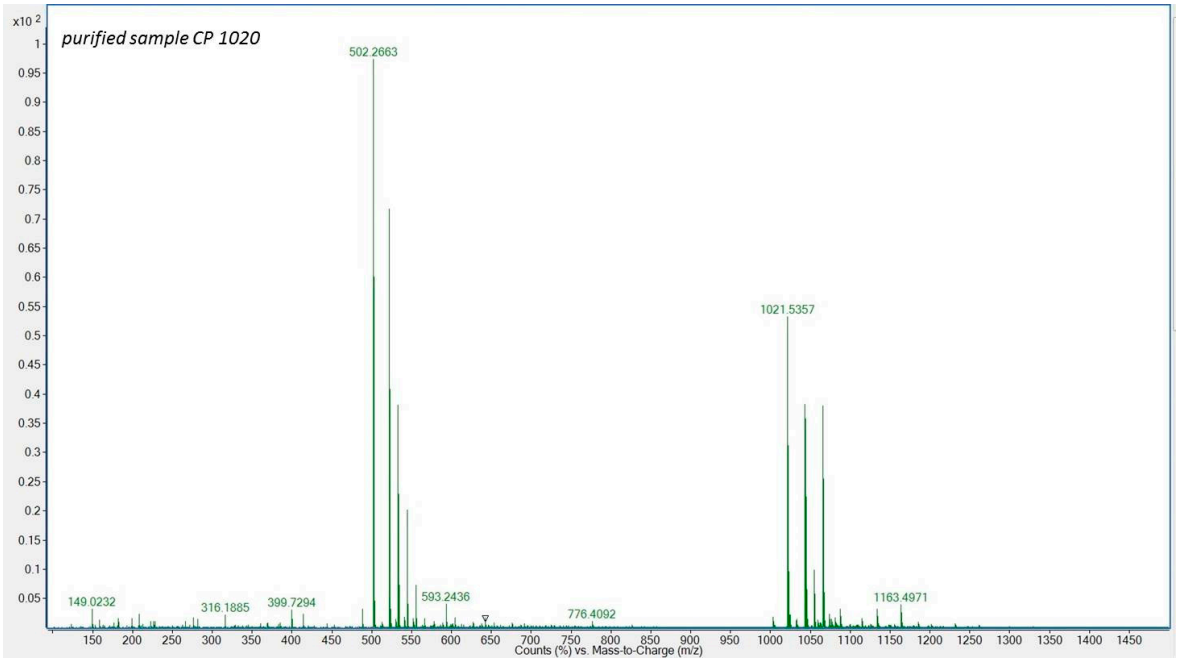


**Figure S16.** MS spectrum of the purified sample CP1020. Mass-to-charge 1021 is the $[M + H]^+$ signal and the base peak *m*/*z* 502 $[M + 2H - H_2O]^{+2}$ of the compound.

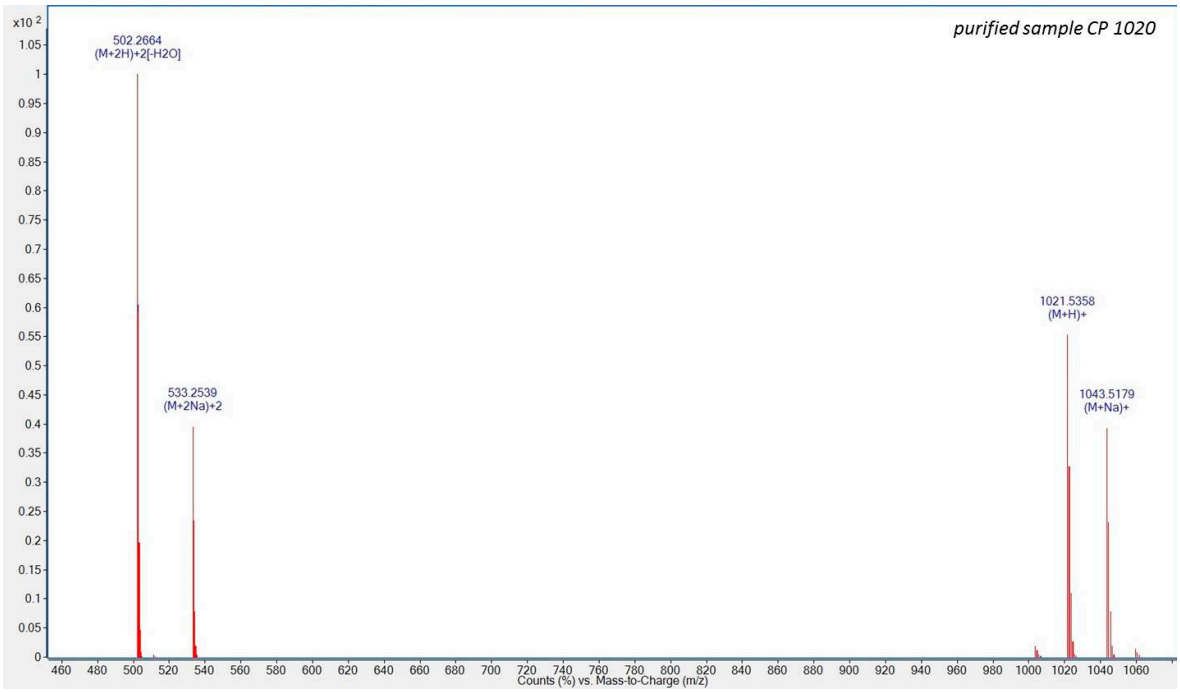


**Figure 17.** MS spectrum with charge carrier, neutral loss annotation and predicted isotope distribution (red bars) of CP1020.

**Table S1.** Characteristics of standard control sample and purified sample.

| Name | Retention Time (min) | Sum Formula | Calculated Mass (Da) | Measured Mass (Da) | Mass Error (ppm) |
|---|---|---|---|---|---|
| control sample AG 828A | 4.2 | $C_{36}H_{53}ClN_6O_{12}S$ | 828.31307 | 828.31339 | 0.39 |
| purified sample AG 828A | 4.2 | $C_{36}H_{53}ClN_6O_{12}S$ | 828.31307 | 828.31342 | 0.43 |
| purified sample CP 1020 | 4.8 | $C_{50}H_{72}N_{10}O_{13}S$ | 1020.5280 | 1020.5287 | 0.69 |

## Primer Sequences of Primers for Transcription Analysis in Huh7 Cells and Zebrafish Eleuthero Embryos, as well as Zebrafish liver Organ Cultures Exposed to Aeruginosin 828A (Tables S2 and S3)

**Table S2.** Primer sequences for analysis in Huh7 cells.

| Huhu 7 cells | Primer | Sequence |
|---|---|---|
| *CYP1a* | forward | AGGAGCTAGACACAGTGATTG |
| *cytochrome P450, 1A* | reverse | GTTCAGGTAGGAACTCAGATG |
| *AHR* | forward | CCAACATCACCTACGCCAGTCG |
| *aryl hydrocarbon receptor* | reverse | ACATCTTGTGGGAAAGGCAGCAG |
| *GSTa1* | forward | ATCCTCCTTCTGCCCGTATGTC |
| *Glutathione S-Transferase a1* | reverse | AAGTTCCACCAGATGAATGTCAGC |
| *UGT1A6* | forward | AGCCCAGACCCTGTGTCCTA |
| *uridine diphospho-glucuronosyltransferase 1A6* | reverse | CCACTCGTTGGGAAAAAGTCA |
| *ODC* | forward | ATGTTGCATCAGCT TTCACG |
| *ornithin-decarboxylase* | reverse | ACTCTCCCAGGCACAAGA CA |
| *NQO* | forward | CGCAGACCTTGTGATATTCCAG |
| *NAD(P)H chinon oxidoreductase* | reverse | CGTTTCTTCCATCCTTCCAGG |
| *BIP* | forward | CGA GGA GGA GGA CAA GAA GG |
| *Binding immunoglobulin protein* | reverse | GAC CTT GAA CGG CAA GAA CT |
| *CHOP* | forward | GGA GCA TCA GTC CCC CAC TT |
| *C/EBP homologous protein* | reverse | TGT GGG ATT GAG GGT CAC ATC |
| *XBP-1s* | forward | TGC TGA GTC CGC AGC AGG TG |
| *spliced X-box binding protein 1* | reverse | GCT GGC AGG CTC TGG GGA AG |
| *GADD34* | forward | CCC AGA AAC CCC TAC TCA TGA TC |
| *growth arrest and DNA damage-inducible protein 34* | reverse | GCC CAG ACA GCC AGG AAA T |
| *HERP* | forward | AAC GGC ATG TTT TGC ATC TG |
| *homocysteine inducible ER protein with ubiquitin like domain 1* | reverse | GGG GAA GAA AGG TTC CGA AG |
| *DR5* | forward | AGA CCC TTG TGC TCG TTG TC |
| *Death receptor 5* | reverse | TTG TTG GGT GAT CAG AGC AG |
| *CJUN* | forward | TCC AAG TGC CGA AAA AGG AAG |
| - | reverse | CGA GTT CTG AGC TTT CAA GGT |
| *CFOS* | forward | CCG GGG ATG CCT CTC TTA CT |
| - | reverse | CCAGGTCCGTGCAGAAGTC |
| *CASP8* | forward | CAG AGC CTG AGA GAG CGA TG |
| *caspase 8* | reverse | AGG CTG AGG CAT CTG TTT CC |
| *IL8* | forward | GAG TGC TAA AGA ACT TAG ATG TCA G |
| *interleukin 8* | reverse | GCT TTA CAA TAA TTT CTG TGT TGG C |
| *TNFα* | forward | CAG CCT CTT CTC CTT CCT GA |
| *Tumor necrosis factor α* | reverse | TGAGGTACAGACCCTCTGAT |
| *uPA* | forward | CAC GCA AGG GGA GAT GAA |
| *Urokinase plasminogen activator* | reverse | ACA GCA TTT TGG TGG TGA CTT |
| *PAI* | forward | TGC TGG TGA ATG CCC TCT ACT |
| *Plasminogen activator inhibitor type 1* | reverse | CGG TCA TTC CCA GGT TCT CTA |
| *GAPDH* | forward | GAAGGTGAAGGTCGGAGTC |
| *Glyceraldehyde 3-phosphate dehydrogenase* | reverse | GAAGATGGTGATGGGATTTC |

**Table S3.** Primer sequences for analysis in zebrafish eleuthero-embryos and liver organ culture.

| Zebrafish | Primer | Sequence |
|---|---|---|
| *bip* | forward | CGA AGA AGC CAG ATA TCG ATG |
| *Binding immunoglobulin protein* | reverse | ACG GCT CTT TTC CGT TGA AC |
| *chop* | forward | GAG TTG GAG GCG TGG TAT GA |
| *C/EBP homologous protein* | reverse | CCT TGG TGG CGA TTG GTG AA |
| *cat* | forward | AGG GCA ACT GGG ATC TTA A |
| *Catalase* | reverse | TTT ATG GGA CCA GAC CTT GG |
| *sod* | forward | GGC CAA CCG ATA GTG TTA GA |
| *Super oxid dismutase* | reverse | CCA GCG TTG CCA GTT TTT AG |
| *c-jun* | forward | ACG TGG GAC TTC TCA AAC TG |
| - | reverse | TCT TGG GAC ACA GAA ACT GG |
| *dusp 5* | forward | TGA AGG TCT CCA GCA TAG |
| *dual specificty phospatase 5* | reverse | GGA ATG ACG AAC TGT AGA G |
| *p53* | forward | GCT TGT CAC AGG GGT CAT TT |
| *tumor suppressor p53* | reverse | ACA AAG GTC CCA GTG GAG TG |

| | | |
|---|---|---|
| *casp3* | forward | CCG CTG CCC ATC ACT A |
| *Caspase 3* | reverse | ATC CTT TCA CGA CCA TCT |
| *bax* | forward | TCA CTC GTT CAG ACC CTC AT |
| *bcl-2-like protein* | reverse | ACG CTT TCC ACG CAC AT |
| *cry5* | forward | CAT GGA GAG AAC GAA CTG GG |
| *cryptochrome 5* | reverse | GTG CAG ACA AGC AGC CGA AC |
| *tnfα* | forward | ACC AGG CCT TTT CTT CAG GT |
| *Tumor necrosis factor α* | reverse | TGC CCA GTC TGT CTC CTT CT |
| *vtg* | forward | AGC TGC TGA GAG GCT TGT TA |
| *vitellogenin* | reverse | GTC CAG GAT TTC CCT CAG T |
| *er* | forward | TGA GCA ACA AAG GAA TGG AG |
| *estrogen receptor* | reverse | GTG GGT GTA GAT GGA GGG TTT |
| *cyp19b* | forward | GGC AGT CTC TGG AGG ATG AC |
| *cytochrome P450, 19b* | reverse | CAG TGT TCT CGA AGT TCT CCA |
| *tshβ* | forward | GCA GAT CCT CAC TTC ACC TAC C |
| *Thyroid stimulating hormone, beta* | reverse | GCA CAG GTT TGG AGC ATC TCA |
| *gst* | forward | CTA TAC ATG CGG CGA AGC |
| *Glutathione S-Transferase* | reverse | CGC ATT GCT CTG GAC GAT |
| *cyp1a* | forward | CCT GGG CGG TTG TCT ATC TA |
| *cytochrome P450, 1a* | reverse | TGA GGA ATG GTG AAG GGA AG |
| *ahr1* | forward | TAG ACA GCG ATA TAC AGC AG |
| *aryl hydrocarbon receptor 1* | reverse | TCTCTCCAACACCATTCATG |
| *ahr 2* | forward | ACGGTGAAGCTCTCCCATA |
| *aryl hydrocarbon receptor 2* | reverse | AGTAGGTTTCTCTGGCCAC |
| *nr1d1* | forward | GTG AAC AAC CAG CTG CAG AA |
| *nuclear receptor subfamily 1, group d, member 1* | reverse | ACT GTA AGG CCT GGA CAT GG |
| *per1* | forward | ATG CGT GCA AGA AGT GGT G |
| *period 1* | reverse | ACG TCC TCA TTT AGC GGA CTC |
| *Ptgds* | forward | CCA TCA AGA CCA AAG GAG GA |
| *prostaglandin D2 synthase* | reverse | TCC ATT TTG TGG AAG CAT GA |
| *esr1* | forward | TGA GCA ACA AAG GAA TGG AG |
| *ER alpha* | reverse | GTG GGT GTA GAT GGA GGG TTT |
| *abcg2a* | forward | TCA TGA AGC CGG GAC TGA AC |
| *ATP-binding cassette, sub-family G* | reverse | GCT CCG TCT ATC AGC ACC TC |
| *rpl13a* | forward | AGC TCA AGA TGC CAA CAC AG |
| *ribosomal protein L13a* | reverse | AAG TTC TTC TCG TCC TCC |