Quaternary biopesticides and disinfectants derived from quinine and amino acids – environmental prospects and risks

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Abstract: The numerous risks associated with the toxicity of conventional quaternary ammonium salts (QASs) to various groups of living organisms have prompted the search for new, safer biologically active compounds synthesized in a sustainable manner from renewable raw materials. Here, we describe new QASs of natural origin containing quinine-based cation and anions derived from proteinogenic amino acids – L-asparagine and L-alanine. The quinine-derived QASs were thoroughly characterized in terms of correctness of chemical structure, physicochemical properties, and biological activity. It was discovered that due to quinine activity, the new salts exhibit strong antifeedant activity toward stored products pests, and in the case of elongation of the alkyl substituent in the 1-alkylquininium cation, they also become potent disinfectants.

The performed analyses also allowed to assess the environmental risk by determining toxicity to monocotyledonous (*Sorghum bicolor*) and dicotyledonous (*Sinapis alba*) terrestial plants, freshwater algae (*Chlorella vulgaris*) and crustaceans (*Daphnia magna*) at various concentrations of the test substance. None of the QASs of natural origin showed phytotoxicity, and salts containing short alkyl substituents in quinine-based cations were noticeably less toxic to aquatic organisms than the other tested compounds. The results indicate that although a trade-off between antimicrobial activity and aquatic toxicity must be made when designing new quinine-based antiseptics, it is possible to obtain potent, naturally-derived and relatively low-toxic biopesticides based on quinine and amino acids.

Keywords: *proteinogenic amino acids, cinchona alkaloids, antifeedants, antimicrobial activity, phytotoxicity, aquatic toxicity*

1. Introduction

Quaternary ammonium salts (QASs) are a group of chemicals manufactured and used on a mass scale around the world. Their favorable surface properties and biological activity have resulted in decades of use as cleaning agents, emulsifiers, preservatives, algicides, cosmetic additives, as well as antiseptics and disinfectants (Arnold et al., 2023; De et al., 2015). However, the increasing use of conventional, fully synthetic QASs, particularly evident in recent years due to the COVID-19 pandemic (Arnold et al., 2023), represents a significant environmental burden due to their limited biodegradability, accumulation tendencies (Pati and Arnold, 2020) and their very high toxicity toward organisms present in water and soil (Badmus et al., 2021; Kaczerewska et al., 2020; Zhang et al., 2015). Due to the persistence of QASs in the environment at constant, low concentrations, there is also a genuine risk of widespread acquisition of resistance by pathogenic microbes to this most commonly used type of disinfectants (Jia et al., 2022). Moreover, due to their good surface properties and ability to form micelles, QASs may also be responsible for the spread of hydrophobic toxic substances in the environment (Badmus et al., 2021).

For the above reasons, a strategy of replacing toxic QASs characterized by low biocompatibility with safer active substances of natural origin and taking into account environmental risks of their production and use is reasonable (Zhang et al., 2021). This approach involves replacing fully synthetic cationic QASs with salts of natural origin, including amphiphilic betaine derivatives (Homa et al., 2024; Merkova et al., 2018; Vonlanthen et al., 2011), which can be obtained in sustainable ways and are characterized by lower ecotoxicity. A particularly interesting variant of this strategy consists in the use of natural compounds with favorable physicochemical or biological properties as a scaffold for new active ingredients. For example, quinine, the oldest, naturally derived antimalarial drug, is currently attracting interest in the scientific community and provides a promising foundation for the structures of active

pharmaceutical ingredients (Jones et al., 2015; Terunuma and Hayashi, 2022). In this regard, it should be noted that recently effective methods have been developed for obtaining QASs with a cation derived from quinine (McNeice et al., 2020; Pernak et al., 2020), for which a number of beneficial applications have been proposed to date. Quinine-based QASs may be applied as catalysts for stereochemical reactions that are stable in alkaline media (McNeice et al., 2020), modifiers for enantiomer separation (Sintra et al., 2019), and plant growth regulators (Rzemieniecki et al., 2021). Due to the confirmed antifeedant properties toward insects exhibited by quinine (Jermy, 1990; Lazzari et al., 2024; Ramaswamy et al., 1992), the prospect of application of quinine-based QASs as biopesticides to reduce insect feeding on plants and stored grain may represent a promising direction (Pernak et al., 2020). The search for new ways to counteract insect feeding in a sustainable and environmentally safe manner is particularly important, since ongoing global warming significantly contributes to the increase in insect metabolism, as well as their need for food. In the case of a 2 °C temperature rise compared to the pre-industrial levels, yield losses caused by pest insects could increase by as much as 46% for wheat, 13% for rice and 31% for corn (Deutsch et al., 2018). Given that the projected increase in global temperature is very likely to reach 2.5–2.9 °C by the end of this century according to the latest UN report (United Nations Environment Programme, 2023), pest activity is also expected to rise faster and to higher levels.

In previous work, no attention was paid to the environmental fate of quinine-based QASs as a new type of pollutant, nor did they consider the toxicity of these compounds to living organisms. In order to mitigate negative environmental effects, in the course of designing the new compounds, the strategy of obtaining new naturally-derived QASs was extended. The cation derived from the plant alkaloid was combined with natural anions of L-asparagine and L-alanine, proteinogenic amino acids that constitute the building blocks of proteins in living organisms. There is no information about harmful effects of these amino acids on human and

animal health (Garlick, 2004). The synthesized quinine-derived QASs were subsequently tested in terms of their lipophilicity, antimicrobial activity, biopesticidal activity toward pest insects, and their toxicity toward terrestrial plants and aquatic organisms. The results obtained in the course of this study allowed a meaningful determination of the application potential of the new quinine derivatives, as well as the risks associated with their possible introduction into the environment.

2. Experimental

2.1. Materials

Quinine monohydrochloride dihydrate (purity ≥95%), 1-bromobutane (purity ≥98%), 1-bromooctane (purity 99%), 1-bromododecane (purity ≥95%), 2-bromoethanol (purity 95%), L-asparagine (purity ≥98%), L-alanine (purity ≥99%), 1-octanol (purity ≥99%) and ion-exchange resin AmberLite™ HPR550 OH were purchased from Merck KGaA, (Darmstadt, Germany). Methanol (purity ≥99.8%), ethanol (purity ≥99.8%), 2-propanol (purity ≥99.7%), diethyl ether (purity ≥99.8%), dimethyl sulfoxide (purity ≥99.9%), dimethylformamide (purity ≥99.8%), acetonitrile (≥99.9%), acetone (purity ≥99.8%), ethyl acetate (purity ≥99.5%) and potassium hydroxide (>98%) were provided by Avantor Performance Materials Poland S.A. (Gliwice, Poland) and used without further purification. Microbiological broth media including Nutrient Broth, Mueller-Hinton Broth, TSB and BHI Broth for bacteria, YPD Broth for yeast and PDB for filamentous fungi were purchased from BioMaxima (Lublin, Poland). The sources of materials used for the preparation of media recommended by OECD guidelines for toxicity studies are disclosed in the Supplementary Data (Table A.1). Water for syntheses, apparatus calibration, solubility studies, octanol-water partition coefficient studies, and biological assay studies was deionized, with a conductivity <0.1 $\mu S \cdot cm^{-1}$, from demineralizer HLP Smart 1000 (Hydrolab, Straszyn, Poland). OECD guidelines-compliant soil and seeds of white mustard

(*Sinapis alba* L.) and sorghum (*Sorghum bicolor* (L.) Moench) were purchased from MicroBioTests Inc. (Gent, Belgium).

2.2. General

^{1}H NMR spectra were acquired using Bruker Avance III™ HD 600 MHz apparatus (Billerica, Massachusetts, USA) or Varian VNMR-S 400 MHz spectrometer (Palo Alto, USA) with TMS as the internal standard, using DMSO-d_6 as a solvent. ^{13}C NMR spectra were obtained with the same instruments at 150 MHz or 100 MHz, respectively. FT-IR spectra were recorded on IFS 66v/S spectrometer (Bruker Optics, Ettlinger, Germany) or collected by using an EasyMax 102 semi-automated system (Mettler Toledo, USA) connected to a ReactIR® 15 (Mettler Toledo, USA) probe equipped with an MCT detector and 9.5 mm AgX probe with a diamond tip. The data were The data were sampled from 4000 to 400 cm^{-1} and visualized using Spectragryph 1.2.13 software (Menges, 2009) (IFS 66v/S spectrometer) or from 3000 to 650 cm^{-1} and processed by iCIR 4.3 software (ReactIR® 15). The water content in all obtained products was measured with a TitroLine 7500 KF coulometer (SI Analytics, Germany) using the Karl Fischer titration method according to the previously described procedure (Stachowiak et al., 2022). Differential scanning calorimeter (DSC) was performed on DSC-XP-10 unit (THASS, Germany). Samples between 5 and 20 mg were placed in platinum pans and were heated from 25 to 120 °C at a heating rate of 10 °C min^{-1} and cooled at a cooling rate of 10 °C min^{-1} to -80 °C. After that, samples were heated again to 120 °C and subsequently cooled to 25 °C. Decomposition temperatures were determined visually using Melting Point System MP90 apparatus (Mettler Toledo, USA).

2.3. Synthesis

2.3.1. Quinine-derived QASs with bromide anion

To perform quaternization, quinine free base of high purity was obtained from quinine monohydrochloride dihydrate of technical grade using the previously developed precipitation

method (Pernak and Rzemieniecki, 2020). First, quinine monohydrochloride dihydrate was dissolved in anhydrous acetonitrile (0.9 dm^3 per 100 g of hydrochloride) at 25 °C, and then, an equimolar amount of potassium hydroxide was added. The precipitate was filtered off and thoroughly washed with warm (40–50 °C) water. In the next stage, the obtained quinine free base was subjected to quaternization with 1-bromoalkanes comprising 4, 8, or 12 carbon atoms in the alkyl chain according to the methodology described previously (Rzemieniecki et al., 2021). 1-(2-Hydroxyethyl)quininium bromide was obtained according to a similar methodology. First, quinine free base (0.03 mol, 9.733 g) was alkylated using 2-bromoethanol (0.0315 mol, 3.936 g). The reaction was conducted for 48 hours at 35 °C in 30 cm^3 of either DMSO or DMF. The post-reaction mixture was transferred to a dropping funnel and added dropwise to vigorously stirred ethyl acetate, and the resulting precipitate was filtered off, dried, and recrystallized twice from methanol-acetone (1:3 volumetric ratio). The resulting quaternary ammonium bromides were pulverized, dried and stored over P_4O_{10}. A semi-automatic EasyMax reactor with a vessel of 100 cm^3 equipped with a reflux condenser, a stir bar and a temperature sensor (Mettler Toledo, USA) was used to perform all quaternization reactions.

2.3.2. Quinine-derived QASs with amino acid-derived anion

In the last stage, L-asparagine and L-alanine QASs with quinine-derived cations were obtained using the previously obtained bromides as cation sources. First, the respective quaternary ammonium bromide (0.01 mol) was dissolved in ethanol (20 cm^3) and placed in a 50 cm^3 EasyMax™ reaction vessel equipped with a stir bar and a temperature sensor. Next, AmberLite™ HPR550 OH highly alkaline anionic resin (15 cm^3) was placed in the vessel, and the system was stirred for the next 15 minutes. The resin was subsequently filtered off from the post-reaction mixture and washed with 10 cm^3 of ethanol. In the next step, 20 cm^3 of aqueous solution containing 0.01 mol of the respective amino acid (L-asparagine, 1.321 g or L-alanine, 0.891 g) in zwitterionic form was added to the filtrate and the mixture was stirred for the next

30 minutes. The solvents were afterwards removed using a rotary vacuum evaporator, and the raw product was dissolved in 15 cm^3 of anhydrous methanol. In the next step, the resulting solution was filtered to remove any potential residue of unreacted amino acid. The solvent was afterwards removed using a rotary vacuum evaporator, and the residue was dried using a Schlenk line (pressure: $2 \cdot 10^{-5}$ bar) while heating to 40 °C for at least 8 hours. The obtained QASs were refrigerated at 4 °C in airtight vessels to prevent moisture absorption and potential decomposition.

2.4. Water solubility

Solubility in water of the obtained compounds at 25 °C was tested using the flask method described in the OECD 105 Guidelines (OECD, 1995a). First, a preliminary test was performed, which consisted in adding increasing amounts of water to a determined amount (0.1 g) of test substance and shaking it for 10 minutes after each addition to determine an approximate solubility. Subsequently, exact solubility was determined for low-soluble compounds. First, a determined amount of pulverized solid, greater than can be dissolved in 10 cm^3 of water, was placed in 3 glass vials, and then water (in 10 cm^3 aliquots) was added to each vial. In addition, 2 more vials containing the same amount of water, but 2 or 3 times more test substance, were prepared to establish the effect of the dissolution of potential impurities on the solubility results. The vessels were stoppered and then stirred at 30 °C for one day. Next, one of the standard vials was equilibrated at 25 °C for 24 hours, and then the aqueous solution was collected from the suspension with a syringe equipped with a filter with a hydrophilic membrane. The concentration of test compounds in water was determined spectrophotometrically using a VWR® UV-5300PC spectrophotometer (VWR International, Belgium) based on calibration curves made previously (at λ_{max} = 235 nm). Second and third vials were tested after being stirred at 30 °C for 2 and 3 days, respectively, using the same procedure. Two additional vials with an

190 increased amount of test substance were tested after 3 days of stirring (alongside the third vial).
191 Finally, the solubility was calculated as the average of 5 results collected for each compound.

192 **2.5. Octanol-water partition coefficient**

193 The octanol-water partition coefficient values (K_{OW}) of the synthesized salts were determined
194 by the shake-flask method according to the OECD Test No. 107 guidelines (OECD, 1995b).
195 Measurements were performed using mutually saturated distilled water and 1-octanol in a glass
196 vial containing a magnetic stir bar. The synthesized products were dissolved in distilled water
197 (or 1-octanol, depending on their water solubility) at chosen concentration corresponding to the
198 values adopted for the phytotoxicity and aquatic toxicity tests (1 g per 1 dm^3 of octanol or
199 water), and then proper amounts of the second solvent (octanol or water) were added. Two
200 duplicate runs were also carried out with different solvent ratios: 2 cm^3 of 1-octanol and 4 cm^3
201 of water (2:1), 4 cm^3 of 1-octanol and 4 cm^3 of water (1:1) and 8 cm^3 of 1-octanol and 4 cm^3 of
202 water (1:2). All vials have been shaken at constant temperature of 25 °C. All obtained samples
203 were subsequently centrifuged after 24 h and the aqueous and octanolic phases were collected
204 with a syringe. The concentrations of compounds in water and in 1-octanol were determined
205 spectrophotometrically using a VWR® UV-5300PC spectrophotometer (VWR International,
206 Belgium) based on calibration curves made previously (at λ_{max} = 235 nm for aqueous solutions,
207 236 nm for 1-octanolic solutions). Two repetitions of each measurement were performed in a
208 specific solvent ratio (1:1, 1:2 and 2:1). Finally, the log K_{OW} was calculated as the average of 6
209 results collected for each compound.
210
211

212 **2.6. Antimicrobial activity**

213 The antimicrobial activity of the tested compounds was determined in relation to Gram-positive
214 bacteria: *Staphylococcus aureus* ATCC 33862, *Enterococcus faecalis* ATCC 19433, *Listeria*

monocytogenes ATCC 19111, *Micrococcus luteus* ATCC 4698, Gram-negative bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Serratia marcescens* ATCC 8100, *Moraxella catarrhalis* ATCC 25238, yeasts *Candida albicans* ATCC 10231, *Rhodotorula mucilaginosa* (PUEB collection) and filamentous fungi *Fusarium graminearum* (PUEB collection). Minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) of the tested substances was determined using the microdilution method based on methodology described previously (Kaczmarek et al., 2021) with some modifications. First, a series of two-fold dilutions of the tested substances in the concentration range of 0.48–1000 mg dm^{-3} were prepared in 96-well microplates in MH broth (for *S. aureus*, *P. aeruginosa* and *E. coli*), TSB (for *S. marcescens*, *M. catarrhalis* and *M. luteus*), BHI (for *L. monocytogenes* and *E. faecalis*), YPD (for *C. albicans* and *R. mucilaginosa*) or PDB (for *F. graminearum*). Then, suspensions prepared from 24-hour cultures of indicator microorganisms in broth media at a final concentration of 10^5 CFU cm^{-3}, were inoculated into the microplates with diluted substances. Incubation was carried out for 24 hours at 30 or 37 °C, depending on the indicator microorganism. The inoculate of *F. graminearum* was prepared in liquid PDB medium from a fresh culture, setting the final spore concentration at 10^4 CFU cm^{-3}, introduced into the prepared microplates and incubated for 5–7 days at 25 °C. After incubation the optical density of microorganisms culturewas determined at 600 nm using BioTek Epoch 2 microplate reader (Agilent, USA). The results are expressed as the average of three replicates. The MIC value was defined as the concentration of tested substances, inhibiting the growth of the microorganism by at least 90%. MBC/MFC values were determined by spot inoculation of 10 mm^3 of cultures from wells with no growth observed in the MIC assay on an agar medium and incubation for 24–48 h at 30 or 37 °C. The lowest concentration of the tested substances supporting no microorganisms growth was defined as the MBC/MFC. In case of *F. graminearum* the MIC and MFC value was evaluated by spotting of 100 mm^3 of cultures from

wells on PDA and incubation for 5–7 days at 25 °C. The lowest concentration of the tested substances supporting no fungal growth was defined as the MIC and MFC. The tested compounds were applied in the form of aqueous solutions at the defined concentration, with the exception of 1-alkylquininium bromides and free base quinine, which were applied in the form of solutions in water-DMSO mixture with volumetric ratio of 7:3. Water-DMSO mixture was used as a control.

2.7. Biopesticidal activity

The bioassay experiments were conducted using adult granary weevil beetles (*Sitophilus granarius* L.), larvae of confused flour beetle (*Tribolium confusum* Duv.) and larvae of khapra beetle (*Trogoderma granarium* Ev.). Insects were grown on a wheat grain or whole-wheat meal diet in laboratory colonies, which were maintained at 26 ± 1 °C and 60 ± 5% relative humidity. Choice and no-choice tests for insect feeding were conducted following a previously described procedure (Klejdysz et al., 2016). Wheat wafer discs (1 cm in diameter and 1 mm thick) were saturated by dipping them either in methanol only (control) or in a methanolic solution of the studied compounds (1%) to be tested. After evaporation of the solvent (30 min of air-drying), the wafers were weighed and offered to the insects in plastic boxes as the sole food source for 5 days. The feeding of insects was recorded under three sets of conditions: (1) on two control discs (CC), (2) on a choice between one treated disc (T) and one control disc (C; choice test), and (3) on two treated discs (TT; no-choice test). Each of the three experiments was repeated five times with 3 adult beetles (*S. granarius*), or 10 larvae (*T. confusum* or *T. granarium*). The number of insects depended on the intensity of their food consumption. The adults used for the experiments were unsexed and 7–10 days old. After 5 days, the discs were reweighed, and the average weight of eaten food was calculated as previously described (Klejdysz et al., 2016). The total deterrence coefficient classifies the total activity of the compounds tested according to the following criteria: 200–151, very good; 150–101, good; 100–51, medium; 50–0, weak;

and <0, attractant. The data were statistically analyzed by means of one-way ANOVA. In the cases where the ANOVA results were statistically significant at the 5% probability level, Tukey's test was performed.

2.8. Influence on germination and early development of terrestial plants

Phytotoxicity tests of the obtained salts were carried out in accordance with OECD 208 guidelines (OECD, 2006), using Phytotoxkit^{TM} plastic containers (MicroBioTests, Belgium). Two species of plants – dicotyledonous (white mustard, *Sinapis alba*) and monocotyledonous (sorghum, *Sorghum bicolor*) were used as test organisms. The designated wells in plastic containers were filled with 90 cm^3 of certified, OECD-compliant dry soil (MicroBioTests, Belgium) and then the soil was saturated with 25 cm^{-3} of aqueous solution of the analyzed compounds. Two concentrations of test solutions were selected: 1 g dm^{-3} and 10 g dm^{-3}. For the compounds insoluble in water at 10 g dm^{-3} only the test at 1 g dm^{-3} was performed, and in the case of 1-dodecylquininium bromide, which was insoluble in water at 1 g dm^{-3}, the experiment was performed using test solutions with lower concentration (0.1 g dm^{-3}). Pure deionized water was used to saturate the soil in the control sample. Subsequently, a sheet of absorbent paper was placed on the saturated soil, and 10 seeds of the selected plant were placed with 1-cm spaces in a single row. Samples were incubated in vertical position, at 26 ±1 °C for 96 hours and the number of germinating seeds in each sample was checked every 24 hours. After the end of the experiment, photos showing plant development were taken and length of roots and shoots of the young plants was measured using ImageJ software. The average growth of the root and stem was calculated in relation to the results recorded for the control sample.

2.9. Aquatic toxicity studies

Three aquatic organisms were chosen to assess the acute toxicity of the synthesized compound: freshwater green alga *Chlorella vulgaris* and a crustacean *Daphnia magna*. In addition, chronic toxicity of a selected compound was assessed for *D. magna* in a reproduction test.

2.9.1. *Acute toxicity toward algae*

The experiments were carried out on model *C. vulgaris* (SAG 211-11b) cells obtained from Culture Collection of Algae at Göttingen University (Germany) according to the methodology described in OECD 201 Guidelines (OECD, 2011). Algae were cultivated in OECD TG medium prepared as described in the Annex 3 of the OECD 201 Guidelines in sterile conditions. To accomodate *C. vulgaris* to test conditions, algae cells have been cultured in OECD medium for 4 days before tests. The inoculum was continuously aerated with sterile air and illuminated with magenta (red + blue) LED grow light with the illuminance of 5500 ±800 lx. Before tests, the inoculum was diluted with pure medium until its absorbance of monochromatic light (688 nm) was in the range of 0.05–0.08 at 10 cm path. To obtain the desired range of concentrations, 19 cm^3 portions of diluted inoculum were mixed with 1 cm^3 of the prepared toxicant solution in OECD medium. In the case of control samples, 1 cm^3 of pure OECD medium was added. The selected compounds were tested in triplicates at a series of geometrically decreasing concentrations: 1000, 100, 10, 1, and 0.1 mg dm^{-3}. For 1-dodecylquininium bromide, due to its low solubility in water and expected high toxicity, a different series was used: 10, 1, 0.1, 0.01, and 0.001 mg dm^{-3}. The absorbance at 688 nm was tested for each sample using 10 cm-long glass cuvettes, and the measurements were conducted at the start of the experiment and after 72 hours. The contents of all test vessels were mixed and illuminated continuously during the test, and their position was randomized to achieve similar levels of illumination for each sample throughout the experiment. The tests were conducted at 22–23 °C. The rate of algae growth inhibition was calculated according to the following equation:

$$\mu = \frac{\ln(\mathrm{OD_{t_2}}) - \ln(\mathrm{OD_{t_1}})}{\mathrm{t_2} - \mathrm{t_1}} \quad (1)$$

where OD_{t_2} and OD_{t_1} are the absorbances of the tested solution at the start (t_1) and at the end (t_2) of the experiment, respectively. Growth rate inhibition was then calculated in relation to the control mean:

$$I_r = 100 \cdot \left(1 - \frac{\mu_{sample}}{\mu_{control}}\right) \quad (2)$$

The EC_{50} range for each of the toxicants tested was determined based on the relationship between its concentration and the calculated I_r value.

2.9.2. Acute toxicity toward D. magna

Freshwater *D. magna* organisms no older than 24 hours were cultured at the Poznan University of Technology in Elendt M4 medium prepared as described in the Annex 3 of the OECD 202 Guidelines (OECD, 2004) and used as test organisms according to the methodology compliant with the OECD 202 Guidelines and ISO 6341 standards. The multi-well Daphtoxkit F plates for the tests were purchased from MicroBioTests Inc. (Gent, Belgium). The Elendt M4 medium was aerated for 1 hour before the tests and the organisms had access to food (*C. vulgaris* suspension) before the start of the test. In the next step, 10 cm^{-3} aliquots of the solution of a given compound in Elendt M4 medium at the specified concentrations: 1000, 100, 10, 1, and 0.1 mg dm^{-3} (with the exception of 1-dodecylquininium bromide, which was tested at 100, 10, 1, 0.1, and 0.01 mg dm^{-3} concentrations) were introduced into each of the 5 cells in a given row of the Daphtoxkit F plate. In the case of the control samples, pure medium without the addition of toxicant was used. Next, no less than 20 daphnia were placed in the first column of cells. Then, from each of the first cells in the first column, 20 daphnia were taken out and divided so that there were 4 cells in each row containing 5 specimens in 10 cm^3 of toxicant solution of the same concentration. Then, the plate was covered with parafilm and closed with a plastic cap. The prepared kits were incubated at 20 °C without light. Number of motionless organisms were counted after 24 h and 48 h. Immobilization rate was then calculated for each concentration in relation to the number of organisms at the start of the test:

$$Im = 100 \cdot \left(1 - \frac{Mob_{t_2}}{Mob_{t_1}}\right) \quad (3)$$

where Mob_{t_2} and Mob_{t_1} are the numbers of the mobile test organisms at the start (t_1) and at the end (t_2) of the experiment, respectively. Next, the dependence between effect and the concentration of the tested compounds was plotted, and on this basis, an EC_{50} range was determined.

2.9.3. Chronic toxicity toward D. magna

For chronic toxicity testing, *D. magna* organisms from the same culture as the individuals used for acute toxicity testing were used. The experiment was carried out according to the semi-static test methodology described in the OECD 211 Guidelines (OECD, 2012). The solutions of a selected compound in non-aerated Elendt M4 medium at sub-lethal concentrations (determined based on the results of the acute toxicity test) were placed in 5 glass beakers for each concentration. Pure medium was used for control samples. In the next step, a single female *D. magna* organism no older than 24 hours was placed in each vessel and the vessels were illuminated with white LED light with the illuminance of 1200 ±600 lx and 16L:8D photoperiod. The range finding experiment was conducted for 21 days. The test medium was renewed 3 times a week, and the organisms were fed living algal cells (*C. vulgaris*) daily. Following the appearance of first brood, living offspring was counted and removed daily from the vessels. The parent animals which died accidentally or inadvertently were excluded from the test. At the end of the experiment, the number of living offspring produced per surviving parental animal was counted and the effect of each concentration of the toxicant on *D. magna* reproduction was calculated and expressed in relation to the results recorded for the controls.

3. Results and discussion

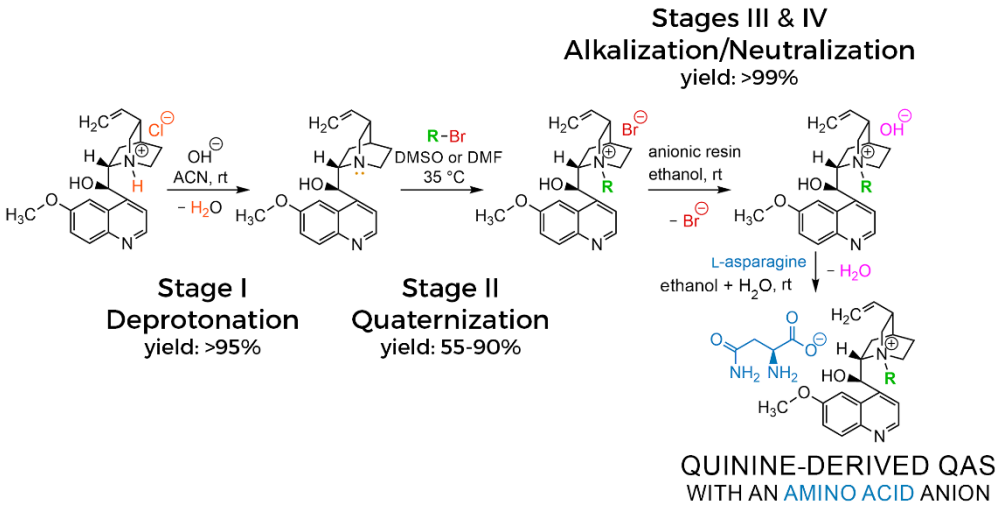
3.1. Synthesis

Currently, the design of methods for the efficient synthesis of new biologically active substances poses a challenge due to the need to adapt the developed method to the principles of green chemistry and sustainable development and prevent waste production, while maintaining the cost-effectiveness of the process, the possibility of scale-up and the possibility of obtaining the desired substances of high purity. The significant scale of this problem is illustrated by the amount of the environmental factor (E-factor, *i.e.*, the ratio of the mass of waste per mass of product). Typical values of this indicator for the processes of obtaining pharmaceuticals and fine chemicals are in the range from 5 to 100 (Sheldon, 2017), which shows the problem associated with the significant amount of waste generated by the above-mentioned chemical industries. In the course of multistage synthesis of compounds with often complex chemical structures, technological problems also cause side reactions that result in impurities that are difficult to separate without chromatographic methods.

The above problem occurs, among others, in the case of the alkylation of quinine, which degrades at elevated temperatures with the formation of numerous derivatives with similar chemical structures (Dawidowicz et al., 2018). In our past research, we proposed a favorable method for highly efficient (95% yield) conversion of technical quinine hydrochloride extracted from natural raw material (cinchona bark) into quinine free base (Pernak and Rzemieniecki, 2020), followed by efficient alkylation of quinine using 1-bromoalkanes and DMSO as solvent at 35 °C to reduce the amount of degradation products of the alkaloid (Pernak et al., 2020). A thorough three-step purification of the crude product yielded QASs with 1-alkylquinium cation in satisfactory yields (>80%) (Scheme 1).

We applied an analogous method to obtain 1-(2-hydroxyethyl)quininium bromide, which to our knowledge has not yet been described previously. However, the introduction of a hydroxyl moiety into the substituent significantly altered the properties of the finished product, thus it was necessary to modify and develop a new method for purifying the reaction mixture to

388 remove the resulting quinine degradation products. In the first stage of purification, the post-
389 reaction mixture was slowly introduced into the ethyl acetate, which acted as an anti-solvent.
390 The precipitate was subsequently recrystallized twice from a mixture of methanol and acetone.
391 Since the yield of the synthesis and purification process of 1-(2-hydroxyethyl)quininium
392 bromide was relatively low (56%), an attempt was made to select more favorable synthesis
393 conditions. When the reaction environment was changed from DMSO to a solvent with similar
394 physicochemical properties – DMF – an increase in total process yield was achieved (up to
395 73%), most likely due to the precipitation of a larger amount of the main product in the first
396 purification step. At the same time, it should be noted that while the use of DMF provides
397 significantly better process efficiency and translates into a reduction in the amount of waste
398 generated per product mass output, this solvent is considered as potential carcinogen and long-
399 term exposure results in harmful effects to human health (Luo et al., 2001). Therefore, further
400 research is warranted to develop more efficient and safer methods for the synthesis and
401 purification of quinine derivatives with a 2-hydroxyethyl substituent. However, the solvents
402 used in the course of purification of 1-(2-hydroxyethyl)quininium bromide belong to the group
403 of media recommended by the CHEM21 solvent selection guide, and moreover, DMSO (unlike
404 DMF) is not considered a hazardous solvent (Prat et al., 2016). In addition, the solvents can be
405 easily recovered and reused in the same process. The results of the syntheses and the basic
406 properties of quinine-based quaternary bromides with butyl (**1Br**), octyl (**2Br**), dodecyl (**3Br**),
407 and 2-hydroxyethyl (**4Br**) are summarized in Table 1.
408



Scheme 1. Process of obtaining quinine-derived QASs with an amino acid anion.

In the next step, the bromide anion was exchanged for the natural anion of a proteinogenic amino acid: L-asparagine or L-alanine. The bromide anion is characterized by limited acute toxicity (van Leeuwen et al., 1987), however, in elevated concentrations it exhibits neurotoxic effects (Thornton and Haws, 2020), and also affects iodine metabolism in the body (Pavelka, 2009). According to the 4th principle of green chemistry ("Designing safer chemicals"), it is reasonable to replace it with anions characterized by the absence of toxic effects on the human body (Garlick, 2004). To incorporate the amino acid anion into quinine-based QASs, the previously described method of converting 1-alkylquininium or 1-(2-hydroxyethyl)quininium bromides into the corresponding quaternary ammonium hydroxides using anionic ion exchange resin in ethanol of natural origin was used. The resulting hydroxides were then neutralized with the appropriate anion source to yield the desired products and water (Pernak et al., 2020). The method is sustainable and conforms to the principles of green chemistry, and its main advantages include low energy input (the process is conducted at atmospheric pressure at room temperature), very high yields (>99%), no contamination of the final product with halides, and the use of safe reaction media that can be easily regenerated and reused. The only by-products are the water formed during neutralization (Scheme 1) and the inorganic salt with a bromide anion obtained after regeneration of the ion exchange resin.

Due to the insolubility of the zwitterionic L-asparagine and L-alanine in ethanol, the amino acids were added in the form of an aqueous solution. After evaporation of the solvents and drying of the residue, quinine-derived QASs with either L-asparaginate or L-alaninate anion were obtained (Table 1). In contrast to **1Br**–**4Br**, which were white crystalline solids, the obtained QASs with the amino acid anions were amorphous solids with glassy appearance at room temperature. According to the results of DSC analyses, new compounds (**4Br**, **1Asp**–**4Asp**, **1Ala**–**4Ala**) did not exhibit first- or second-order phase transitions in the measurement range from -80 to 120 °C, although at temperatures exceeding 128 °C, the release of gaseous thermal decomposition products could be observed for QASs containing the amino acid anion. The exact results of the above-mentioned analyses are summarized in Table A.2 (Supplementary Data).

Taking into account the need to assess the environmental impact of the new methods of chemical synthesis, we calculated Green Chemistry Metrics (GCM) values for the quinine quaternization (**1Br**–**4Br**) and for the anion exchange reaction from bromide to amino acid anion (**1Asp**–**4Asp**, **1Ala**–**4Ala**). The results are summarized in Table 1. Despite the 100% atom economy of the quinine conversion reaction to **1Br**–**4Br**, the need for additional media in the course of purification of the finished salt results in an increase of the E-Factor values (8.58–11.13). These values are within the range typical for industrial synthesis of fine chemicals (Sheldon, 2017). The lower efficiency of the **4Br** synthesis process results in higher E-Factor values compared to the more efficient syntheses of **1Br**–**3Br** compounds. The E-Factors for the aforementioned reactions may be lowered by improving the selectivity of the process to lower the amount of by-products requires to be separated.

Table 1. Synthesized quinine-derived QASs with bromide (**1Br**–**4Br**) and amino acid (**1Asp**–**4Asp**, **1Ala**–**4Ala**) anions

Compound	**R**	Anion	Atom economy[a]	Yield[a] [%]	E-Factor[a] [kg kg^{-1}]	Appearance at 25 °C	Water content	Water solubility[b]

			[%]				[%]	[g dm^{-3}]
1Br	C_4H_9	bromide	100	84	8.58	crystalline solid	0.77	9.3 ±0.2
2Br	C_8H_{17}	bromide	100	83	8.68	crystalline solid	0.20	2.3 ±0.1
3Br	$C_{12}H_{25}$	bromide	100	82	8.81	crystalline solid	0.75	0.1 ±0.004
4Br	C_2H_4OH	bromide	100	56[c] 73[d]	14.51[c] 11.13[d]	crystalline solid	1.00	>250
1Asp	C_4H_9	L-asparaginate	84	>99	1.94	amorphous solid	2.09	>1000
2Asp	C_8H_{17}	L-asparaginate	85	>99	1.92	amorphous solid	1.83	>1000
3Asp	$C_{12}H_{25}$	L-asparaginate	86	>99	1.90	amorphous solid	1.53	>1000
4Asp	C_2H_4OH	L-asparaginate	84	>99	1.95	amorphous solid	-[e]	>1000
1Ala	C_4H_9	L-alaninate	83	>99	1.97	amorphous solid	2.66	>1000
2Ala	C_8H_{17}	L-alaninate	84	>99	1.94	amorphous solid	1.44	>1000
3Ala	$C_{12}H_{25}$	L-alaninate	86	>99	1.91	amorphous solid	2.69	>500
4Ala	C_2H_4OH	L-alaninate	82	>99	1.97	amorphous solid	-[e]	>1000

[a] partial value for a given synthesis stage, [b] OECD 105, [c] synthesis conducted in DMSO, [d] synthesis conducted in DMF, [e] highly hygroscopic compound

The anion exchange process is characterized by more favorable GCM values compared to the syntheses of **1Br**–**4Br**. Despite the lower atom economy (82–86%) resulting from the nature of exchange reaction, the total alkalization and neutralization efficiency is almost equal to the theoretical one (>99%), which translates into a reduction in waste generation and lowers the negative environmental impact. This is indicated by E-Factor values ranging from 1.90 to 1.97, more than twice as low as the lower end of the E-Factor range characteristic of fine chemicals synthesis (5–50).

The new amino acid anion QASs (**1Asp**–**4Asp**, **1Ala**–**4Ala**) are characterized by higher water content (from 1.44% for **2Ala** to 2.69% for **3Ala**) compared to the values determined for bromides (0.2–1.0%). This is a typical phenomenon for QASs with the amino acid anion

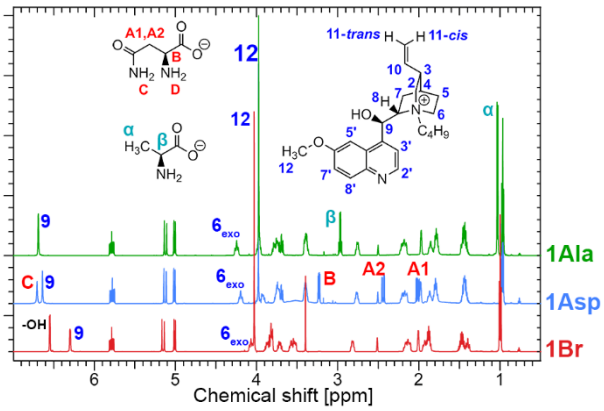
(Kaczmarek et al., 2022). More thorough drying would require a dry environment in a glovebox and the use of multi-stage azeotropic distillation. For **4Asp** and **4Ala** salts with a 2-hydroxyethyl substituent, it was impossible to determine the exact water content due to the high hygroscopicity of these compounds; the water content values recorded during the analyses (9.6 ±0.24% for **4Asp** and 8.2 ±0.15% for **4Ala**) were characterized by two orders of magnitude greater measurement error than for the other compounds tested – the error value for other compounds ranged from 0.002 to 0.005%.

Low solubility in water may be the reason for the low bioavailability of biologically active substances (Bhalani et al., 2022). Quinine in its free base form is a poorly water-soluble substance (0.05 g dm^{-3}) (Yalkowsky et al., 2010), and the solubility of QASs **1Br**–**4Br** is strongly correlated with the length of the alkyl substituent: **1Br** with a short (butyl) substituent (9.3 g dm^{-3}, Table 1) is dissolved more than 70 times better than its analog **3Br** with a long (dodecyl) substituent (0.1 g dm^{-3}). In contrast, the presence of a hydroxyl group in the short substituent of **4Br** increases the solubility in water to more than 250 g dm^{-3}. It should be noted here that the chemical structure of the anion has an even more significant effect on the solubility of quinine-based QASs in water, and the replacement of the bromide anion with either an L-asparaginate or L-alaninate anion results in ready solubility (more than 1000 g dm^{-3}), with the exception of **3Ala**, which still showed very good solubility (more than 500 g dm^{-3}). Given the relationship between the solubility of active substances in water and their bioavailability (Bhalani et al., 2022), combining quaternary cations with low affinity for water with amino acid anions may result in increased bioavailability of new QASs, although more thorough studies are required to confirm the validity of utilizing such a strategy.

3.2. Spectral analysis

For all new quinine-based QASs (**4Br**, **1Asp**–**4Asp**, **1Ala**–**4Ala**), spectral analysis was performed using FT-IR, ^{1}H NMR and ^{13}C NMR techniques. The results of the spectral characterization of the products are shown in Supplementary Data (Figs. A.1–A.27). In the ^{1}H NMR spectrum of **4Br**, a triplet occurred at a chemical shift of 5.59 ppm, which was not visible in the spectra of 1-alkylquininium bromides (**1Br**–**3Br**). This signal was identified as originating from the hydroxyl group in the substituent in **4Br**. Moreover, in the ^{13}C NMR spectrum of the same compound, a signal at 61.8 ppm can be observed, originating from the –CH_2– group in the immediate vicinity of the hydroxyl group. Both these signals confirm the successful 2-hydroxyethylation process and the correct chemical structure of **4Br**. A comparison of the ^{1}H NMR spectra of **4Br** and 1-ethylquininium bromide described in the literature (Pernak et al., 2020) is shown in Fig. A.28 (Supplementary Data).

The successful anion exchange for either the L-asparaginate anion (**1Asp**–**4Asp**) or the L-alaninate anion (**1Ala**–**4Ala**) was also confirmed. The appearance of characteristic signals from the newly introduced amino acid anion was observed, and the integration of the new signals in the ^{1}H NMR spectra indicated an equimolar ratio of cation and anion in the new QASs. Characteristic signals from the anions in the proton spectra of **1Br**, **1Asp** and **1Ala** are shown in Fig. 1. It should be noted that the hydrogen atoms in the –CH_2– group in L-asparagine differ in terms of chemical environment due to the chirality of the amino acid (Wishart et al., 2009). It should be noted that the diastereotopic hydrogen atoms in the CH_2 group in the L-asparaginate anion are characterized by different chemical environment due to the chirality of the anion. The difference between the chemical shifts of those atoms reaches approximately 0.4 ppm (see signals A1 and A2 in Fig. 1) in the spectra of the obtained L-asparaginates (**1Asp**–**4Asp**).



511

512 **Figure 1.** Comparison of **1Br**, **1Asp** and **1Ala** ^{1}H NMR spectra (0.5–7.0 ppm)

513

514 All characteristic signals originating from the quinine-based cations, which were present in the
515 NMR and FT-IR spectra of **1Br**–**4Br**, occurred in the spectra of the L-asparaginate and L-
516 alaninate salts as well, which indicates that no significant changes in the structure of the cation
517 occurred as a result of the ion exchange reaction. The triplet (0.77–0.78 ppm) occurring at the
518 baseline of the ^{1}H NMR spectra, as well as the corresponding signal at approximately 10 ppm
519 in the ^{13}C NMR spectra, originate from the –CH_3 group in the ethyl substituent of
520 dihydroquinine, a minor residue of which is present in quinine extracted from natural sources.
521 It should be noted that methods for complete removal of dihydroquinine prior to derivatization
522 of the alkaloid are known, although they require expensive and waste-generating preparative
523 techniques (e.g., HPLC or UPLC with special stationary phases (Borges, 2015)). No significant
524 signals from other organic impurities were observed in the NMR spectra. Nevertheless, it was
525 noted that the introduction of proteinogenic amino acid anions notably alters the chemical shifts
526 of hydrogen atoms in 1-alkylquininium or 1-(2-hydroxyethyl)quininium cations, particularly in
527 the quinuclidine group, compared to the spectra of the corresponding bromides (Fig. 1).
528 To assess the chemical stability of new QASs, additional ^{1}H and ^{13}C NMR spectra of 2
529 exemplary salts: **1Asp** and **1Ala** were acquired 5 and 7 months following their synthesis,
530 respectively. From the comparison of the obtained spectra (Fig. A.29, Supplementary Data), it

can be observed that the same signals originating from cations and anions of QASs are present in the spectra obtained both immediately after synthesis and after long-term storage. This result implies that the new QASs can be stored in pure form for at least 5 months without the appearance of any decomposition products in noticeable amounts.

3.3. Octanol-water partition coefficient

As a measure of a lipophilicity, the octanol-water partition coefficient (K_{OW}) makes it possible to estimate the interaction of a chemical compound with a variety of biological interfaces. For example, there is a correlation between the values of log K_{OW} and skin permeability coefficient (K_p) – a parameter describing the ability of an active ingredient to penetrate the skin, which is important for the design of new active pharmaceutical ingredients (Olejniczak et al., 2024). It is also possible to estimate the bioavailability of new pesticides based on their log K_{OW} value (Akamatsu, 2011). In addition, log K_{OW} is useful in predicting the environmental fate of new compounds, for example, by estimating the probability of bioaccumulation of the analyzed substance in tissues or soil. Determination of log K_{OW} values is required by REACH regulations, and a value of this parameter higher than 4 indicates a high probability of bioaccumulation (The European Parliament and the Council, 2006).

The new quinine-based QASs are characterized by moderate hydrophilicity or lipophilicity, and their log K_{OW} values range from -1.34 (**4Ala**) to 1.84 (**3Br**). The dependence of the log K_{OW} values on the anion and the substituent in the cation is shown in Fig. 2, while the exact values are summarized in Table A.3 (Supplementary Data).

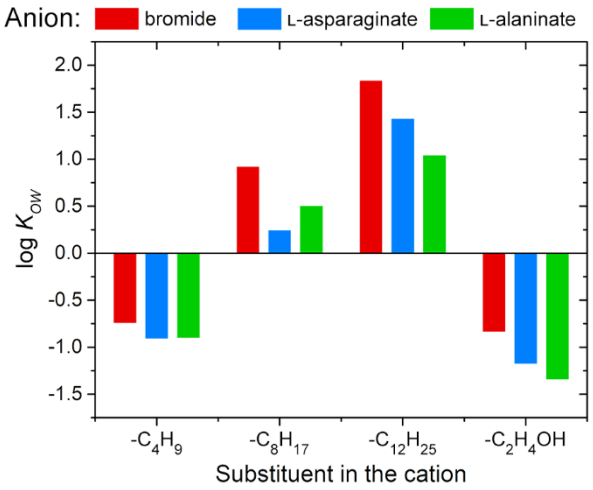


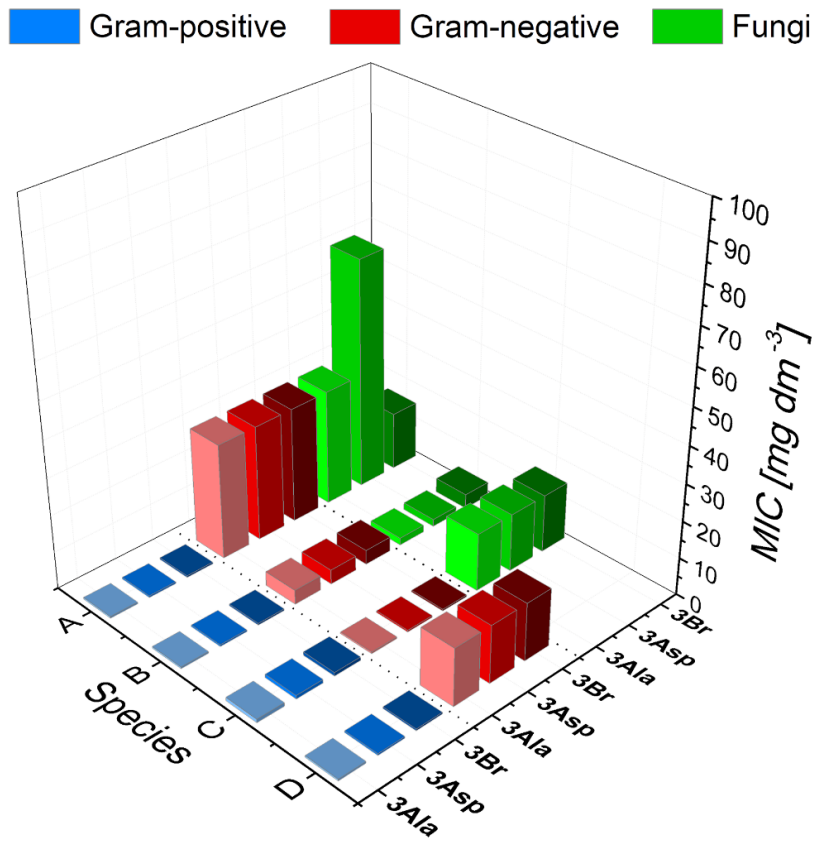
Figure 2. Octanol–water partition coefficient determined for the quinine-based QASs

Expectedly, the length of the alkyl substituent in the quinine-based cation is the most important parameter affecting the log K_{OW}. The presence of a long dodecyl substituent is the main reason for the increased lipophilicity of compounds **3Br**, **3Asp**, and **3Ala**, even despite the presence of a highly hydrophilic amino acid-derived anion in the case of the latter two. In contrast, compounds with a short butyl substituent show significant hydrophilicity (log K_{OW} < -0.5). The introduction of a hydroxyl group into the short alkyl chain does not increase the hydrophilicity of **4Br** compared to **1Br**, although both **4Asp** and **4Ala** are characterized by lower log K_{OW} values (-1.18 and -1.34, respectively) compared to **1Asp** (-0.91) and **1Ala** (-0.90). In all analyzed cases, the exchange of the bromide anion for the proteinogenic amino acid anion resulted in a significant reduction in log K_{OW}. It should also be noted that all quinine-based QASs have significantly higher hydrophilicity compared to quinine free base (log K_{OW} = 3.44) (Hansch et al., 1995).

3.4. Antimicrobial activity

In the context of acquiring multidirectional resistance by strains of pathogenic microorganisms against commonly used disinfectants (*e.g.*, benzalkonium chloride) (Maillard, 2022; Merchel

572 Piovesan Pereira and Tagkopoulos, 2019; Nordholt et al., 2021), as well as the risks associated
573 with the penetration of synthetic cationic surfactants into the environment and their high
574 toxicity toward aquatic organisms (Tezel and Pavlostathis, 2015), it becomes necessary to
575 search for new, safer active ingredients with potent antimicrobial activity among compounds
576 of natural origin. Therefore, the biological activity of the obtained quinine-based QASs against
577 various microbes that pose a serious threat to human health, especially to immunocompromised
578 patients in hospital settings, was investigated. The microorganisms tested included pathogenic
579 foodborne bacteria (*L. monocytogenes*) and microbes responsible for opportunistic infections
580 with potentially serious consequences (*e.g.*, *S. aureus*, *P. aeruginosa*, *E. coli, C. albicans*).
581 Moreover, the influence of new QASs toward *F. graminearum*, a pathogen of numerous
582 important crop plants (*e.g.*, barley or rice) at all stages of their development was determined.
583 In addition to salts with bromide or amino acid anion, the antimicrobial activity of quinine in
584 the form of hydrochloride and free base was also examined. All tested microorganisms were
585 susceptible to QAS-type disinfectants and none of the bacterial strains selected contained any
586 genes determining resistance to the antimicrobial activity of this group of compounds (*e.g.*,
587 qacE or qacEΔ1). Determined values of minimum inhibitory concentration (MIC), as well as
588 minimum bactericidal/fungicidal concentration (MBC/MFC) are summarized in Table A.4
589 (Supplementary Data).
590



591

592 **Figure 3.** Comparison of MICs of quinine-based QASs with a dodecyl substituent in the cation
593 (**3Br**, **3Asp**, **3Ala**). For Gram-positive bacteria: A – *L. monocytogenes*, B – *M. luteus*, C – *E.*
594 *faecalis*, D – *S. aureus*; for Gram-negative bacteria: A – *P. aeruginosa*, B – *E. coli*, C – *M.*
595 *catarrhalis*, D – *S. marcescens*; for fungi: A – *C. albicans*, B – *R. mucilaginosa*, C – *F.*
596 *graminearum*

597

598 The results indicate that **3Br**, **3Asp** and **3Ala** comprising a dodecyl substituent showed the
599 highest antimicrobial activity. They were characterized by MIC values of <1 mg dm^{-3} for the
600 tested Gram-positive bacteria and Gram-negative *M. catarrhalis*, while MIC determined for the
601 other Gram-negative bacteria (*P. aeruginosa*, *E. coli*, *S. marcescens*) and the fungi *R.*
602 *mucilaginosa* and *F. graminearum* ranged from 3.9 to 31.25 mg dm^{-3} (Fig. 3). MBC/MFC
603 values against the mentioned microorganisms were equal or twice as high (<0.48–62.5 mg dm^{-3}).
604 These results indicate that **3Br**, **3Asp** and **3Ala** demonstrate activity against pathogens at the

level of benzalkonium chloride, commonly applied as a potent disinfectant (Rzemieniecki et al., 2019). Relatively low susceptibility to the tested dodecyl substituted QASs was noted only in the case of *C. albicans* (MIC 15.62–62.5 mg dm^{-3}, MFC 62.5–250 mg dm^{-3}).

QASs with a short substituent (butyl or 2-hydroxyethyl) in the quinine-derived cation, on the other hand, exhibited low antagonistic activity towards the tested microorganisms (MIC and MBC/MFC up to above 1000 mg dm^{-3}), comparable to that of pure quinine. On the other hand, **2Br**, **2Asp** and **2Ala** comprising a medium-length substituent (octyl) in the cation showed noticeable levels of activity toward most of the microorganisms tested, although they were less active compared to their counterparts with a dodecyl substituent.

Based on the obtained results, it can be concluded that the length of the alkyl substituent and the associated amphiphilicity of the quinine-based cation is the main factor affecting the level of antibacterial activity. Hence, it can be stated that obtained compounds most likely exhibit antimicrobial activity according to the same mechanism as other amphiphilic QASs (Tischer et al., 2012) – this is also indicated by the higher susceptibility of Gram-positive bacteria to the tested compounds compared to other groups of microorganisms. MIC and MBC/MFC values were in the vast majority of cases independent of the chemical structure of the anion. Only in the case of *E. faecalis* **2Asp** and **2Ala** were characterized by significantly reduced activity compared to **2Br**, but such differences were not observed between **3Asp** or **3Ala**, and **3Br**. Thus, it can be concluded that the strategy of introducing a non-toxic amino acid anion into QASs does not cause significant changes in the antimicrobial activity of quinine-based salts.

3.5. Biopesticidal activity

Taking into account recent reports on the antifeedant activity of QASs comprising anions derived from amino acids (Kaczmarek et al., 2024, 2022) and the confirmation of the biopesticidal activity of quinine (Pernak et al., 2020), we analyzed the antifeedant properties of

the obtained bromides and amino acid salts comprising quinine-based cations toward adult forms of granary weevil (*S. granarius*) and larvae of 2 species: confused flour beetle (*T. confusum*) and khapra beetle (*T. granarium*). As in the case of antimicrobial activity tests, quinine free base and quinine hydrochloride were used as reference substances. The results of the tests are summarized in Fig. 4, while the exact values are summarized in Table A.5 (Supplementary Data).

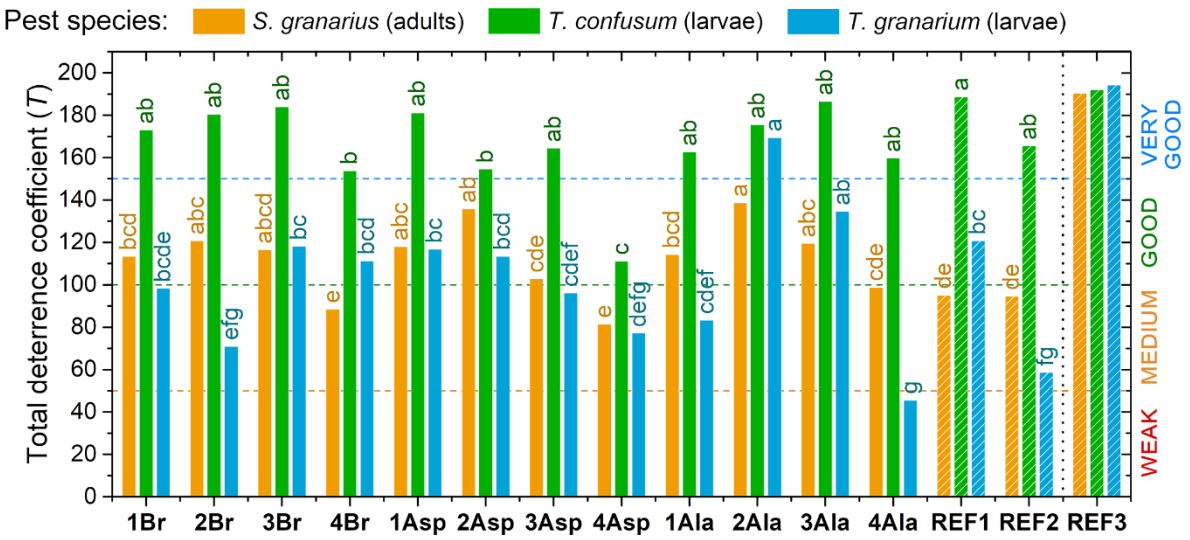


Figure 4. Antifeedant activity of the synthesized QASs toward granary weevil adults (*S. granarius*), confused flour beetle larvae (*T. confusum*), and khapra beetle larvae (*T. granarium*); **REF1** – quinine free base, **REF2** – quinine hydrochloride, **REF3** – azadirachtin, results reported previously (Łozowicka et al., 2007)

Analysis of the results showed that the majority of the tested QASs exhibited good antifeedant activity toward adult granary weevils as well as khapra beetle larvae, and very good activity toward confused flour beetle larvae. A deviation from this rule was observed only in the case of **4Br**, **4Asp** and **4Ala** containing a 2-hydroxyl substituent, for which the total deterrence coefficient *T* toward granary weevil showed values ranging from 81 to 99, comparable to the

values achieved for the reference compounds (95). **4Asp** was further characterized by significantly worse activity toward confused flour beetle larvae than the rest of the tested compounds and reference substances. Also in our previous studies, we observed that excessive hydrophilicity of the cation can negatively affect deterrent activity – choline analogs (Pernak et al., 2007) and compounds containing hydroxyl (Stachowiak et al., 2020) or ester groups (Kaczmarek et al., 2022) in the cations are generally characterized by worse *T* values compared to QASs with cations based on synthetic surfactants with long alkyl chains (Niemczak et al., 2019).

The introduction of an alkyl substituent into the quinine cation resulted in an increase in antifeedant activity against adult granary weevils compared to the reference substances; for 5 QASs (**2Br**, **1Asp**, **2Asp**, **2Ala**, **3Ala**) the difference to the references was statistically significant (Fig. 4). The length of the substituent in the 1-alkylquininium cation had no effect on the performance of the compounds with bromide anion, while in the case of L-asparaginate and L-alaninate, **2Asp** and **2Ala** containing an intermediate-length (octyl) substituent had the best effect against adult weevils (*T* equal to 136 and 139, respectively). However, these results were not significantly different from the *T* values determined for most compounds with either butyl or dodecyl chains.

The antifeedant activity of the tested compounds toward larvae of khapra beetle (*T. granarium*) was highly varied, with *T* values ranging from 45 (**4Ala**) to 169 (**2Ala**). In the case of this pest, no clear dependence of biopesticidal activity on the type of cation or anion was observed. Interestingly, an incidental, statistically significant increase in the activity was observed for **2Ala** compared to the other salts. Like in the case of *S. granarius*, many of the quinine-based QAS obtained were characterized by at least good antifeedant properties and showed significantly better activity toward khapra beetle larvae compared to quinine hydrochloride (*T* = 59). Interestingly, quinine in the form of the free base also demonstrated significantly better

activity toward khapra beetle (T = 121) compared to the hydrochloride form – such discrepancies between the selected reference substances were not observed for the other species tested.

With the exception of **4Asp**, all quinine-based QASs showed very good (T > 150) antifeedant properties toward confused flour beetle larvae regardless of the type of substituent in the cation or anion structure (Fig. 4). This is most likely due to the very strong effect of quinine structure on inhibiting the feeding of this species – the T value determined for quinine free base in confused flour beetle tests was equal to 189. Thus, it can be concluded that the activity of quinine against *T. confusum* larvae is comparable to that of azadirachtin (T = 192, see Fig. 4), a substance known for its very potent biopesticidal properties. Although quinine is characterized by potent antifeedant activity toward some mosquito species (Lukenge et al., 2023), its activity toward stored product pests is reported to be significantly lower compared to azadirachtin or neem oil formulations (Sanané et al., 2021). This is also true in the case of most of the obtained quinine-based QASs, which did not reach the effectiveness of pure azadirachtin (T = 194) to khapra beetles, and only **2Ala** (T = 169) exhibited similar antifeedant activity toward larvae of this species. In the case of adult granary weevils, the differences were even more pronounced and pure azadirachtin (T = 190) was noticeably a superior feeding deterrent than any of the quinine-based compounds tested. However, the very good antifeedant activity of quinine (in the form of the free base and hydrochloride) toward *T. confusum* larvae, that also determines high efficacy of the majority of quinine-based QASs, is a surprising result and indicates the legitimacy of further research into the use of this alkaloid and its derivatives in the protection of plants and seeds against pests. It should also be emphasized that despite the exceptional biopesticidal activity of azadirachtin to a broad spectrum of stored product pests, this substance cannot be profitably obtained by synthetic methods due to its complex chemical structure (Jauch, 2008). In addition, azadirachtin is difficult to extract in large quantities from

Azadirachta indica plant matter which contributes to its high price. Therefore, the search for feeding deterrents derived from other, more readily available natural resources remains a legitimate approach. Moreover, given the high antimicrobial activity of salts **3Br**, **3Asp**, and **3Ala** toward *F. graminearum* (a fungal pathogen of seeds and plants) and other microorganisms (see Fig. 3), there is some potential for using the quinine-based QASs with longer alkyl substituents for comprehensive biopesticidal protection of stored grain against both pest insects and harmful plant pathogens. However, further research in this area needs to be preceded by analyses of the toxicity of the new QASs, as well as their environmental fate and effects on stored grain quality.

3.6. Phytotoxicity

Both the pesticide residues in the soil (Bragança et al., 2018) and the presence of antifeedant on seeds (de Paulo Barbosa et al., 2023) may have a negative effect on plant germination and development. Therefore, the effect of quinine-based QASs and a reference substance (quinine hydrochloride) on seeds of a dicotyledonous (white mustard, *S. alba*) and a monocotyledonous plant (sorghum, *S. bicolor*) was studied at different concentrations. The effect of the application on the germination capacity of the plants is shown in Table A.6 (Supplementary Data), while Fig. 5 shows the effect of the obtained compounds on the growth of shoots and roots of white mustard. An analogous graph for sorghum (Fig. A.30) and a summary of the exact results of the phytotoxicity tests (Tables A.7 and A.8) can be found in Supplementary Data.

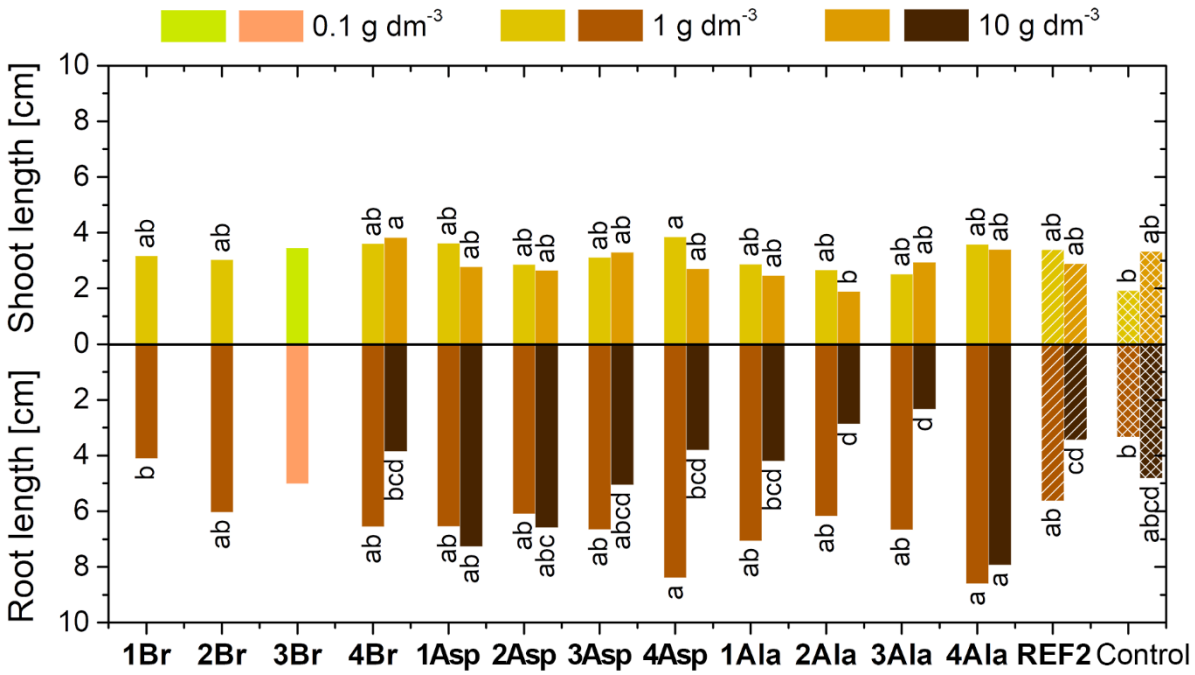


Figure 5. Effect of quinine-based QASs on the early development of white mustard (*S. alba*). **REF2** – quinine hydrochloride

Regardless of the chemical structure of the test compound, 80–100% of seeds of both plants germinated after 96 h after treatment. This range coincides with the germination capacity of untreated seeds according to the manufacturer's specifications. Thus, none of the applied QASs or the reference formulation showed significant phytotoxicity at concentrations up to 10 g dm^{-3} at the earliest stage of white mustard and sorghum development. Also, no significant germination delays were observed during the experiment compared to the control.

Analysis of young white mustard plants revealed an interesting effect of the new quinine-based QASs on the growth rate of roots and shoots (Fig. 5). At a concentration of 1 g dm^{-3}, the tested salts caused an increase in shoot length by 31–100% compared to the control, while roots were elongated by 24–158%. **4Asp** and **4Ala** had the strongest stimulating effect on root growth, and in their case the differences in white mustard root length (152% and 158%, respectively) were statistically significant compared to the control. Regarding the shoot elongation, a significant

difference to the control was also recorded for **4Asp** (100% increase). The beneficial effect of **4Asp** and **4Ala** may be due to the high hydrophilicity of these compounds (log K_{OW} < -1), which may positively influence the bioavailability of quinine-based cation in the soil. At the same time, it should be remembered that the cation and anion of QASs behave differently in the soil and their effects on plants should be considered separately (Homa et al., 2024). Given that **4Br** with the same cation stimulated root and shoot growth to a lesser extent, it can be predicted that the plant growth-stimulating effect observed for **4Asp** and **4Ala** is a product of the beneficial effect of the 1-(2-hydroxyethyl)quinium cation and the enhancement of metabolism caused by exogenous application of proteinogenic amino acids (Sowmya et al., 2023). It should also be emphasized that the growth-stimulating activity of quinine in terrestrial plants has not yet been studied in detail, and thus further research of this phenomenon is recommended.

At elevated concentrations (10 g dm^{-3}), a difference in the effect of QASs with L-asparaginate and L-alaninate anions was observed. The addition of **1Asp**–**4Asp** continued to stimulate white mustard root growth up to 79% compared to the control, although it did not stimulate shoot elongation. In contrast, for compounds **1Ala**–**3Ala** an inhibition of root (up to -43%) and shoot growth (up to -28%) was observed. Conversely, the addition of **4Ala** at 10 g dm^{-3} resulted in a 95% and 29% increase in root and stem length, respectively. The observed phenomenon can be explained by the fact that L-alanine in high concentrations can cause phytotoxic effects in some dicotyledonous plants (Alfosea-Simón et al., 2021).

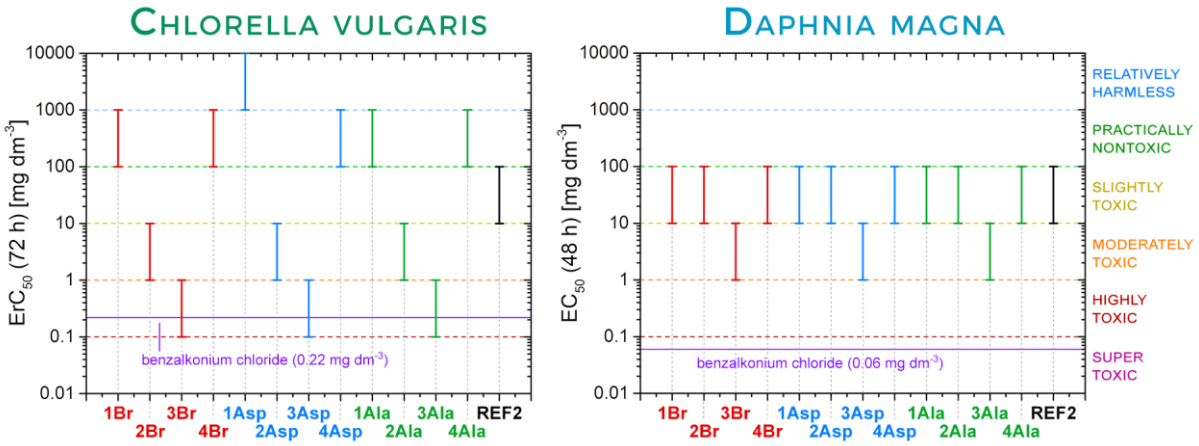
In the case of young sorghum plants, the addition of quinine hydrochloride or quinine-based QASs at a concentration of 10 g dm^{-3} or lower did not cause statistically significant changes in root or shoot length compared to the control. Thus, no phytotoxic effect of the obtained compounds was observed toward sorghum. Since growth inhibition was not observed at a statistically significant level for white mustard either, it can be concluded that application of the obtained QASs on the stored grain for pest protection will not cause phytotoxic effects.

However, more detailed studies on a wider spectrum of monocotyledonous and dicotyledonous plants are needed to assess the risk of quinine-derived biopesticides application in the agrochemical operations.

3.7. Aquatic toxicity

3.7.1. Freshwater green algae (*Chlorella vulgaris*)

Proteinogenic amino acids are relatively harmless to many groups of aquatic organisms, and their exogenous addition can have beneficial effects on algal growth. Among other things, positive effects of amino acid addition have been observed on the increase in biomass of freshwater algal cultures of *C. vulgaris* (Koochi et al., 2023). On the other hand, surface-active QASs containing long alkyl substituents are characterized by high toxicity to aquatic organisms, including microalgae (Zhu et al., 2010). This is due to the same mechanism of interaction with cell membranes, which is responsible for the antibacterial activity of these compounds (Sanches et al., 2023), as well as adverse interactions with organelles, including chloroplasts (Pawłowska et al., 2019). Given the important role of algae as an essential source of food and oxygen in aquatic biocoenoses, assessing toxicity of new chemicals to this group of organisms is important in the course of the environmental risk analysis. According to the studies we performed on *C. vulgaris*, the obtained quinine-based QASs are characterized by a diverse range of acute toxicity. The estimated ErC_{50} values ranged from 0.1 (**3Br**, **3Asp**, **3Ala**) to more than 1000 mg dm^{-3} (**1Asp**). The determined ErC_{50} ranges are summarized in Fig. 6, while the growth inhibition values of *C. vulgaris* for the corresponding toxicant concentration are summarized in Table A.9 (Supplementary Data).



782

783 **Figure 6.** 72 h ErC_{50} (the concentration of test substance which results in a 50 percent reduction
784 in growth rate) of tested QASs on green algae *C. vulgaris* and 48 h EC_{50} on freshwater
785 crustacean *D. magna*. **REF2** – quinine hydrochloride. Toxicity categories according to (El-
786 Harbawi, 2014)

787

788 As in the case of antimicrobial activity, toxicity toward algae was determined by the type of
789 substituent in the quinine-derived cation. All compounds with a dodecyl substituent (**3Br**,
790 **3Asp**, **3Ala**) exhibited the same ErC_{50} range (0.1–1 mg dm^{-3}), comparable to the toxicity toward
791 *C. vulgaris* recorded for other QASs with a dodecyl chain, such as benzalkonium chloride
792 (ErC_{50}: 0.22 mg dm^{-3}) (Zhu et al., 2010) or salts with an imidazolium cation (0.37 mg dm^{-3})
793 (Zhang et al., 2017). No differences were also noticed between the toxicity of the 3 salts with
794 an octyl substituent (**2Br**, **2Asp**, **2Ala**, ErC50: 1–10 mg dm^{-3}). It should therefore be
795 emphasized that optimization of the quinine-derived cation structure for antimicrobial activity
796 (especially in the case of **3Br**, **3Asp** and **3Ala**) also leads to a significant increase in acute
797 toxicity against *C. vulgaris*, which presumably results from the analogous mechanism of action
798 of amphiphilic QASs against cells of both pathogenic microorganisms and algae (Sanches et
799 al., 2023). Thus, this means that despite their natural origin and enhanced biocompatibility,
800 potential quinine-based quaternary disinfectants containing a long alkyl substituent should still
801 be treated as a potential pollutant, and their release into the environment and concentration in

wastewater should be closely monitored analogously to "conventional" QASs of synthetic origin (Arnold et al., 2023).

Compounds with a short butyl or 2-(hydroxyethyl) substituent were "practically nontoxic" (El-Harbawi, 2014) and exhibited ErC_{50} values exceeding 100 mg dm^{-3}. Thus, they were less toxic than quinine hydrochloride (10–100 mg dm^{-3}). Interestingly, a decrease in the toxicity against *C. vulgaris* (ErC_{50} >1000 mg dm^{-3}) to a level where the chemical can be considered "relatively harmless" (El-Harbawi, 2014) was noted for **1Asp**. Thus, it can be concluded that the introduction of an amino acid anion into the structure of QASs could reduce the toxicity of the quinine structure toward *C. vulgaris* in some cases, although it had no effect on reducing the adverse effects caused by QASs comprising a quinine-based cation with a longer alkyl substituent.

3.7.2. Freshwater crustaceans (*Daphnia magna*)

Freshwater crustaceans of the *D. magna* species are highly sensitive to the presence of a wide range of chemicals in water, including cationic surfactants. QASs of synthetic origin such as benzalkonium chloride, didecyldimethylammonium chloride or cetyltrimethylammonium bromide are characterized by acute toxicity toward *D. magna* at very low concentrations (EC_{50} <0.1 mg dm^{-3}) (Fuchsman et al., 2022; Homa et al., 2024; Nowacka et al., 2023), which qualifies them in the "super toxic" category. This forces special caution in the disposal of conventional disinfecting agents and prompts the search for active ingredients characterized by lower toxicity to aquatic animals.

The acute toxicity analysis revealed that among the obtained quinine-based QASs, compounds with cations containing either a short (**1Br**, **1Asp**, **1Ala**, **4Br**, **4Asp**, **4Ala**) or a medium-length (**2Br**, **2Asp**, **2Ala**) substituent were characterized by EC_{50} values against *D. magna* in the range of 10–100 mg dm^{-3} ("slightly toxic", Fig. 6). The harmful effect of the listed salts is presumably

due to the aquatic toxicity of quinine, since the EC_{50} range estimated for the reference compound (quinine hydrochloride) is identical. As with the toxicity analyses conducted for *C. vulgaris*, extension of the alkyl chain to 12 carbon atoms resulted in a significant increase in toxicity to crustaceans (EC_{50}: 0.1–1 mg dm^{-3}). These results indicate that **3Br**, **3Asp** and **3Ala** containing 1-dodecylquininium cation, which determines their potent antimicrobial properties are at least moderately toxic to multiple classes of freshwater organisms and should be thoroughly controlled as substances potentially hazardous to the aquatic environment in the case of their potential application. However, quinine-based QASs with dodecyl substituent were still less toxic than conventional QASs used in disinfectant formulations. The exact results of the acute toxicity tests toward *D. magna* are summarized in Table A.10 (Supplementary Data). Of all the compounds tested, **1Asp** was characterized by good or very good antifeedant activity, and its toxicity toward test organisms from a wide range of systematic groups (bacteria, fungi, terrestrial plants, freshwater algae, freshwater crustaceans) was the lowest. Due to the low value of log K_{OW} (-0.91) it was also characterized by a low risk of bioaccumulation. Therefore, an additional chronic toxicity study was conducted to determine the ability of *D. magna* to reproduce in an environment contaminated with **1Asp** at sublethal concentrations ($\leq$ 10 mg dm^{-3}). The results of the study are summarized in Fig A.31 and Table A.11 (Supplementary Data). The data obtained show that at low concentrations (0.01 mg dm^{-3} and 0.1 mg dm^{-3}), **1Asp** did not affect the reproduction of *D. magna* females, and the number of parthenogenetic offspring did not differ significantly from control organisms. Conversely, the applied QAS caused a reduction in the number of offspring by an average of 48% as well as other adverse effects (occurrence of aborted eggs and dead neonates) at a concentration of 1 mg dm^{-3}, indicating the presence of toxic effects. It should be noted that no relationship was observed between the concentration of **1Asp** and the mortality of *D. magna*; only four parent organisms (one in

851 control, one at 0.01 mg dm^{-3}, one at 1 mg dm^{-3}, and one at 10 mg dm^{-3}) died inadvertently
852 during the experiment.
853 Fascinatingly, increasing the concentration of **1Asp** to sublethal levels (10 mg dm^{-3}) did not
854 result in a further increase in toxicity toward *D. magna*, but instead caused a biostimulatory
855 effect. At the highest concentration of **1Asp** tested, the reproductive output increased by 32%
856 compared to the control, and no mortality or signs of stress were observed during the test. This
857 unexpected phenomenon may stem from compensation for the toxic effects of 1-butylquininium
858 cation due to biostimulation induced by elevated concentrations of L-asparagine in the medium.
859 Despite the lack of data on the effect of exogenous addition of L-asparagine and other on *D.*
860 *magna*, there are reports that indicate a beneficial effect of proteinogenic amino acid addition
861 on the reproduction of the genetically similar *Daphnia pulex* (Fink et al., 2011). In the context
862 of this result, further, more thorough studies are warranted to test whether the addition of
863 proteinogenic amino acids can reduce the negative effects of chemicals exhibiting chronic
864 toxicity on aquatic crustaceans.
865

866 ## 4. Conclusions

867 In this study, new naturally-derived quaternary ammonium salts (QASs) with quinine-based
868 cation and two amino acid anions: L-asparaginate and L-alaninate were obtained for the first
869 time using a sustainable, environmentally friendly synthesis method. and the acquired FT-IR
870 and NMR spectra allowed confirmation of their chemical structures. The new compounds
871 exhibited many times higher solubility in water compared to known 1-alkylquininium
872 bromides. The antimicrobial activity of the analyzed quinine-based QASs increased with the
873 elongation of the alkyl chain in the cation, and the chemical structure of an anion had no
874 influence on this effect. The 1-dodecylquinium salts inhibited the growth of most of the
875 microbes tested at concentrations below 16 mg dm^{-3}, thus showing activity at the level of

commercially available disinfectants. Most quinine derivatives also proved to be good or very good feeding deterrents active toward stored product pests, particularly effective against *T. confusum* larvae. In view of this high activity, and due the low availability and high price of the most effective natural antifeedants for storage pests (*i.e.* azadirachtin), further research to determine the optimal chemical structure and dose of quinine-based QASs to achieve optimal biopesticidal activity is warranted.

No significant phytotoxic effects were observed for any of the tested compounds in concentrations below 10 mg dm^{-3}, which is a promising preliminary result in the context of the potential application of new compounds. Interestingly, although QASs with an amino acid anion and 1-(2-hydroxyethyl)quininium cation were characterized by weak antifeedant activity and had no antimicrobial properties, their application on seeds caused accelerated growth of white mustard plants compared to the control. This result implies the potential use of more hydrophilic forms of quinine-based QASs. As with conventional QASs, the aquatic toxicity of the analyzed quinine derivatives was related to the length of the alkyl substituent in the cation. Despite the lack of clear toxicity of salts with 1-(2-hydroxyethyl)quininium and 1-butylquininium cations toward algae (ErC_{50} > 100 mg dm^{-3}), QASs containing a longer alkyl chain were moderately or highly toxic toward *C. vulgaris*. Toxicity toward *D. magna* was mainly due to the adverse effects of quinine. An additional chronic toxicity test against *D. magna*, performed for the least harmful QAS with a 1-butylquinium cation and an L-asparaginate anion, showed no long-term effects at concentrations of 0.1 mg dm^{-3} and below. Surprisingly, at sublethal concentration (10 mg dm^{-3}), inhibition of the chronic toxicity of this salt and positive effects on the reproduction of test organisms were observed.

The results of the conducted studies indicate that quinine is an interesting raw material for the synthesis of new biologically active compounds, and its quaternary salts containing short alkyl substituents exhibit low or mild toxicity to microbes, terrestrial plants and aquatic organisms.

In addition, they are effective biopesticides that prevent stored product pests from feeding with moderate or high efficiency. Moreover, the elongation of the alkyl chain in the quinine-derived cation, allows for synthesis of new QASs with potent disinfectant properties, albeit at the expense of their increased toxicity to aquatic organisms. Therefore, it should be emphasized that quinine-based cations that determine antimicrobial activity are also toxic to multiple classes of aquatic organisms and, despite their higher biocompatibility, should be treated as emerging pollutants like conventional QAS disinfectants currently in use. From the above, it also follows that new QASs such as 1-butylquininium L-asparaginate (**1Asp**) – inactive against microorganisms and low-toxic, but with favorable antifeedant activity – offer a potentially safer alternative as dedicated biopesticides in applications where the disinfectant activity of new QASs is not required.

Author contributions: CRediT

Tomasz Rzemieniecki: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft. **Krzysztof Juś**: Data curation, Investigation, Methodology. **Tomasz Klejdysz**: Investigation, Data curation, Formal analysis, Funding Acquisition, Resources. **Daniela Gwiazdowska**: Data curation, Funding Acquisition, Investigation, Methodology, Resources.

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Appendices

Appendix A: Supplementary data; Materials for OECD media preparation (OECD 201 TG medium, Elendt M4 medium), spectral analyses (FT-IR, NMR), additional data and exact values (physicochemical parameters, antimicrobial activity, biopesticidal activity, germination capacity, phytotoxicity, acute toxicity toward *C. vulgaris* and *D. magna*, chronic toxicity toward *D. magna*), references.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors did not use any generative AI and AI-assisted technologies.

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