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RESEARCH ARTICLE

Synthesis and Biological Evaluations of Granulatamide B and its Structural Analogues

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Abstract: ***Background*:** While granulatamides A and B have been previously isolated, their biological activities have been only partially examined. The aim of this study was to synthesize granulatamide B (**4b**), a tryptamine-derivative naturally occurring in *Eunicella* coral species, using the well-known procedure of Sun and Fürstner and its 12 structural analogues by modifying the side chain, which differs in length, degree of saturation as well as number and conjugation of double bonds.

***Method*:** The prepared library of compounds underwent comprehensive assessment for their biological activities, encompassing antioxidative, antiproliferative, and antibacterial properties, in addition to *in vivo* toxicity evaluation using a Zebrafish model. Compound 4i, which consists of a retinoic acid moiety, exhibited the strongest scavenging activity against ABTS radicals (IC_{50} = 36 ± 2 μM). In addition, **4b** and some of the analogues (**4a**, **4c** and **4i**), mostly containing an unsaturated chain and conjugated double bonds, showed moderate but non-selective activity with certain IC_{50} values in the range of 20-40 μM.

***Result*:** In contrast, the analogue 4l, a derivative of alpha-linolenic acid, was the least toxic towards normal cell lines. Moreover, 4b was also highly active against Gram-positive Bacillus subtilis with an MIC of 125 μM. Nevertheless, both **4b** and **4i**, known for the best-observed effects, caused remarkable developmental abnormalities in the zebrafish model *Danio rerio*.

***Conclusion*:** Since modification of the side chain did not significantly alter the change in biological activities compared to the parent compound, granulatamide B (**4b**), the substitution of the indole ring needs to be considered. Our group is currently carrying out new syntheses focusing on the functionalization of the indole core.

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1. INTRODUCTION

The extreme physical and chemical conditions in the marine environment have led macro- and microorganisms to adapt by synthesizing secondary metabolites for purposes of defense, attack, or signaling [1]. These compounds are often characterized by unique chemical structures, higher oxygen content, and the presence of halogen atoms and thus differ significantly from compounds isolated from terrestrial organisms [2]. Because marine metabolites exhibit a wide range of biological activities, including anticancer [3], anti-in-

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flammatory [4], antimicrobial [5], antioxidant [6] and others, they have attracted the attention of the scientific community, particularly with regard to the discovery of new compounds as potential drugs that are more potent and less toxic. Although the upsurge of marine (blue) biotechnology did not begin until the middle of the last century, more than 30,000 compounds have been isolated to date, largely due to the implementation of extensive marine bioprospecting programs [7-9]. As a result, a large number of steroids, terpenes, phenols, polypeptides, glycosides, and alkaloids have been extracted, characterized, synthesized, and subjected to numerous studies for their biological effects, with a focus on anticancer activity [10].

Marine indole alkaloids include a group of different heterocyclic products that exhibit various pharmacological activities, as already described in the recent literature [11, 12]. So far, they have been isolated mainly from marine microorganisms (fungi and bacteria), sponges, bryozoans, tunicates, molluscs, red algae, mangroves, and corals [11]. However, the latter is not a prolific source of these chemicals, as alkaloids account for only 4% of all metabolites studied that have been isolated from cnidarians, according to Rocha *et al*. [13]. Moreover, the coral microbiome may be the actual producer of indole compounds, as the indole synthesis pathway from tryptophan is well-known in bacteria [14], and 3-hydroxy-L-kynurenine, a tryptophan metabolite, has been detected in the coral genus *Eunicella* [15]. Nevertheless, there are more than 100 indole alkaloids obtained from the coral extracts that exhibit remarkable biological properties such as antibacterial, insecticidal and cytotoxic activities [16]. Notable among them are two *N*-acyl tryptamines, granulatamides A and B, isolated in 2006 from the soft coral *Eunicella granulata* (Grasshoff, 1992) and previously studied only on a few cell lines for their antiproliferative activities [17]. Such *N*-fatty acyl tryptamines are a rare group of natural products that have been isolated mainly from terrestrial organisms [18-20] and for which few biological studies were conducted [21-24]. In addition to isolation from natural organisms, total synthetic approaches to the preparation of granulatamides A [25] and B [26] have also been published. Therefore, both granulatamides are important because of their interesting chemical structure consisting of 3 main components: indole nucleus, amide bond, and long fatty acyl chain.

The indole nucleus is a planar and aromatic heterocyclic motif that is considered a privileged structure in organic and medicinal chemistry [27-29]. Moreover, it is a pharmacophore of numerous natural and synthetic compounds that exhibit various biological activities with the potential to treat different diseases, as observed in preclinical and clinical studies [30]. The versatility of pharmacological activities is the result of the chemical nature of indoles, particularly the hydrogen bond donation, high π-electron density, and high HOMO (highest occupied molecular orbital) energy. Consequently, indole compounds interact with various target proteins and nucleobases [12, 24]. Due to the delocalization of the nitrogen lone-pair to the π-electron system, the majority of organic reactions occur at the C-3 position (protonation, oxidation, electrophilic substitutions, cycloaddition), whereas the substitution of the N-H bond in the ring occurs under basic conditions [29, 30]. An example of C-3 substituted indoles are indolamines, such as the neurotransmitters tryptamine and its 5-hydroxy derivative (serotonin) or the neurohormone melatonin (5-methoxy-N-acetyltryptamine) [28]. Their potential is shown by their ability to form an amide bond when conjugated, for example, with certain organic acids, leading to more effective biological properties and binding to various biological targets [31]. In addition, such compounds tend to be more stable due to the resonance stability of the amide bond and lower reactivity and susceptibility to nucleophilic attack [32]. Among fatty acids, polyunsaturated fatty acids (PUFAs) play an important role because the presence of a long hydrocarbon chain and a reactive carboxylic group enables the preparation of pharmacologically heterogeneous compounds. In 2020, Jóźwiak *et al*. extensively investigated the advantages of saturated and unsaturated fatty acids and their condensation with heterocyclic compounds leading to increased tumour selectivity and lower toxicity.

Interestingly, ω-3 PUFAs have been shown to modulate inflammatory processes and act as antibacterial, neuroprotective or anticancer agents. The latter results from the fact that tumour cells use fatty acids more extensively due to increased metabolism, indicating the possibility of increasing the selectivity of compounds conjugated to such acids [33]. Furthermore, the anticancer effect of PUFAs may be related to the modulation of membrane integrity and interference with ion channels in the membranes of cancer cells, as has been widely discussed by Mukerjee *et al*. [34]. Adding the assumption that ω-3 PUFAs may be antioxidants rather than prooxidants, the potential of these acids becomes even clearer [35, 36].

Considering the above-mentioned properties of indole-containing compounds, the insufficient data on biological properties and as part of our project Bioprospecting of the Adriatic Sea in relation to the soft

corals of genus *Eunicella* [37, 38], the marine natural product granulatamide B was synthesized alongside with twelve structural analogues. Furthermore, the compounds were screened for their antioxidant, antiproliferative and antibacterial activities and complemented with *in vivo* toxicity data. Our strategy was based on modulating the biological properties by modifying the side chain using fatty acids that differ in length, degree of saturation, number and position of double bonds and their conjugation. Our initial screening involved variation of the above-mentioned parameters by use of commercially available compounds in order to facilitate a controlled and systematic exploration of their influence on biological activities. This decision allowed us to efficiently access a diverse range of compounds with distinct side chain characteristics, enabling a comprehensive study of the impact of these variations on the compound's activities.

2. MATERIALS AND METHODS

2.1. Chemicals

Tryptamine (98%), alpha-linolenic acid (99%), palmitoleic acid (> 99%) and trolox (97%) were obtained from Acros Organics (Belgium). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (> 98%), retinoic acid (97%) and triethylamine were purchased from Alfa Aesar (Germany). 1-hydroxybenzotriazole hydrate (>97%), arachidonic acid (>95%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (Switzerland). Ethyl acetate, *c*-hexane, potassium persulfate, *N*,*N*-dimethylformamide, oleic acid, citric acid, and sodium bicarbonate were purchased from BDH Prolabo (United Kingdom). Dichloromethane, methanol, sodium chloride and sodium sulphate were obtained from VWR Chemicals (USA). The human carcinoma cell lines: colorectal adenocarcinoma (SW620), ductal pancreatic adenocarcinoma (CFPAC-1), metastatic breast cancer (MCF7), hepatocellular carcinoma (HepG2) as well as normal skin fibroblasts (HFF) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM) in addition to foetal bovine serum (FBS, 10% aq.), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) was used as a growth medium. All reagents were obtained from Lonza Group (Basel, Switzerland). All chemicals used for the preparation of artificial water (AW): $CaCl_2 \times 2H_2O$, $MgSO_4 \times 7H_2O$, $NaHCO_3$ and KCl were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. General Information

All chemicals were used without further purification. Monitoring of reaction progress was carried out by thin layer chromatography (TLC) on pre-coated Merck silica gel plates (60F-254) and visualized by UV_{254} lamp. Silica gel (Silica 60 for column chromatography; 0.063 - 0.2 mm, Macherey-Nagel, Germany) was used for the purification of the compounds by column chromatography. Melting points (m.p.) were determined by the Fisher-Jones melting point apparatus. Infrared spectroscopy was performed by Agilent Cary 630 ATR-FTIR spectrometer. Absorption spectra were recorded by using an Agilent Cary 60 UV-Vis spectrophotometer. ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded in deuterated solvent ($CDCl_3$) on a Varian INOVA 400 spectrometer (Varian, Palo Alto, CA, USA) with TMS as an internal standard. LC-QqQ analysis was performed by using an Agilent 1260 series high-performance liquid chromatograph coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with a jet stream electrospray (AJS ESI) source and UV detection at λ 254 nm (Agilent Technologies, Palo Alto, CA, USA). Zorbax Extend C-18 column was used (4.6 mm × 100 mm, 3.5 µm, 80A). The mobile phases were composed of miliQ water (A) and acetonitrile (B), both containing 0.1% formic acid. Compounds were anlysed by using three different methods: (i) 0 - 20 min (30% B), 20 - 30 min (95% B): (ii) 0 - 20 min (50% B), 20 - 30 min (95% B) or (iii) 0 - 25 min (10% B), 25 - 30 min (95% B). HRMS was performed using an SYNAPT G2-Si Q-TOF hybrid quadrupole time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with an electrospray (ESI) ionisation source (Z-spray) and an additional sprayer for the reference compound (Lock Spray). The samples were prepared by dissolving 1 mg of the different fractions in MeOH. The sample (1 µL of injection volume) was loaded on a KITETEX C18 (1.3 µm, 50 x 2.1 mm) column (Phenomenex, Torrance, CA, USA) heated at 50 °C. The effluent was fully directed toward the ESI source of the Q-TOF instrument. ESI-HRMS data were recorded in the positive ion modes. The source and desolvation temperatures were 120 and 450 °C, respectively. Nitrogen was used as a drying and nebulizing gas at flow rates of 50 and 900 L/h, respectively. Typically, the capillary voltage was 3 kV, the sampling cone voltage 20 V and the source offset 20 V. Lock mass corrections using $[M+H]+$, $[M-H]-$ ions at m/z 556.2771 and 554.2615 of a leucine-enkephalin solution (50 pg µL−1 in 50:50 acetonitrile/water + 0.1% formic acid) were applied for accurate mass measurements (elemental composition determination). The mass range was 50- 1150 Da, and

spectra were recorded at 0.2 s/scan in the centroid mode at a resolution of 20,000 (FWHM) in the sensitivity mode. Data acquisition and processing were performed with MassLynx 4.1 software.

2.3. Synthesis of Granulatamide B (4b) and its Structural Analogues (4a, 4c-4l and 5).

2.3.1. General Procedure for the Synthesis of Pyrone 2a-2c.

A mixture of ethyl-2,3-dimethylacrilate (**1**) (5 mmol, 0.695 mL) and corresponding acyl chloride (6 mmol) in dichloromethane (5 mL) was added to a suspension of $AlCl_3$ (1.60 g, 12 mmol) and dichloromethane (15 mL) under reflux for 3 hours. The mixture was then poured into excess ice-water, and the aqueous layer was extracted with dichloromethane (3 × 10 mL). The residue, obtained after drying and evaporation, was added to a mixture of H_2SO_4 (10 mL) and HOAc (20 mL) and stirred at 40 °C until complete conversion followed by TLC. The mixture was then poured into ice and neutralized with Na_2CO_3. The aqueous layer was extracted with EtOAc (3 × 30 mL), and combined organic layers were dried and evaporated to obtain a residue, which was purified by column chromatography (*c*-hexane/EtOAc = 5/1).

4-methyl-6-propyl-2H-pyran-2-one (**2a**). Compound **2a** was prepared by applying the above-mentioned procedure using starting material **1** and butyryl chloride (6 mmol, 0.621 mL) to obtain **2a** as a yellow liquid (563.5 mg, 74%). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (3.79), 297 (3.40). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.94 (t, *J* = 7.4 Hz, 3H), 1.67 (m, 2H), 2.10 (s, 3H), 2.41 (t, *J* = 7.5 Hz, 2H), 5.82 (s, 1H), 5.93 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 13.46, 20.19, 21.43, 35.49, 105.82, 110.59, 156.20, 163.37, 164.70. IR (ν_{max}): 2972, 2935, 2877, 1719, 1646, 1562, 1460, 1441, 1380, 1227, 1153, 1130, 1095, 1028, 963, 843, 817, 687 cm^{-1}. ESI-MS (+): *m/z* 153.0 $[M+H]^+$. HRESIMS (+) *m/z* 153.0929 (calcd for $C_9H_{13}O_2$, 153.0916).

4-methyl-6-heptyl-2H-pyran-2-one (**2b**). Compound **2b** was prepared by applying the above-mentioned procedure using starting material **1** and octanoyl chloride (6 mmol, 1.024 mL) to obtain **2b** as a yellow liquid (575 mg, 84%). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (3.12), 297 (3.51).^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87 (t, *J* = 6.8 Hz, 3H), 1.23-1.33 (m, 8H), 1.61-1.68 (m, 3H), 2.11 (s, 3H), 2.44 (t, *J* = 7.7 Hz, 2H), 5.82 (s, 1H), 5.94 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.04, 21.43, 22.58, 26.86, 28.90, 28.94, 31.63, 33.64, 105.66, 110.57, 156.20, 163.41, 165.03. IR (ν_{max}): 2957, 2928, 2859, 1723, 1648, 1564, 1462, 1441, 1408, 1380, 1227, 1154, 1134, 1108, 1028, 964, 838, 817, 728, 683 cm^{-1}. ESI-MS (+): *m/z* 209.0 $[M+H]^+$. HRESIMS (+) *m/z* 209.1554 (calcd for $C_{13}H_{21}O_2$, 209.1541).

4-methyl-6-undecyl-2H-pyran-2-one (**2c**). Compound **2c** was prepared by applying the above-mentioned procedure using starting material **1** and dodecanoyl chloride (6 mmol, 1.424 mL) to obtain **2c** as a yellow liquid (1132.8 mg, 85%). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (3.51), 297 (3.90).^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87 (t, *J* = 6.5 Hz, 3H), 1.21-1.37 (m, 16H), 1.57-1.67 (m, 2H), 2.11 (s, 3H), 2.43 (t, *J* = 7.7 Hz, 2H), 5.83 (s, 1H), 5.94 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.06, 21.39, 22.64, 24.70, 26.85, 28.97, 29.23, 29.29, 29.42, 29.58, 31.87, 33.64, 105.63, 110.56, 156.15, 163.36, 165.03. IR (ν_{max}): 2955, 2920, 2853, 1709, 1646, 1564, 1477, 1439, 1402, 1389, 1222, 1156, 1130, 1098, 1024, 953, 862, 836, 815, 732 cm^{-1}. ESI-MS (+): *m/z* 265.1 $[M+H]^+$. HRESIMS (+) *m/z* 265.2181 (calcd for $C_{17}H_{29}O_2$, 265.2168).

2.3.2. General Procedure for the Synthesis of Acids 3a-3c.

A solution of $Fe(acac)_3$ (44.5 mg, 0.125 mmol) and corresponding pyrone **2** (2.5 mmol) in diethylether (25 mL) and toluene (25 mL) was rapidly stirred under nitrogen atmosphere at −30 °C. Then, the addition of MeMgBr (3 M in Et_2O, 7.5 mmol, 2.5 mL) followed, and the stirring was continued for 20 min. The reaction was quenched with a saturated aqueous solution of NH_4Cl, followed by the addition of HCl (1 M) in order to set the pH of the aqueous layer to 2-3. The aqueous layer was extracted with EtOAc (5 × 20 mL), and the resulting organic layers were combined, dried over Na_2SO_4 and evaporated. Finally, the residue was purified by column chromatography (*c*-hexane/EtOAc = 10/1).

(2Z,4E)-3,5-dimethylocta-2,4-dienoic acid (**3a**). Compound **3a** was prepared by applying the above--mentioned procedure using MeMgBr and pyrone **2a** (2.5 mmol, 382 mg) to obtain **3a** as a pale yellow liquid (307 mg, 73%). UV (CH_2Cl_2): λ_{max} (log ε) = 280 (3.94). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.91 (t, *J* = 7.3 Hz, 3H), 1.49 (sext, *J* = 7.4 Hz, 2H), 1.73 (s, 3H), 2.04 (s, 3H), 2.09 (t, *J* = 7.8 Hz, 2H), 5.69 (s, 1H), 6.38 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 13.58, 18.46, 20.88, 25.59, 42.75, 116.68, 123.50,

143.10, 156.01, 170.22. IR (ν_{max}): ~3500-2400 (br), 2920, 2853, 1699, 1573, 1469, 1439, 1357, 1305, 1253, 1223, 1089, 1022, 947, 895, 732, 694 cm^{-1}. ESI-MS (+): *m/z* 169.1 $[M+H]^+$. HRESIMS (−) *m/z* 167.1072 (calcd for $C_{10}H_{15}O_2$, 167.1072).

(2Z,4E)-3,5-dimethyldodeca-2,4-dienoic acid (**3b**). Compound **3b** was prepared by applying the above--mentioned procedure using MeMgBr and pyrone **2b** (2.5 mmol, 520 mg) to obtain **3b** as a pale yellow liquid (455 mg, 82%). UV (CH_2Cl_2): λ_{max} (log ε) = 280 (3.95). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.6 Hz, 3H), 1.24-1.29 (m, 8H), 1.39-1.51 (m, 2H), 1.73 (s, 3H), 2.04 (s, 3H), 2.10 (t, *J* = 7.5 Hz, 2H), 5.68 (s, 1H), 6.39 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.11, 18.62, 22.68, 25.69, 27.81, 29.20, 31.82, 40.84, 116.60, 123.24, 143.88, 156.09, 170.12. IR (ν_{max}): ~3500-2400 (br), 2928, 2859, 1689, 1628, 1594, 1441, 1380, 1292, 1253, 1115, 1040, 935, 858, 726, 709 cm^{-1}. ESI-MS (+): *m/z* 225.1 $[M+H]^+$. HRESIMS (−) *m/z* 223.1700 (calcd for $C_{14}H_{23}O_2$, 223.1698).

(2Z,4E)-3,5-dimethylhexadeca-2,4-dienoic acid (**3c**). Compound **3c** was prepared by applying the above-mentioned procedure using MeMgBr and pyrone **2c** (2.5 mmol, 661 mg) to obtain **3c** as a pale yellow liquid (435 mg, 62%). UV (CH_2Cl_2): λ_{max} (log ε) = 280 (3.85). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.7 Hz, 3H), 1.23-1.29 (m, 16H), 1.41-1.50 (m, 2H), 1.73 (s, 3H), 2.04 (s, 3H), 2.11 (t, *J* = 7.7 Hz, 2H), 5.70 (s, 1H), 6.37 (s, 1H). ^{13}C NMR (100 MHz, CDCl3) δ (ppm) 14.12, 18.62, 22.69, 25.68, 27.81, 29.24, 29.37, 29.54, 29.62, 29.64, 29.69, 31.93, 40.85, 116.63, 123.29, 143.77, 156.14, 170.49. IR (ν_{max}): ~3500-2400 (br), 2925, 2854, 1689, 1629, 1596, 1443, 1420, 1380, 1294, 1257, 1204, 1167, 1033, 933, 858, 799, 713 cm^{-1}. ESI-MS (+): *m/z* 281.2 $[M+H]^+$. HRESIMS (−) *m/z* 279.2336 (calcd for $C_{18}H_{31}O_2$, 279.2324).

2.3.3. General Procedure for the Synthesis of Granulatamide B and Analogues (4a-4l).

Triethylamine (45.4 mg, 0.45 mmol), 1-hydroxybenzotriazole hydrate (44.6 mg, 0.33 mmol) and *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (86.3 mg, 0.45 mmol) were added to a solution of tryptamine (52.8 mg, 0.33 mmol) and corresponding acid (**3a-3c** or commercially available ones) in DMF (0.3 mL) and CH_2Cl_2 (5 mL). After stirring overnight, the solvents were evaporated while the residue was treated with water (15 mL) and extracted with EtOAc (3 × 20 mL). The organic layers were combined and washed with aqueous citric acid (5%, 3 × 20 mL), saturated aqueous $NaHCO_3$ (3 × 30 mL) and brine (50 mL). The organic layer was then dried over Na_2SO_4 and evaporated to obtain the residue, which was purified by column chromatography (*c*-hexane/EtOAc = 4/1).

(2Z,4E)-N-(2-(1H-indol-3-yl)ethyl)-3,5-dimethylocta-2,4-dienamide (**4a**). Compound **4a** was prepared by applying the above-mentioned procedure using acid **3a** (0.30 mmol, 52.8 mg) to obtain **4a** as a light yellow oil (76 mg, 83%). UV (CH_2Cl_2): λ_{max} (log ε) = 221 (4.35), 280 (3.79), 290 (3.66). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.80 (t, *J* = 7.3 Hz, 3H), 1.28 (sext, *J* = 7.4 Hz, 2H), 1.54 (s, 3H), 1.80 (t, *J* = 7.7 Hz, 2H), 1.86 (s, 3H), 2.95 (t, *J* = 6.9 Hz, 2H), 3.63 (q, *J* = 6.6 Hz, 2H), 5.67 (s, 1H), 5.83 (s, 1H), 6.16 (s, 1H), 7.01 (d, *J* = 1.9 Hz, 1H), 7.11 (t, *J* = 7.5 Hz, 1H), 7.15-7.24 (m, 1H), 7.37(d, *J* = 8.1 Hz, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 8.17 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 13.70, 17.65, 20.86, 25.32, 25.44, 39.51, 41.61, 111.14, 113.26, 118.74, 119.41, 121.84, 122.12, 122.49, 123.06, 127.42, 136.42, 141.93, 145.57, 166.75. IR (ν_{max}): 3404, 3270, 3062, 2935, 2876, 1647, 1603, 1528, 1439, 1349, 1268, 1238, 1201, 1104, 1044, 933, 851, 813, 746 cm^{-1}. ESI-MS (+): *m/z* 311.1 $[M+H]^+$. HRESIMS (−) *m/z* 309.1971 (calcd for $C_{20}H_{25}N_2O$, 309.1967).

(2Z,4E)-N-(2-(1H-indol-3-yl)ethyl)-3,5-dimethyldodeca-2,4-dienamide (**4b**). Compound **4b** (granulatamide B) was prepared by applying the above-mentioned procedure using acid **3b** (0.30 mmol, 67.2 mg) to obtain **4b** as a light yellow oil (88 mg, 80%). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.47), 274 (3.97), 290 (3.85). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.8 Hz, 3H), 1.06 - 1.40 (m, 10H), 1.54 (s, 3H), 1.81 (d, *J* = 7.3 Hz, 2H), 1.85 (s, 3H), 2.95 (t, *J* = 6.8 Hz, 2H), 3.63 (q, *J* = 6.6 Hz, 2H), 5.64 - 5.69 (m, 1H), 5.82 (s, 1H), 6.16 (s, 1H), 7.01 (d, *J* = 2.3 Hz, 1H), 7.11 (ddd, *J* = 8.1, 7.1, 1.1 Hz, 1H), 7.19 (ddd, *J* = 8.2, 7.0, 1.2 Hz, 1H), 7.36 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.59 (d, *J* = 7.9 Hz, 1H), 8.08 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.12, 17.74, 22.68, 25.41, 25.43, 27.85, 29.17, 29.35, 31.84, 39.49, 39.60, 111.16, 113.21, 118.76, 119.41, 121.84, 122.14, 122.43, 122.82, 127.38, 136.36, 142.34, 145.69, 166.79. IR (ν_{max}): 3403, 3278, 3056, 2925, 2853, 1643, 1598, 1522, 1456, 1435, 1375, 1339, 1260, 1229, 1097, 1031, 1010, 923, 841, 804, 737 cm^{-1}. ESI-MS (+): *m/z* 367.2 $[M+H]^+$. HRESIMS (+) *m/z* 367.2758 (calcd for $C_{24}H_{35}N_2O$, 367.2758).

(2Z,4E)-N-(2-(1H-indol-3-yl)ethyl)-3,5-dimethylhexadeca-2,4-dienamide (**4c**). Compound **4c** was prepared by applying the above-mentioned procedure using acid **3c** (0.30 mmol, 84.1 mg) to obtain **4c** as a light yellow oil (111 mg, 87%). UV (CH_2Cl_2): λ_{max} (log ε) = 221 (4.67), 280 (4.10), 290 (3.97). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.7 Hz, 3H), 1.21-1.30 (m, 18H), 1.54 (s, 3H), 1.81 (t, *J* = 7.6 Hz, 2H), 1.85 (s, 3H), 2.95 (t, *J* = 6.9 Hz, 2H), 3.63 (q, *J* = 6.6 Hz, 2H), 5.67 (s, 1H), 5.82 (s, 1H), 6.16 (s, 1H), 7.01 (d, *J* = 2.4 Hz, 1H), 7.11 (t, *J* = 7.5 Hz, 1H), 7.20 (t, *J* = 7.3 Hz, 1H), 7.36(d, *J* = 8.2 Hz, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 8.04 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.08, 17.72, 22.67, 25.34, 25.43, 27.85, 29.28, 29.33, 29.38, 29.49, 29.62, 29.66, 31.90, 39.49, 39.59, 111.12, 113.30, 118.76, 119.43, 121.79, 122.15, 122.48, 122.86, 127.41, 136.38, 142.27, 145.59, 166.76. IR (ν_{max}): 3404, 3285, 3064, 2928, 2861, 1655, 1610, 1528, 1446, 1349, 1268, 1238, 1104, 1044, 934, 851, 813, 746 cm^{-1}. ESI-MS (+): *m/z* 423.2 $[M+H]^+$. HRESIMS (−) *m/z* 421.3222 (calcd for $C_{28}H_{41}N_2O$, 421.3219).

N-(2-(1H-indol-3-yl)ethyl)octanamide (**4d**). Compound **4d** was prepared by applying the above-mentioned procedure using octanoic (caprylic) acid (0.30 mmol, 43.2 mg, 0.047 mL) to obtain **4d** as a white solid (62 mg, 72%, m.p. = 93-94 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.28), 280 (3.83), 290 (3.75). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87 (t, *J* = 6.7 Hz, 3H), 1.22-1.32 (m, 8H), 1.58 (t, *J* = 7.4 Hz, 2H), 2.10 (t, *J* = 7.6 Hz, 2H), 2.97 (t, *J* = 6.7 Hz, 2H), 3.61 (q, *J* = 6.4 Hz, 2H), 5.54 (s, 1H), 7.02 (d, *J* = 2.3 Hz, 1H), 7.12 (t, *J* = 7.4 Hz, 1H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 8.27 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.04, 22.58, 25.37, 25.73, 28.98, 29.23, 31.66, 36.90, 39.67, 111.26, 113.06, 118.71, 119.45, 122.00, 122.17, 127.38, 136.44, 173.15. IR (ν_{max}): 3404, 3255, 3084, 2920, 2853, 1632, 1573, 1461, 1431, 1379, 1282, 1230, 1097, 1074, 1014, 925, 860, 784, 746 cm^{-1}. ESI-MS (+): *m/z* 287.1 $[M+H]^+$. HRESIMS (−) *m/z* 285.1972 (calcd for $C_{18}H_{25}N_2O$, 285.1967).

N-(2-(1H-indol-3-yl)ethyl)dodecanamide (**4e**). Compound **4e** was prepared by applying the above-mentioned procedure using dodecanoic (lauric) acid (0.30 mmol, 60.1 mg) to obtain **4e** as a white solid (84 mg, 82%, m.p. = 104-105 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.23), 280 (3.67), 290 (3.58). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.7 Hz, 3H), 1.22-1.29 (m, 16H), 1.57 (t, *J* = 6.8 Hz, 2H), 2.09 (t, *J* = 7.6 Hz, 2H), 2.97 (t, *J* = 6.7 Hz, 2H), 3.60 (q, *J* = 6.5 Hz, 2H), 5.53 (s, 1H), 7.02 (d, *J* = 2.3 Hz, 1H), 7.13 (t, *J* = 7.4 Hz, 1H), 7.22 (t, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 8.22 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.09, 22.66, 25.37, 25.74, 29.29, 29.32, 29.34, 29.47, 29.59, 29.60, 31.89, 36.91, 39.66, 111.24, 113.10, 118.72, 119.47, 121.98, 122.19, 127.38, 136.43, 173.13. IR (ν_{max}): 3397, 3263, 3084, 2920, 2853, 1632, 1573, 1469, 1431, 1379, 1290, 1223, 1126, 1074, 1014, 895, 806, 746 cm^{-1}. ESI-MS (−): *m/z* 341.1 $[M-H]^-$. HRESIMS (−) *m/z* 341.2593 (calcd for $C_{22}H_{33}N_2O$, 341.2593).

N-(2-(1H-indol-3-yl)ethyl)hexadecanamide (**4f**). Compound **4f** was prepared by applying the above--mentioned procedure using hexadecenoic (palmitic) acid (0.30 mmol, 76.9 mg) to obtain **4e** as a white solid (100.3 mg, 84%, m.p. = 107-109 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (3.84), 280 (3.55), 290 (3.45). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.5 Hz, 3H), 1.22-1.33 (m, 24H), 1.52-1.62 (m, 2H), 2.10 (t, *J* = 7.7 Hz, 2H), 2.98 (t, *J* = 6.7 Hz, 2H), 3.61 (q, *J* = 6.5 Hz, 2H), 5.53 (s, 1H), 7.03 (d, *J* = 2.3 Hz, 1H), 7.13 (t, *J* = 7.5 Hz, 1H), 7.21 (t, *J* = 7.2 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.61 (d, *J* = 7.8 Hz, 1H), 8.21 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.09, 22.67, 25.38, 25.74, 29.29, 29.29, 29.34, 29.34, 29.48, 29.61, 29.64, 29.68, 29.68, 30.00, 31.91, 36.91, 39.66, 111.24, 113.11, 118.72, 119.48, 121.98, 122.20, 127.38, 136.43, 173.13. IR (ν_{max}): 3389, 3265, 3084, 2920, 2853, 1631, 1553, 1473, 1458, 1426, 1380, 1343, 1280, 1227, 1097, 1071, 1013, 933, 804, 741 cm^{-1}. ESI-MS (+): *m/z* 399.2 $[M+H]^+$. HRESIMS (−) *m/z* 397.3227 (calcd for $C_{26}H_{41}N_2O$, 397.3219).

(9Z,12Z)-N-(2-(1H-indol-3-yl)ethyl)octadeca-9,12--dienamide (**4g**). Compound **4g** was prepared by applying the above-mentioned procedure using linoleic acid (0.30 mmol, 84.1 mg) to obtain **4g** as a light yellow solid (102 mg, 80%, m.p. = 55-57 °C)UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.28), 280 (3.85), 290 (3.77). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.9 Hz, 3H), 1.25-1.38 (m, 14H), 1.53-1.61 (m, 2H), 2.04 (m, 4H), 2.12 (t, *J* = 7.7 Hz, 2H), 2.77 (t, *J* = 6.6 Hz, 2H), 3.61 (q, *J* = 6.6 Hz, 2H), 5.28-5.43 (m, 4H), 5.73 (s, 1H), 7.03 (s, 1H), 7.12 (t, *J* = 7.4 Hz, 1H), 7.21 (t, *J* = 7.8 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 8.16 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.04, 22.55, 25.37, 25.62, 25.70, 27.19, 29.11, 29.24, 29.32, 29.43, 29.59, 31.50, 31.88, 36.88, 39.65, 111.22, 113.13, 118.73, 119.49, 121.95, 122.21,

127.38, 127.89, 128.04, 130.06, 130.24, 136.42, 173.07. IR (v_{max}): 3419, 3292, 3017, 2928, 2861, 1655, 1558, 1461, 1349, 1271, 1227, 1104, 1044, 918, 806, 746 cm^{-1}. ESI-MS (+): *m/z* 423.2 $[M+H]^+$. HRESIMS (−) *m/z* 421.3219 (calcd for $C_{28}H_{41}N_2O$, 421.3219).

(5Z,8Z,11Z,14Z)-N-(2-(1H-indol-3-yl)ethyl)icosa-5,8,11,14-tetraenamide (**4h**). Compound **4h** was prepared by applying the above-mentioned procedure using arachidonic acid (0.30 mmol, 91.3 mg) to obtain **4h** as a light yellow solid (85.7 mg, 64%, m.p. = 48-50 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.24), 280 (3.79), 290 (3.68). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.89 (t, *J* = 7.0 Hz, 3H), 1.24-1.41 (m, 6H), 1.62-1.74 (m, 2H), 2.00-2.15 (m, 6H), 2.74-2.86 (m, 6H), 2.97 (t, *J* = 6.8 Hz, 2H), 3.61 (q, *J* = 6.6 Hz, 2H), 5.27-5.44 (m, 8H), 5.50 (s, 1H), 7.03 (s, 1H), 7.13 (t, *J* = 7.6 Hz, 1H), 7.21 (t, *J* = 8.1 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 1H), 7.61 (d, *J* = 8.0 Hz, 1H), 8.13 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.03, 22.54, 25.37, 25.51, 25.63, 25.63, 26.67, 27.21, 29.30, 31.49, 31.50, 36.19, 39.67, 111.22, 113.13, 118.72, 119.51, 121.92, 122.23, 127.38, 127.52, 127.86, 128.18, 128.19, 128.60, 128.67, 129.15, 130.51, 136.42, 172.72. IR (v_{max}): 3412, 3285, 3017, 2928, 2861, 1655, 1528, 1461, 1349, 1260, 1104, 1044, 925, 810, 746 cm^{-1}. ESI-MS (+): *m/z* 447.1 $[M+H]^+$. HRESIMS (−) *m/z* 445.3223 (calcd for $C_{30}H_{41}N_2O$, 445.3219).

(2E,4E,6E,8E)-N-(2-(1H-indol-3-yl)ethyl)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenamide (**4i**). Compound **4i** was prepared by applying the above-mentioned procedure using *all-trans*-retinoic acid (ATRA) (0.30 mmol, 90.1 mg) to obtain **4i** as a yellow solid (85.7 mg, 64%, m.p. = 177-180 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.54), 280 (4.21), 290 (4.23), 356 (4.75). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.03 (s, 6H), 1.41-1.50 (m, 2H), 1.55-1.67 (m, 2H), 1.68-1.74 (m, 3H), 1.98 (s, 3H), 2.02 (t, *J* = 6.5 Hz, 2H), 2.36 (d, *J* = 1.2 Hz, 3H), 3.01 (t, *J* = 6.7 Hz, 2H), 3.66 (q, *J* = 6.7 Hz, 2H), 5.54-5.62 (m, 2H), 6.05-6.29 (m, 4H), 6.90 (dd, *J* = 15.1, 11.4, Hz, 1H), 7.04 (d, *J* = 2.4 Hz, 1H), 7.12 (t, *J* = 7.5 Hz, 1H), 7.21 (t, *J* = 7.8 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 7.62 (d, *J* = 7.0 Hz, 1H), 8.20 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 12.83, 13.56, 19.23, 21.70, 25.41, 28.94, 28.94, 33.08, 34.25, 39.57, 39.62, 111.22, 113.17, 118.78, 119.48, 121.56, 122.06, 122.17, 127.39, 128.15, 129.56, 129.60, 129.74, 135.57, 136.44, 137.33, 137.75, 138.61, 148.34, 167.06. IR (v_{max}): 3285, 3047, 2928, 2868, 1610, 1536, 1439, 1394, 1364, 1290, 1260, 1230, 1201, 1163, 1134, 1044, 977, 828, 746 cm^{-1}. ESI-MS (+): *m/z* 443.2 $[M+H]^+$. HRESIMS (−) *m/z* 441.2911 (calcd for $C_{30}H_{37}N_2O$, 441.2906).

(Z)-N-(2-(1H-indol-3-yl)ethyl)hexadec-9-enamide (**4j**). Compound **4j** was prepared by applying the above-mentioned procedure using palmitoleic acid (0.30 mmol, 76.3 mg) to obtain **4j** as a yellow solid (77.3 mg, 65%, m.p. = 64-66 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.19), 280 (3.70), 290 (3.62). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.9 Hz, 3H), 1.24-1.35 (m, 16H), 1.52-1.62 (m, 2H), 1.96-2.06 (m, 4H), 2.09 (t, *J* = 7.6 Hz, 2H), 2.98 (t, *J* = 6.7 Hz, 2H), 3.61 (q, *J* = 6.6 Hz, 2H), 5.29-5.40 (m, 2H), 5.50 (s, 1H), 7.03 (d, *J* = 2.5 Hz, 1H), 7.13 (t, *J* = 7.5 Hz, 1H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 8.14 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.06, 22.62, 25.37, 25.71, 27.16, 27.21, 28.96, 29.11, 29.23, 29.24, 29.69, 29.71, 31.75, 36.88, 39.65, 111.22, 113.15, 118.73, 119.49, 121.94, 122.21, 127.39, 129.76, 129.98, 136.42, 173.06. IR (v_{max}): 3397, 3270, 3099, 2928, 2861, 1640, 1553, 1461, 1431, 1387, 1349, 1305, 1275, 1230, 1134, 1104, 1074, 1014, 977, 940, 806, 746 cm^{-1}. ESI-MS (+): *m/z* 397.3 $[M+H]^+$. HRESIMS (−) *m/z* 395.3071 (calcd for $C_{26}H_{39}N_2O$, 395.3062).

(Z)-N-(2-(1H-indol-3-yl)ethyl)octadec-9-enamide (**4k**). Compound **4k** was prepared by applying the above-mentioned procedure using oleic acid (0.30 mmol, 84.7 mg) to obtain **4k** as a light yellow solid (101.1 mg, 79%, m.p. = 68-70 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 222 (4.17), 280 (3.66), 290 (3.59). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, *J* = 6.9 Hz, 3H), 1.18-1.38 (m, 20H), 1.49-1.60 (m, 2H), 1.93-2.06 (m, 4H), 2.09 (t, *J* = 7.7 Hz, 2H), 2.98 (t, *J* = 6.6 Hz, 2H), 3.61 (q, *J* = 6.7 Hz, 2H), 5.27-5.41 (m, 2H), 5.49 (s, 1H), 7.04 (d, *J* = 2.5 Hz, 1H), 7.13 (t, *J* = 6.9 Hz, 1H), 7.21 (t, *J* = 6.9 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 1H), 7.61 (d, *J* = 7.1 Hz, 1H), 8.10 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.06, 22.64, 25.37, 25.70, 27.16, 27.21, 29.11, 29.22, 29.23, 29.27, 29.30, 29.49, 29.69, 29.74, 31.87, 36.88, 39.63, 111.19, 113.22, 118.74, 119.52, 121.91, 122.24, 127.39, 129.75, 129.99, 136.42, 173.01. IR (v_{max}): 3397, 3270, 3091, 3010, 2928, 2861, 1632, 1558, 1461, 1431, 1387, 1349, 1305, 1275, 1230, 1134, 1104, 1074, 1014, 925, 858, 806, 746 cm^{-1}. ESI-MS (+): *m/z* 425.2 $[M+H]^+$. HRESIMS (−) *m/z* 423.3391 (calcd for $C_{28}H_{43}N_2O$, 423.3375).

(9Z,12Z,15Z)-N-(2-(1H-indol-3-yl)ethyl)octadeca-9,12,15-trienamide (**4l**). Compound **4l** was prepared by applying the above-mentioned procedure us-

ing alpha-linolenic acid (0.30 mmol, 83.5 mg) to obtain **4l** as a light yellow solid (100 mg, 79%, m.p. = 56-58 °C). UV (CH_2Cl_2): λ_{max} (log ε) = 222 (4.12), 280 (3.60), 290 (3.50). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.97 (t, *J* = 7.6 Hz, 3H), 1.25-1.41 (m, 8H), 1.58 (t, *J* = 6.9 Hz, 2H), 1.99-2.14 (m, 6H), 2.81 (t, *J* = 6.4 Hz, 4H), 2.98 (t, *J* = 6.8 Hz, 2H), 3.61 (q, *J* = 6.5 Hz, 2H), 5.24-5.42 (m, 6H), 5.49 (s, 1H), 7.03 (s, 1H), 7.13 (t, *J* = 7.4 Hz, 1H), 7.21 (t, *J* = 7.5 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 1H), 7.61 (d, *J* = 8.0 Hz, 1H), 8.13 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 14.22, 20.52, 25.37, 25.52, 25.61, 25.68, 27.18, 29.09, 29.21, 29.23, 29.56, 36.87, 39.64, 111.20, 113.18, 118.73, 119.51, 121.92, 122.23, 127.10, 127.40, 127.72, 128.24, 128.30, 130.27, 131.96, 136.43, 173.02. IR (ν_{max}): 3397, 3270, 3091, 3017, 2928, 2861, 1632, 1558, 1461, 1431, 1387, 1349, 1305, 1275, 1230, 1134, 1104, 1074, 1014, 940, 873, 746 cm^{-1}. ESI-MS (+): *m/z* 421.2 $[M+H]^+$. HRESIMS (−) *m/z* 419.3068 (calcd for $C_{28}H_{39}N_2O$, 419.3062).

2.3.4. Synthesis of N-(2-(1H-indol-3-yl)ethyl)Acetamide (5)

Compound **5** was synthesized according to a procedure of Yoshida *et al*. [38, 39] Tryptamine (52.8 mg, 0.33 mmol) was added to acetic acid (19.8 mg, 0.33 mmol) and 10 mL of EtOAc, and the reaction was heated at 70 °C for 24h. The reaction mixture was evaporated while the residue was treated with water (15 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were successively washed with aqueous citric acid (5%, 3 × 20 mL), saturated aqueous $NaHCO_3$ (3 × 30 mL) and brine (50 mL). The organic layer was then dried over Na_2SO_4 and evaporated to obtain the residue, which was purified by column chromatography (*c*-hexane/EtOAc = 4/1) to give the title compound as yellow-orange solid (65.5 mg, 98%, m.p. = 70 °C).

UV (CH_2Cl_2): λ_{max} (log ε) = 228 (4.21), 280 (3.65), 290 (3.56). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.92 (s, 3H), 2.98 (t, *J* = 6.7 Hz, 2H), 3.60 (q, *J* = 6.6 Hz, 2H), 5.55 (s, 1H), 7.04 (d, *J* = 2.3 Hz, 1H), 7.13 (t, *J* = 7.5 Hz, 1H), 7.22 (t, *J* = 7.6 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 1H), 7.61 (d, *J* = 7.7 Hz, 1H), 8.17 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 23.37, 25.27, 39.81, 111.25, 113.06, 118.70, 119.53, 121.98, 122.24, 127.36, 136.42, 170.04. IR (ν_{max}): 3404, 3270, 3062, 2980, 2935, 1640, 1536, 1439, 1372, 1275, 1238, 1208, 1104, 1044, 1014, 813, 746 cm^{-1}. ESI-MS (−): *m/z* 200.9 $[M-H]^-$. HRESIMS (−) *m/z* 201.1034 (calcd for $C_{12}H_{13}N_2O$, 201.1028).

2.4. Evaluation of Antioxidative Activity

The *in vitro* antioxidative potential of synthesized compounds was investigated by means of ABTS·+ radical cation assays according to Zheleva-Dimitrova *et al*. [40] with minor modifications. The ABTS·+ radical cation was firstly prepared by overnight incubation of the mixture of ABTS+ solution (7 mM) and potassium persulfate solution (2.4 mM) in the dark at room temperature. The radical solution was then diluted with methanol until the absorbance value at 734 nm was around 0.700. Samples dissolved in methanol (100 µL) were mixed with 100 µL of the diluted ABTS-radical cation solution in a 96-well plate and incubated for 7 min in the dark. The absorbance values were measured at 734 nm by using a microplate reader. The final concentration of tested samples was within the range of 1000-15.6 µM). The ABTS·+-solution mixed with the same amount of methanol and Trolox at the same concentration range were used as negative and positive controls, respectively. Radical scavenging activity was calculated according to the formula:

$$\% \text{ inhibition} = 100 \times (1 - AS/AC) \quad (1)$$

where AC and AS stand for the negative control and sample absorbance values, respectively. The concentration resulting in 50% inhibition of ABTS·+ radical cation (IC_{50}) was determined from the graph, which presents the inhibition percentage plotted against the concentration of the samples. All measurements were performed in three independent experiments and for each, a fresh solution of ABTS·+ radical cation was prepared.

2.5. Evaluation of Antiproliferative Activity

Investigation of antiproliferative activity was carried out as described in our previous paper [38]. The cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C in a humidified atmosphere. Carcinoma cell lines and normal human fibroblasts were seeded in 96 well plates at densities of 3000 and 5000 cells per well for transformed and normal cell lines, respectively. The next day, cells were incubated with test samples for 72 h while 5-fluorouracil (5-FU) was used as a positive control. Test samples were dissolved in dimethyl sulfoxide (DMSO) additionally with the use of vortexing and ultrasonic bath, and the final concentration range was 100-0.01 µM. The final amount of DMSO did not exceed 0.1%. The cell growth rate was investigated using MTT assay according to the manufacturer's guidelines. Using the formulas proposed by the National Institutes of Health (NIH, Bethesda, MD, USA), the percentage of cell growth was calculated by transforming the experimentally determined absor-

bance values [41]. Concentrations of samples needed to inhibit 50% of cell growth (IC_{50} values) were calculated from dose-response curves using linear regression analysis. All measurements were performed in three independent experiments.

The selectivity index (SI), as a measure of the cytotoxic selectivity of the tested samples against cancer cells, was calculated according to the following formula [42]:

SI = IC_{50} (non-transformed cell line)/IC_{50} (cancer cell line)(2)

2.6. Evaluation of Antibacterial Activity

The antibacterial activity of the compounds was evaluated by broth microdilution assay according to our previous report [38], using *Escherichia coli* NCTC 12241, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* NCTC 12903, and *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 as indicator strains. All assays employed positive (inoculated media without the tested sample) and negative (sterile media) controls and quality control using chloramphenicol (CMP), norfloxacin (NRL) and amikacin (AMK). All assays were performed in duplicates at 35 °C aerobically. The final concentration range of tested compounds was 0.97 - 1000 μM. Results were interpreted both visually and spectrophotometrically at wavelength of 600 nm using a microplate reader, and minimal inhibitory concentrations, if observed, were confirmed by culture isolation from the wells.

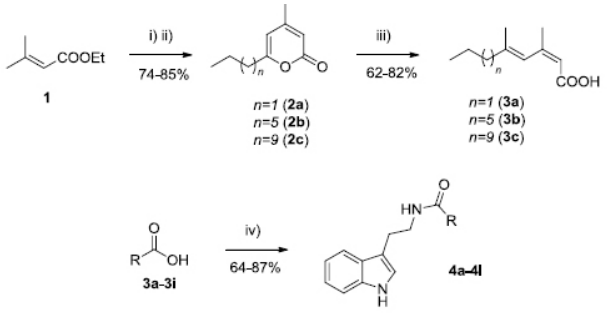
2.7. Evaluation of Toxicity *in vivo*

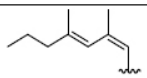
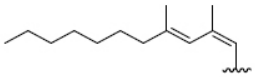
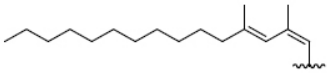
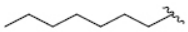
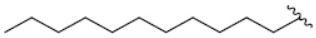
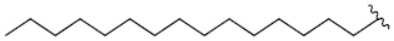
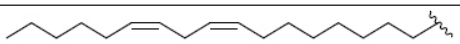
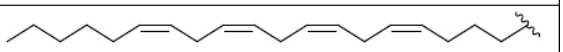
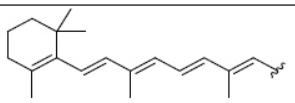
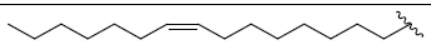
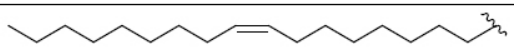
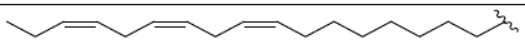
Zebrafish *D. rerio* adults of wild-type strain were obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology. Zebrafish maintenance and embryo production are described in detail in our previous study [43]. The stock solutions of tested individual compounds were prepared by diluting obtained dry samples in DMSO. From the stock solutions, a wide range of concentrations was prepared to determine acute toxicity, as follows: **4a** (399.36-0.78 μM), **4b** (152.0-9.5 μM), **4c** (283.5-17.72 μM), **4d** (100.0-3.13 μM), **4e** (613-19.15 μM), **4f** (370-11.56 μM), **4g** (521-16.28 μM), **4h** (200-25 μM), **4i** (100.0-1.50 μM), **4j** (400-25 μM), **4k** (400-50 μM), **4l** (400-12.5 μM), **5** (563-35 μM), tryptamine (998.5-62.40 μM). As a dilution medium, artificial water was used (ISO, 1996). The embryotoxic potential of tested samples was determined by performing the zebrafish embryotoxicity test (ZET) following the OECD 203 Guideline (2013). During the experiment, DMSO concentration did not exceed 1%. The test was conducted in 24-well plates containing 1 ml of tested sample per well. The control group was exposed to 1 mL of artificial water containing 1% of DMSO. During 96 h of development, specimens were incubated at 27.50 ± 0.5°C (Innova 42 incubator shaker; New Brunswick, Canada). At 96 hours post fertilization (hpf), mortality and developmental abnormalities were recorded using an inverted microscope (Olympus CKX41) equipped with Leica EC3 digital camera and LAS EZ 3.2.0 digitizing software. Zebrafish maintenance and spawning were performed in aquaria units approved by the Croatian Ministry of Agriculture and according to Directive 2010/63/EU [44].

3. RESULTS AND DISCUSSION

3.1. Chemistry

The synthesis of the tryptamine derivative, granulatamide B (**4b**) was carried out by applying Sun and Fürstner procedure published in 2013 [26]. The authors developed a three-step total synthesis consisting of obtaining multigram amounts of 2-pyrone (**2b**) in the reaction of crotonate (ethyl-2,3-dimethylacrilate, **1**) and acyl halide (octanoyl chloride) in 84% yield. Subsequently, iron-catalyzed ring-opening/cross-coupling of **2b** with a Grignard reagent MeMgBr yielded the (2*Z*,4*E*)-dienoic acid **3b** in 82%. The role of the iron catalyst and the mechanism of this cross-coupling were detailed by Legros and Figadère [45]. The acid **3b** was then condensed with tryptamine under standard conditions by using HOBt (1-hydroxy benzotriazole)/EDAC(1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) coupling protocol to furnish **4b** in 80%. Analogously, butyryl chloride and dodecanoyl chloride gave corresponding 2-pyrones **2a** and **2c** differing in the side chain lengths, in 74% and 85% yields, respectively. Further Fe-catalyzed coupling of obtained **2a** and **2c** with MeMgCl afforded corresponding acids **3a** (73%) and **3c** (62%). The latter was used to obtain analogues of granulatamide B with a yield higher than 80%, containing a shorter (**4a**) and a longer (**4c**) side chain with the same configuration of double bonds. Caprylic, lauric, and palmitic acids were used to prepare counterparts **4d**-**4f**, respectively, containing saturated side chains of the same length. Condensation of tryptamine with mono- or polyunsaturated acids, such as linoleic, arachidonic, retinoic, palmitoleic, oleic and alpha-linolenic acid provided analogues **4g**-**4l**, respectively, in yields ranging from 64% to 80%. Finally, compound **5** was obtained *via N*-acetylation of tryptamine in excellent yield (98%), as described in the literature [39], using acetic acid as promoter and ethyl acetate as both solvent and acetyl group donor (Scheme **1** and **2**).



Compound	R
4a	
4b (granulatamide B)	
4c	
4d	
4e	
4f	
4g	
4h	
4i	
4j	
4k	
4l	

Scheme (1). Synthesis of granulatamide B (**4b**) and its structural analogues (**4a**, **4c**-**4l**). Reagents and conditions: (**i**) acyl chloride, $AlCl_3$, CH_2Cl_2, reflux; (**ii**) H_2SO_4, HOAc, 40 °C; (**iii**) MeMgBr (in Et_2O), [Fe(acac)$_3$] (5 mol %), Et_2O/toluene ((1/1), 0.05 M), −30 °C; (iv) tryptamine, acids **3a**-**3c**/or commercially available mentioned in the section 2.3.3, HOBt, EDC·HCl, Et_3N, CH_2Cl_2/DMF,).

5

Scheme (2). Synthesis of analogue *N*-acetyltryptamine (**5**).

All compounds were characterized by extensive spectroscopic analysis and chemical methods (the recorded spectra are included in Supplementary materials). The absorption spectra were typical indole chromophores with λmax (DCM) at 290, 280 and 228 nm [46, 47]. Furthermore, the infrared spectrum showed signals at about 3400 cm-1 characteristic for N-H stretching of the secondary amides and strong bands at about 1640 cm-1, typical of the N-C=O stretching region of the amides [46]. The NMR spectra demonstrated that compounds **4a**-**4l** and **5** were tryptamine amides that exhibited typical shifts for the benzene ring and the pyrrole part of the indole system, as well as for the ethylamine moiety with amide proton, as previously reported in the literature [46, 47]. Analogues **4f** and **4g** were previously synthesized by Schmidt *et al.* and had identical spectroscopic data to those [21], whereas the analogues **4a**, **4c**, **4d**, **4e** and **4h**-**4l** were synthesized and characterized for the first time, even though *N*-lauroyl tryptamine (**4e**) was found in a culture produced by *Eubacterium rectale* [24]. *N*-Palmitoyl tryptamine (**4f**) and *N*-linoleoyl tryptamine were previously isolated from the Annonaceae plants [18, 21]. All compounds used for further biological evaluation had a purity of more than 95%.

3.2. Biological Evaluations

3.2.1. Radical-scavenging Activity

Several previous studies have been performed to investigate the antioxidant activity of tryptamine derivatives and their ability to scavenge free-radicals [48-53]. The latter is promoted by the formation of resonance-stabilized indolyl radicals upon electron or hydrogen transfer, as well as by the formation of radical adducts [[54-56]]. In addition, the importance of indole ring substitution and its contribution to the overall antiradical activity has been highlighted [28]. However, not many studies have been performed with *N*-fatty acyl derivatives of tryptamine. Therefore, the evaluation of the antioxidant potential of the synthesized granulatamide B (**4b**) and its structural analogues (**4a**, **4c**-**4l** and **5**) was performed by applying the *in vitro* ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay and measuring the decrease in absorbance values after the reduction of the ABTS·+ cation-radical by potential antioxidants.

Table 1. Inhibition percentage (%) at the highest concentration (1 mM) and IC_{50} values of $ABTS^{\cdot+}$ radical-scavenging activity of the samples 4a-4l, 5 and tryptamine. Trolox was used as a positive control.

Sample	inhibition (%) at c = 1 mM	IC_{50} (μM)
4a	53 ± 1	862 ± 53
4b	58 ± 2	704 ± 66
4c	51 ± 2	950 ± 59
4d	44 ± 1	>1000
4e	51 ± 2	964 ± 85
4f	43 ± 2	>1000
4g	59 ± 2	669 ± 6
4h	59 ± 3	450 ± 18
4i	84 ± 2	36 ± 2
4j	70 ± 2	333 ± 4
4k	58 ± 3	667 ± 40
4l	61 ± 2	461 ± 12
5	49 ± 1	>1000
Tryptamine	88 ± 0.5	105 ± 6
Trolox	91 ± 1	14 ± 1

Values are expressed as mean value ± standard deviation of triplicate measurements.

The results are presented in Table **1**, as the percentage of radical inhibition at the highest concentration tested (1 mM) and as the amount of compounds required to inhibit ABTS·+ by 50% (IC_{50}). The results were compared with the positive standard, Trolox and tryptamine, which has been shown to possess antioxidant activity [57, 58]. The strongest free-radical scavenging activity was exhibited by analogue **4i** with an IC_{50} value of 36 ± 2 μM and 84% of ABTS·+ inhibition at 1 mM, comparable to Trolox (IC_{50} = 14 ± 1 μM, 91%). On the opposite, all other *N*-acyl tryptamines showed rather weak activity in inhibiting the radical, with **4d**, **4f** and **5** failing to inhibit 50% of the radical at the highest concentration. Our results for compound **4f** (IC_{50} > 1000 μM) are in agreement with that reported in previous research by Schmidt and colleagues, but the opposite for compound **4g,** which authors found to be weakly active, only at the concentration above 1 mM [21]. Furthermore, we did not detect any significant effect on scavenging activity by either the chain length or number of double bonds in the aliphatic chain, with the exception of highly conjugated **4i**.

It should be noted that conjugation of tryptamine with certain fatty acids, except retinoic acid in **4i**, results in a decrease of anti-radical activity compared to tryptamine activity. Therefore, it is possible that the free secondary amino group of tryptamine contributes to the reduction of the ABTS·+ radical cation, as previously discussed by Schmidt *et al*. [21] and Gürkök *et al*. [59]. In contrast, better antioxidant activity was measured for *N*-fatty acyl derivatives of 4,5-dimethoxy tryptamines [22] and melatonin analogues [60] when an acyl residue (nonanoyl group) was introduced. However, in some cases, the presence of a side chain was both favourable and unfavourable in terms of radical scavenging [61], which can also be observed in our granulatamide B analogues. Nevertheless, the N-H bond of the indole ring is also crucial for radical scavenging activity, as previously noted [28, 54, 55], but it was not altered in granulatamide B and the structural analogues in our study. Moreover, the presence of unsaturated acyl residues in compounds **4a**-**4c** resulted in quite similar scavenging activity compared with their saturated counterparts (**4d**-**4f**). However, a slightly better effect was shown by compounds containing one or more double bonds in the fatty acyl side chain (**4g**, **4h**, **4j**-**4l**), which may be explained by the antioxidant properties of mono- or polyunsaturated fatty acids, especially ω-3 acids, which could act as antioxidants as suggested by Richard *et al*. [35].

Similarly, the antiradical activity of terpenes was also shown to depend on the presence of π bonds [62]. Although analogue **4l** consisted of an ω-3 fatty acid chain, compound **4j** (*N*-palmitoleoly tryptamine) had the lowest IC_{50} values (333 ± 4 μM) among commercial fatty acid conjugates. Interestingly, *N*-palmitoyl-5-hydroxytryptamine was shown to upregulate the expression of antioxidant enzymes in immortalized mouse hippocampal cell lines (HT-22) [63]. In 2002, Henry and co-workers used a model liposome oxidation to investigate the antioxidant activity of many commercial saturated and unsaturated fatty acids, with the former showing higher activities [64].

On the other hand, it is important to note the chemical instability and susceptibility of unsaturated fatty acids to oxidation processes due to light or air exposure, which could also influence the overall results [65]. Interestingly, Fagala and Catalá pointed out that conjugated linoleic acid has a higher capacity to quench DPPH radicals compared to linoleic acid through a facilitated donation of an electron or hydrogen due to the formation of more stable fatty acid radicals through resonance and electron delocalization [66]. The same effect explains why carotenes with multiple conjugated double bonds exhibit a high capacity for electron donation and, thus, higher radical scavenging activities [67], as observed with the analogue with retinoyl moiety **4i**. *All-trans* retinoic acid is already known for its potent antioxidant activity and is widely studied [68-71]. In contrast, conjugation of the double bonds in the side chain of **4a**-**4c** did not contribute to better inhibition of the ABTS·+ radical cation. That assumption is consistent with the study conducted by Chambers *et al.* [72]. The authors recently reported that conjugation of the original molecules (various flavonolignans) with retinoic acid improved antioxidant activity only in some of the hybrids, possibly because of the ester bond [72]. The same was observed in another study, assuming the importance of the amide bond in tryptamine-based conjugates [31].

Moreover, an improvement in activity was obtained after the amide function was reduced [21]. On the other hand, functionalization of the indole ring strongly affects the antioxidant potential of such compounds, leading to changes in ring stability and electronic distribution [55, 59]. This is evident in the research of Venepally *et al*., who synthesized compounds similar to ours and showed a stronger inhibitory effect against radicals *in vitro* [22]. This could be due to the presence of two methoxy groups in the C-4 and C-5 positions of the indole ring, which are electron-donating and, therefore, enhance inhibition activity [73]. Another study showed that the presence of a methoxy group in the C-4 position did not alter the antioxidant capacity of

Table 2. ***In vitro*** **growth inhibitory activity of 4a-4l, 5, tryptamine and 5-FU tested against four human cancer cell lines: SW620, CFPAC-1, MCF-7 and HepG2 and non-transformed fibroblast cell line (HFF-1).**

Sample	IC_{50} (μM)				
	MCF-7	**SW620**	**CFPAC-1**	**HepG2**	**HFF-1**
4a	39.51 ± 11.61	72.95 ± 11.31	37.36 ± 0.13	36.64 ± 3.94	35.75 ± 18.81
4b	25.42 ± 0.35	33.87 ± 1.26	34.79 ± 2.04	25.45 ± 2.04	16.00 ± 0.28
4c	33.56 ± 0.27	39.44 ± 3.97	35.34 ± 7.52	33.18 ± 0.54	28.24 ± 3.65
4d	>100	>100	>100	>100	23.83 ± 2.82
4e	>100	>100	>100	>100	22.52 ± 1.26
4f	>100	>100	>100	>100	1.51 ± 0.41
4g	48.57 ± 0.22	66.81 ± 0.39	34.61 ± 1.90	39.42 ± 2.30	16.64 ± 0.57
4h	50.24 ± 2.88	95.65 ± 18.16	>100	41.40 ± 1.67	41.83 ± 10.73
4i	25.44 ± 1.35	32.42 ± 2.13	39.31 ± 1.54	20.67 ± 1.31	20.07 ± 2.37
4j	31.06 ± 5.43	37.64 ± 6.28	53.13 ± 14.97	29.00 ± 4.35	35.75 ± 6.02
4k	58.04 ± 9.86	81.37 ± 22.97	>100	42.75 ± 15.13	34.21 ± 2.80
4l	33.06 ± 3.61	28.64 ± 8.73	60.21 ± 24.97	47.13 ± 15.84	75.42 ± 5.96
5	>100	>100	>100	>100	75.42 ± 20.66
Tryptamine	>100	>100	>100	>100	62.03 ± 27.98
5-FU	0.01 ± 0.001	0.83 ± 0.03	0.84 ± 0.09	7.18 ± 1.95	0.12 ± 0.02 *

Results obtained on lung fibroblast WI38. Values are expressed as mean value ± standard deviation of triplicate measurement.

the indole derivatives [59]. Therefore, even though the anti-radical mechanisms of indole-derived compounds based on a single electron or hydrogen atom donation have been hypothesized [28, 61], the exact mechanisms need further investigation.

3.2.2. Antiproliferative Activity

As highlighted in the recent reviews, many naturally occurring or synthetic indole derivatives exhibited anticancer properties, both *in vitro* and *in vivo,* due to physio-chemical characteristics of indole ring and are therefore being used to design novel anticancer agents [12, 27, 74, 75]. In accordance with this observation, the antiproliferative activity of granulatamide B and its structural analogues was evaluated using the MTT assay against four human tumour cell lines (breast, colorectal or pancreatic adenocarcinoma and hepatocellular carcinoma) as well as non-transformed cell lines (human fibroblasts). Tryptamine and the standard compound, 5-fluorouracil (5-FU), were used to compare the results obtained. The results are depicted in Table **2**, as the concentration of specific compounds required for 50% inhibition of cell growth (IC_{50} values).

The data show predominantly moderate and non-selective inhibitory activity against the tested cell lines, with the exception of analogues **4d**-**4f** and **5**, which did not show a value comparable to 5-FU, but IC_{50} values in the sub-micromolar and micromolar range. The determined IC_{50} values for granulatamide B (**4b**) against MCF-7, SW620, CFPAC-1 and HepG2 cells were 25.42 ± 0.35, 33.87 ± 1.26, 34.79 ± 2.04 and 25.45 ± 2.04 μM, respectively. Its shorter (**4a**) and longer side chain (**4c**) analogues with the same double bond configuration exhibited similar activity, so chain length did not play a significant role in altering growth inhibition. In 2006, Reyes and his team [17] reported slightly higher activities of granulatamides A and B against 16 human tumour cell lines, but they used a different assay based on the sulforhodamine B reaction, which could be the reason for the differently determined inhibitory activities. Indeed, the mentioned assay does not measure the metabolic activity of the cells as the MTT assay used in our study but is based on the property of sulforhodamine B dye to bind to proteins [76]. However, we also used other cell lines, in particular, nontransformed human fibroblasts (HFF-1) and determined the inhibitory activities against normal cell lines for the

first time. Finally, large variations in the same samples could be explained by different cell cycle synchronization, which could affect the sensitivity of the cells to the tested compounds [77].

Compared with tryptamine, only condensation with unsaturated fatty acids (FA) resulted in antiproliferative activity, with the exception of analogues **4h** and **4k,** which showed no activity against CFPAC-1 cells (IC_{50} > 100 μM). Our results are in agreement with those of Chávez and co-workers, who also observed no activity of *N*-acyltryptamine bearing saturated chain, including our analogue **4f** against cancer cells [18]. Numerous studies have demonstrated the anticancer properties of various fatty acids and their conjugates [33, 78-80]. It has been shown that only saturated fatty acids with short-carbon chains (less than 10C) exhibit anticancer activity, whereas the opposite effect was observed for unsaturated acids, where the position of double bonds plays an important part as well [33]. However, in our study, neither the chain length nor the number of double bonds had a strong influence on the results. It should be noted, however, that only two ω-6 acids (arachidonic and linoleic acids) and one ω-3 acid (alpha-linolenic acid) were involved, so more diverse unsaturated acids should be included in the future to conclude with certainty to what extent the structural differences of the acids contribute to the activity.

Importantly, conjugation of double bonds, as presented by analogue **4i** with retinoyl moiety alongside analogues **4a**-**4c** with conjugated double bonds, does not appear to contribute remarkably to antiproliferative activity compared to compounds with an unsaturated side chain. Nevertheless, **4i** exhibited IC_{50} values comparable to those of granulatamide B (**4b**), being 25.44 ± 1.35, 32.42 ± 2.13, 39.31 ± 1.54, and 20.67 ± 1.31 μM for MCF-7, SW620, CFPAC-1 and HepG2, respectively. In addition, the anticancer activity of all-*trans* retinoic acid (ATRA) against a number of different cell lines *in vitro* and *in vivo* is already known and has been extensively studied [81-83]. The activity of ATRA based on the binding to the nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR) results in various pharmacological effects, ranging from reduction of angiogenesis, inhibition of metastasis, secretion of cytotoxic cytokines, promotion of apoptosis and inhibition of cancer cell proliferation [84]. Despite numerous studies and a broad knowledge of its anticancer activity, ATRA is currently being used only in the treatment of promyelocytic leukemia [85]. One of the main disadvantages of ATRA is its poor water solubility, low bioavailability and potential precipitation in the gastrointestinal tract, which can be avoided by conjugation with other molecules (such as tryptamine in our study), contributing to better biological activity [81].

Interestingly, *N*-(4-Hydroxyphenyl)retinamide, which is also a synthetic amide of ATRA, has been used in numerous clinical trials against various cancers with some promising results [86-88]. Such modifications of ATRA usually lead to self-assembly and formation of micelles, which opens a new direction in the field of nanotechnology and targeted drug delivery [81]. In addition, a recent study by a Czech group found a correlation between cell toxicity and aggregation of tryptamine amides of oleanolic acid [89].

Although acetamides have been demonstrated to have anticancer properties [90], analogue **5** (*N*-acetyltryptamine) was found to be inactive against the cancer cell lines tested in our studies. This may be due to the fact that the indole ring was not modified, as melatonin, a 5-methoxy derivative of **5,** has been shown to stimulate apoptosis, regulate survival signaling, and suppress angiogenesis and metastasis, both *in vivo* and *in vitro* [91-93]. Research data from Venepally and co-workers [22] support this assumption, as they synthesized eight *N*-acylamide derivatives of 4,5-dimethoxy tryptamine. The derivative containing an octanoyl moiety (similar to our **4d,** which showed no inhibition of cancer cell growth) exhibited cytostatic activity against 3 cancer cell lines, including HepG2, which was also used in this study, while the derivative containing an amide bond with oleic acid (similar to our **4k**) showed the highest activity (IC_{50} < 15 μM). Moreover, their compounds were also less active against normal cell lines. Therefore, functionalization of the indole ring, especially with electron-donating groups, could be crucial for better activity and/or selectivity against cancer cells.

As shown in Table **2**, all tested compounds were also active against HFF-1, with analogues **4l** and **5** being the least active (IC_{50} values: 75.42 ± 5.96 and 75.42 ± 20.66 μM, respectively). Accordingly, a selectivity index (SI) was determined and presented in Table **3**, with values below 2 indicating general toxic effects [42]. SI for compounds **4a**-**4c** and **4g**-**4k** was below than 1 for all cell lines tested, except for analogue **4l** which had the highest indexes of 2.28 and 2.63 for MCF-7 and SW620, respectively. Thus, the selectivity of **4l** over MCF-7 was lower than that of the standard drug, 5-FU (SI=12), whereas it was higher for SW620. However, the activity of 5-FU against another type of fibroblast (WI38) must be taken into account.

Table 3. Selectivity index (SI) of 4a-4l, 5, tryptamine and 5-FU tested against four human cancer cell lines: SW620, CFPAC-1, MCF-7 and HepG2.

Sample	SI			
	MCF-7	**SW620**	**CFPAC-1**	**HepG2**
4a	0.90	0.49	0.96	0.98
4b	0.63	0.47	0.46	0.63
4c	0.84	0.72	0.80	0.85
4d	-	-	-	-
4e	-	-	-	-
4f	-	-	-	-
4g	0.34	0.25	0.48	0.42
4h	0.83	0.44	-	1.01
4i	0.79	0.62	0.51	0.69
4j	1.15	0.95	0.67	1.23
4k	0.59	0.42	-	0.80
4l	2.28	2.63	1.25	1.60
5	-	-	-	-
Tryptamine	-	-	-	-
5-FU	12	0.14	0.14	0.02

3.2.3. Antibacterial Activity

In addition to anticancer properties, tryptamine-based compounds have also shown promising antibacterial activities due to the presence of the pyrrolidine ring of the indole backbone [57, 94, 95]. Moreover, the secondary amide group of tryptamides is crucial for the formation of hydrogen bonds in the active site and resulting interaction with the bacterial cell [94]. To date, several tryptamine-containing antibiotics have been studied and reported, such as nematophin [94, 96, 97], but melatonin is also known to exhibit antibacterial activity *via* some known biological mechanisms, as recently discussed in a comprehensive review by He and co-workers [98].

In our study, tryptamine-based compounds showed mostly partial inhibition against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*). Nevertheless, the minimum inhibitory concentrations (MICs) were observed for **4a** against *B. subtilis* (1000 μM) and granulatamide B (**4b**) against *B. subtilis* and *S. aureus* (125 and 1000 μM, respectively) (Table **4**). Therefore, **4b** was more selective towards Gram-positive bacteria. This is consistent with the fact that coral extracts were more active against non-marine and Gram-positive bacteria [99], considering that **4b** was originally isolated from the coral species *Eunicella granulata*. In our previous study, extracts and fractions from *E. cavolini* were also more selective towards Gram-positive bacterial strains [38]. Additionally, non-proteinaceous, which include indoles, are known to be more active against Gram-positive than Gram-negative bacteria [96, 100]. However, a broader bacterial panel needs to be included in the future in order to confirm the above-discussed observations. Contrary, the MIC for **4c** was not observed to be within the reported concentration range against any of the bacterial strains tested. However, it has the same double bond configuration as its shorter counterparts, **4a** and **4b**. It appears that even the conjugation of the double bonds did not significantly contribute to the improvement of antibacterial activity, as the MICs for **4i** were above the highest concentration tested (>1000 μM). Nevertheless, the orientation of the double bonds is of great importance since naturally occurring fatty acids with *cis*-orientation have greater antibacterial properties than those with *trans*-orientation [101], such as retinoic acid used to obtain compound **4i**. On the other hand, *N*-acetyltryptamine (**5**) was observed to have little chemical activity, although some studies [102, 103] showed antibacterial activity of this molecule. However, in these studies, the authors used the disc diffusion bioassay, so no MIC values were obtained.

Table 4. Minimum inhibitory concentrations (MICs) of 4a-4l, 5 and tryptamine (TR) against *Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Sample	Minimum inhibitory Concentration (μM)			
	B. subtilis	***S. aureus***	***P. aeruginosa***	***E. coli***
4a	1000	>1000	>1000	>1000
4b	125	1000	>1000	>1000
4c	>1000	>1000	>1000	>1000
4d	>1000	>1000	>1000	>1000
4e	>1000	>1000	>1000	>1000
4f	>1000	>1000	>1000	>1000
4g	>1000	>1000	>1000	>1000
4h	>1000	>1000	>1000	>1000
4i	>1000	>1000	>1000	>1000
4j	>1000	>1000	>1000	>1000
4k	>1000	>1000	>1000	>1000
4l	>1000	>1000	>1000	>1000
5	>1000	>1000	>1000	>1000
TR	>1000	>1000	>1000	>1000
NRL	0.25	0.5	-	-
AMK	-	-	8	-
CLP	-	-	-	4

Table 5. Classification of radionuclides and co-ordinating ligand groups, as a function of their chemical nature: hard, soft, or intermediate [1, 4].

a)					
	Sample	**LC_{50} (μM)**	**96% CI**	**Hill Slope**	**R^2**
	4a	6.25	5.900-6.624	4.822	0.9904
	4b	44.34	42.46-46.32	4.839	0.9924
	4d	28.64	25.96-32.35	4.167	0.9807
	4i	36.51	34.82-38.29	6.962	0.9981
b)					
	Sample	**EC_{50} (μM)**	**96% CI**	**Hill Slope**	**R^2**
	4a	4.758	4.172-5.427	4.557	0.9878
	4b	39.90	34.67-52.92	5.328	0.9603
	4d	22.62	19.82-25.62	4.465	0.9786
	4i	10.59	8.007-12.18	2.933	0.9841

Furthermore, fatty acids are known to have remarkable antibacterial activity against both Gram-positive and Gram-negative bacteria [101, 104]. In addition, due to their amphiphilic nature, fatty acids attack the cell membrane by reacting with phospholipids, encapsulating them, and eventually causing a violation of membrane stability, leading to growth inhibition and death [104]. However, the efficacy and selectivity of this activity depend on several parameters that affect the structure and shape, such as chain length of the chain or the presence of double bonds. Nevertheless, the conclusions cannot be drawn straightforwardly since there is a discrepancy between results showing in some cases better antibacterial and/or antimicrobial activity with increasing chain length in some cases, whereas in others, they show a more moderate chain length [105-107]. Finally, as already pointed out, the hydroxyl group of the free fatty acids seems to be cru-

cial for the antibacterial activity [101], possibly explaining the lower activities of our compounds.

Similar compounds, fatty acyl derivatives of dimethoxy-tryptamine also showed low activities against a group of seven different pathogenic bacterial strains, with the exception of two derivatives with MIC < 50 µg/mL against *B. subtilis* [22]. The main difference is the modification of the indole ring, as already discussed in sections 2.2.1 and 2.2.2. This may be precisely the key to enhancing antibacterial activity, considering that the substitution of the indole nitrogen with alkyl, aryl or benzyl groups resulted in higher antistaphylococcal activity [97]. Moreover, an antibiotic nematophin, which has a high structural similarity to the compounds tested in our study but differs in the presence of the α-ketoamide structural motif, showed excellent activity against *S. aureus* [94]. Interestingly, cationic amphiphiles have been shown to have higher activity against Gram-negative bacteria due to the electrostatic interactions with the anionic bacterial cells [108]. Therefore, we can assume that a positively charged indole ring in our compounds could contribute to better activity. Thus, interventions in the indole core and side chain are key to improving antibacterial properties in the future.

3.2.3. Evaluation of Embryotoxicity in vivo

The zebrafish (*Danio rerio*) model is increasingly used to evaluate the toxicity of novel molecules in the early stages of drug development because of numerous advantages, as Cassar and co-workers have detailed [109]. Of the compounds tested in our study, 4 of 13 harmed the survival and embryonic development of the zebrafish *D. rerio* within the concentration range tested. The highest toxicity in terms of mortality was observed at **4a** (LC_{50}=6.25 µM) and **4d** (LC_{50}=28.64 µM), followed by **4i** (LC_{50}=36.51 µM) and **4b** (LC_{50}=44.34 µM). The highest incidence of developmental abnormalities was observed in samples **4a** and **4i**, which had EC_{50} values of 4.76 and 10.59 µM, respectively (Fig. **1**, Table **2**). The most common abnormalities observed during exposure to **4a**, **4b**, **4d**, and **4i** were edema (Fig. **2B**, **c**), scoliosis (Fig. **2B**), and blood accumulation (Fig **2c**). The lethality of the negative control group was less than 5%.

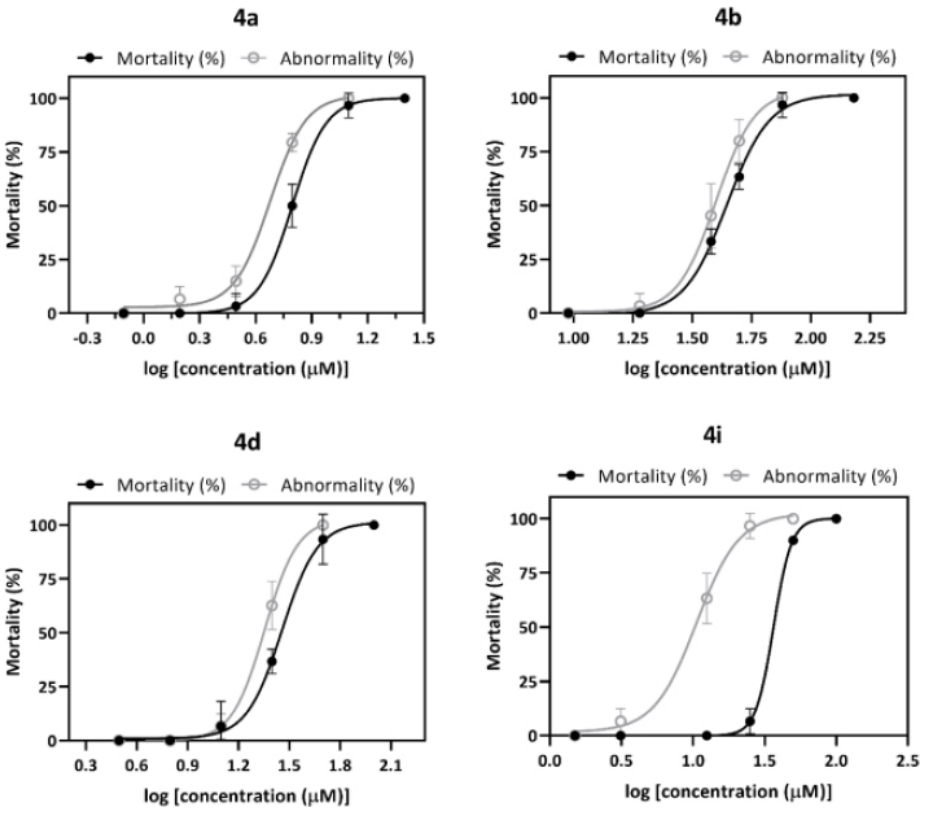


Fig. (1). Concentration-response curves used for the calculations of the *D. rerio* (n=30): mortality and abnormality rates after 96 h of exposure to **4a**, **4b**, **4d**, and **4i**. Error bars indicate standard deviations (SD). Dose-response curves were generated using GraphPad Prism software version 6.0.

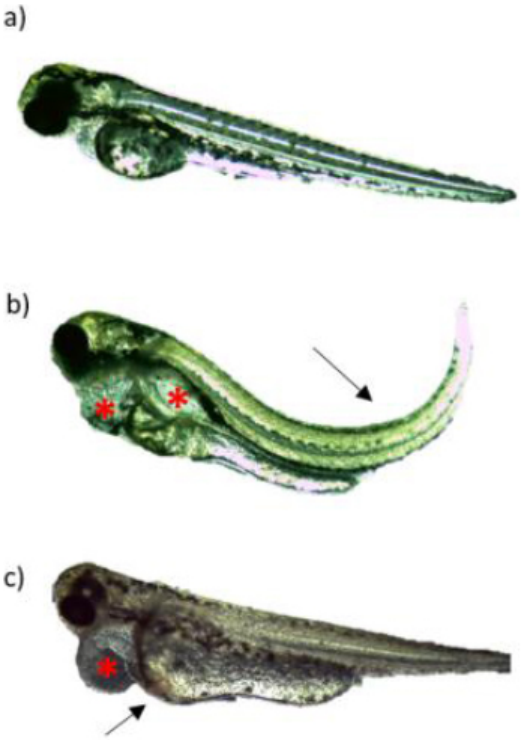


Fig. (2). The most commonly observed morphological changes during a 96-h exposure to **4a**, **4b**, **4d**, and **4i. a**) normally developed larva in the control treatment group; **b**) edema (asterisks), scoliosis (arrow); c) edema (asterisk), blood accumulation (arrow), decreased yolk sac consumption. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

To date, little research has been conducted on the *in vivo* toxicity of *N*-fatty acyl tryptamines, especially using the zebrafish model, making our study of even greater importance. A mixture of 8 tryptaminamides, including our compound **4f,** was tested on the brine shrimp *Artemia salina* and showed no toxicity (LC_{50} > 500 μg/mL) [18]. Interestingly, three of the four most toxic compounds we studied contained an unsaturated aliphatic chain composed of conjugated double bonds, including ATRA. In addition, few studies have been performed to evaluate the effects of retinoic acid and its derivatives on zebrafish embryonic development, including some other consequent biological activities [110-113]. The teratogenic effects of ATRA and related compounds on zebrafish embryos were previously investigated by Herrmannin 1995. The author reported that ATRA at the highest concentration of 1 μM caused several malformations, including brain deformations oedema and a shortened and bent tail [110]. In addition, we have observed no developmental abnormalities with an ATRA derivative, **4i**, at a concentration of 1.6 μM (data not shown). Furthermore, a 2014 Chinese study found that treatment of zebrafish embryos with a higher dose of retinoic acid resulted in dose-dependent malformations associated with cell apoptosis in the eye and tail regions, while nanomolar concentrations of the same acid produced neurotoxicity and behavioral changes [112]. The authors also found LC_{50} and EC_{50} values of retinoic acid to be 17.31 and 2.47 nM, respectively, which are much lower than the values we found for the retinoic acid derivative **4i**.

The toxicity of unsaturated and saturated fatty acids was studied using the brine shrimp model. This revealed the high toxicity of linoleic and linolenic acids used for the synthesis of compounds **4g** and **4l**, with reported LC_{50} values around 10 μM [114]. In contrast, in our study, these acids showed no toxic consequences when conjugated with tryptamine, even at the highest concentrations of 521 and 400 μM, respectively. Furthermore, lipids, including mono- or polyunsaturated fatty acids, affect a variety of physiological processes and are known for their beneficial effects on both embryonic and larval zebrafish development, as detailed by Quinlivan and Farber [115]. Therefore, the zebrafish model was used to examine the dietary effects and consequences of treatment with various fatty acids, focusing with an emphasis on omega-6 and omega-3 fatty acids that resulted in alterations in gene expression and cell signaling [116]. For instance, an omega-6 arachidonic acid (AA) used to prepare compound **4h** displayed resorptive effects by stimulating matrix metalloproteinases important for skeletal formation [117] accompanied by an improvement in immunity and resistance to *Streptococcal* infection [118] in zebrafish. Contrary, one-hour treatment of zebrafish larvae with 80 μM AA resulted in moderate thrombosis and cardiovascular toxicity [119] or oxidative stress and lipid peroxidation were observed even after AA-enriched diets [120], clearly indicating the need for further research of the biological activities of omega-6/omega-3 unsaturated fatty acids.

On the other hand, zebrafish have been used to study other biological properties of tryptamine-like derivatives. For instance, Savoldi and colleagues observed behavioral changes in zebrafish induced by ayahuasca brew, which is known to contain *N,N*-dimethyltryptamine (DMT). Moreover, ayahuasca reduced anxiety-like behaviors in zebrafish at low concentrations but reduced locomotion and increased anxiety at higher concentrations [121]. Furthermore, tryptamides were shown to exhibit neuroprotective properties in zebrafish larvae by reversing locomotion deficiency and neurotoxicity after incubation with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [122]. Another study also showed potentially neuroprotective

properties of *N*-fatty acyl tryptamines in conjunction with antioxidant activity [21]. This is related to the findings of Kumari and co-workers [123] of a potential antiepileptic activity after treatment of zebrafish embryos with omega-3, α-linolenic acid, whose aliphatic chain is presented in **4l,** and suggests a new avenue to study neurodegenerative diseases.

CONCLUSION

In this study, a naturally occurring marine secondary metabolite, granulatamide B (**4b**), was synthesized along with 12 structural analogues and screened for antiproliferative, antioxidant, and antibacterial activities *in vitro* and embryotoxicity *in vivo*. All the compounds consisted of the same indole ring (tryptamine moiety) and an amide bond but differed in side-chain structure (chain length, degree of saturation and conjugation of double bonds where possible). The results revealed that the conjugation of different acyl moieties with tryptamine resulted in a decrease in antioxidant activity except for the compound that is a derivative of retinoic acid (**4i**). Subsequently, **4b** and some of the analogues (**4a**, **4c** and **4i**), mostly containing unsaturated chains and conjugated double bonds, exhibited moderate but not selective activity against both cancer and normal cell lines with determined IC_{50} values in the range of 20-40 μM. In addition, the growth of Gram--positive and Gram-negative bacteria was only partially inhibited by the tested compounds, with only the MIC value of 125 μM for **4b** standing out. Finally, despite their potential in terms of antioxidant, antiproliferative and antibacterial activities, compounds **4b** and its analogue **4i** also caused remarkable developmental abnormalities in zebrafish *D. rerio*. Therefore, a compound such as **4l** could be used as a derivative of alpha-linolenic acid and could be used for further development and testing, especially as an anticancer agent, since it has been shown to be least active against normal cell lines and more selective against cancer cells. In conclusion, the substitution of the indole ring, especially by electron-donating groups, needs to be considered as it might be crucial for improved biological activity and lower toxicity. Furthermore, our group is currently carrying out new syntheses focusing on the functionalization of the indole ring.

LIST OF ABBREVIATION

TLC	=	Thin layer chromatography
m.p.	=	Melting points
DMSO	=	dimethyl sulfoxide
CMP	=	chloramphenicol

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Croatian Ministry of Agriculture (HR-POK-023) and according to the Directive 2010/63/EU.

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. The embryotoxic potential of tested samples was determined by performing the zebrafish embryotoxicity test (ZET) following the OECD 203 Guideline (2013).

All methods are reported in accordance with ARRIVE guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data are available from the corresponding authors upon reasonable request. Samples are available from the corresponding authors for a limited period.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Information regarding spectroscopic analyses (^{1}H and ^{13}C NMR, HRMS, LC-MS and IR) for all the compounds (**2a-2c**, **3a-3c**, **4a-4l** and **5**) can be found in the supplementary document.

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