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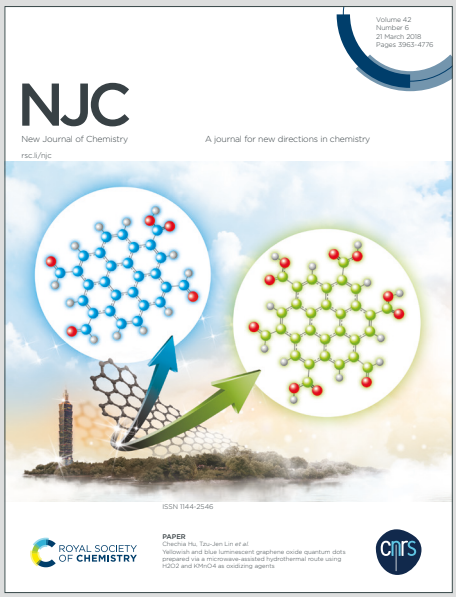

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ARTICLE

Design and functionalization of bioactive benzoxazines. An unexpected *ortho*-substitution effect

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A family of *o*, *p*- and N-functionalized dihydro-2H-benzo-1,3-oxazines was synthesized and characterized. In vitro antimicrobial activity of the new benzoxazines was assessed against pathogenic fungi and Gram-negative and Gram-positive bacteria. The screened compounds showed significant in vitro antimicrobial effect, but introduction of the bulky substituent in the *ortho* position of the aryl ring caused a loss of their antibacterial efficacy. Cytotoxic assay results revealed that a molecule containing long alkyl chain substituents offered a remarkable viability of mouse fibroblast cells. Additionally, the liquid nature of alkyl-chain-modified monomers and the high processing windows indicate their high application potential.

Introduction

A search for new antibacterial compounds is a challenging task since bacteria continuously develop resistance to such compounds. Infections due to such bacterial strains are infrequent but potentially dangerous or fatal, especially for immunosuppressed patients. Similarly, fungal infections are a commonly observed complication in the course of many diseases. Therefore, it is still essential to modify the structural motif of biologically active compounds or design novel motifs showing a similar or better activity. Compounds based on the 1,3-benzoxazine architecture show different antimicrobial properties, e.g. bactericidal or fungicidal, and exhibit a wide spectrum of pharmacological features, such as antitumor, anti-tuberculosis, antimalarial, or anthelmintic activity.[1] Therefore, these types of compounds have been an unceasingly important subject of interdisciplinary investigations. In addition, N-substituted 3,4-dihydro-2H-1,3-benzoxazines are monomers to form polybenzoxazines, which have gained immense attention because of their excellent mechanical and thermal properties.[2] Hence, the search for new functional benzoxazines is still of great importance. Here, we report on the synthesis and characterization of new benzoxazines as potential antibacterial agents and valuable monomers for polybenzoxazine formation.

Experimental section

General

Solvents for reactions were purified by standard methods: THF and triethylamine were distilled from Na/benzophenone. Other solvents (also for workup) were used as received. Column chromatography was performed on silica gel 60 Å 230-400 mesh (Macherey Nagel) and Macherey Nagel TLC plates (silica gel 60 Å, UV 254). All chemicals were obtained from commercial sources and used without further purification: 4-*tert*-buthylphenol (99%), 2,4-di-*tert*-buthylphenol (99%), dodecylamine (98%), *p*-cresol (98%), 4-(2,4,4-trimethylpentan-2-yl)phenol (98%), *p*-bromophenethylamine (98%), formaldehyde (37% solution in H_2O) were purchased from Aldrich. $[Pd(PPh_3)_2]Cl_2$ (Aldrich, 98%), CuI (Aldrich, 99.9%), ethynyl(trimethyl)silane (Fluorochem, 97%), K_2CO_3 (anhydrous, Aldrich, 99%). The ^1H, ^{13}C NMR spectra were obtained using Bruker Avance 500 MHz spectrometer. The chemical shifts are given in ppm relative to the residual signals of the solvent ($CDCl_3$, ^1H: 7.26 ppm, ^{13}C: 77.2 ppm). HRMS spectra were recorded using Bruker MicOTOF-Q spectrometers with ESI ion source and time-of-flight mass analyzer. IR spectra were recorded using a Bruker 66/s FTIR spectrometer. Microanalyses were conducted with an Elementar CHNS Vario EL III analyzer. Melting points were characterized by differential scanning calorimetry conducted using a Mettler-Toledo DSC 3 from 30 to 250 °C at a heating rate of 10 °C/min

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Electronic Supplementary Information (ESI) available: spectroscopic data (^1H, $^{13}C\{^1H\}$ NMR, spectra, X-ray experimental data and refinement, antimicrobial data. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/x0xx00000x

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under nitrogen. Thermogravimetric analysis (TGA) was determined with a SETARAM Setsys TG-DTA 16/18 at a heating rate of 10 °C/min under N_2.

X-ray data collection and reduction

X-ray diffraction data for a suitable monocrystal of the measured samples were collected using a KUMA KM4 CCD and Xcalibur CCD Onyx or Ruby (see ESI, Table S1) with ω scan technique. The data collection and processing utilized CrysAlis suit of programs.[3] The space groups were determined based on systematic absences and intensity statistics. Lorentz polarization corrections were applied. The structures were solved by direct methods and refined by full-matrix least-squares on F^2. All calculations were performed using the SHELXTL-2013 suite of programs.[4] All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atom positions were calculated with geometry and fixed. Thermal ellipsoid plots were prepared with 30% probability displacements for non-hydrogen atoms using Mercury 3.1 program.[5] All data have been deposited with the Cambridge Crystallographic Data Centre CCDC-1547956 for **1**, -1547957 for **2**, -1547955 for **3**, -1835726 for **4**, -1860484 for **8**, -1835932 for **12**, -1860482 for **13**, -1860480 for **15**, -1835916 for **16**, -1860481 for **20**, and -1860483 for **21**. Copies of the data can be obtained free of charge by application to CCDC, 12 Union Road, Cambridge CB21EZ, UK or e-mail: deposit@ccdc.cam.ac.uk.

Biological assay, Antimicrobial screening , Test microorganisms

The compounds **1-8** were tested against a panel of microorganisms including Gram-positive cocci: *Staphylococcus aureus* PCM 2054, *S. pseudintermedius* KP-Spi1, *Streptococcus agalactiae* KP-Sag1, *S. canis* KP-Sac1; Gram-positive endospore forming rods: *Bacillus megaterium* PCM 1400, *B. subtilis* PCM 1949, *B. cereus* PCM 1948, *B. mycoides* PCM 2024; Gram-negative rods: *Escherichia coli* PCM 2057, *Pseudomonas aeruginosa* PCM 2058, *Salmonella galinarum* KP-Sg1, *Proteus vulgaris* PCM 542; plant pataogenes: *Pectobacterium carotovora* sbp. *carotovora* IOR (strain 1822 and 1815), *P. atrosepticum* IOR (strain 1826 and 1825); yeasts: *Candida krusei* KW-F117, *C. albicans* KP-Ca1, *C. glabrata* KP-Cg1; filamentous fungi: *Fusarium culmorum* KW-F, *F. moniliforme* KW-Fm1, *Aspergillus niger* KW-An1, *A. flavus* KW-Af1, *Trichoderma harzianum* sbp. *aggressivum* KW-Tha1. The strains came from the following collections: KW - own collection (Agricultural Microbiology Lab, Department of Plant Protection, University of Environmental and Life Sciences, Wrocław, Poland), KP - Microbiology Lab, Department of Pathology (University of Environmental and Life Sciences, Wrocław, Poland), PCM - Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland), IOR - Culture Collection of Plant Pathogens at Institute of Plant Protection (Poznań, Poland). Cultures of bacteria were maintained on TSA (Tryptic Soy Agar, Fluka, Sigma-Aldrich), yeast and filamentous fungi on PDA (Potato Dextrose Agar, Emapol).

Cells

Mouse embryo fibroblasts (BALB/3T3 clone A31) were purchased from Sigma-Aldrich.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal (MCB) and fungicidal (MFC) concentration

The minimum inhibitory concentrations (MIC) were determined by a serial dilution method in 48-well plates (Tissue Culture Plates VWR European).[6a] The bacterial strains were grown on Mueller-Hinton Agar (MHA, Sigma-Aldrich) at 35±2 °C for 24h but plant pathogens of *Pectobacterium* spp. were incubated at 28 °C. The fungal species were grown on Sabouraud Dextrose agar (SDA, Oxoid, Thermo Scientific) at 28±2 °C for 24 h (yeast) or 48 h (filamentous fungi). Optical density of bacterial and yeast suspension was standardized to 0.5 McFarland (spectrophotometer VIS-723G, Rayleigh, Beijing). Suspensions turbidity of the molds were confirmed by viable counting in a Thoma chamber. The final inoculum size was 5×10^5 cfu×cm^{-3} for the antibacterial assay and 1×10^4 cfu×cm^{-3} for the antifungal assay. Compounds were dissolved in dimethyl sulfoxide (DMSO) and tested at a concentration range from 400 to 6,25 μg×cm^{-3}. Mueller-Hinton Broth (for bacteria) or Sabouraud Dextrose Broth (for fungi) were used for serial dilution. Tetracycline (Sigma-Aldrich) and Aphotericin B (Gibco, Life Technologies) were used as negative controls (at the dose of 30 μg×cm^{-3}) and solvent (DMSO) as positive control. The microbial growth was visualized by adding 50 μl of rezasurine aqueous solution on well (pink colour of medium indicates growth of microorganisms and blue means inhibition of growth).[6b] Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the tested chemical compounds that inhibited visible growth, while minimum bactericidal/fungicidal concentration (MBC/MFB) was defined as the lowest compounds concentration that killed 99% of microorganisms cells.[6c] To determine MBC/MFC, cultures were taken from each well without visible growth and inoculated in MHA for bacteria or in SDA for yeasts and molds (time and temperature of incubation as described above). The experiments were done in 3 series at 3 repetition.
The data were subjected to analysis of variance using the Tukey test ($p > 0.05$) using STATISTICA 12.0 software for Windows. All data were presented as the mean values ± standard deviation (SD).

Preparation of resazurin solution

300 mg resazurin sodium salt (Sigma-Aldrich) was dissolved in 40 ml sterile water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.[6b]

Agar disk diffusion assay

The antimicrobial activity of **1-8** was evaluated by the disc diffusion technique by determination of growth inhibition zones.[7] Briefly, a volume of 100 μL of suspension of the test microorganisms containing 1.5×10^8 μg×cm^{-3} of bacteria or 1.5×10^6 μg×cm^{-3} of yeast was spread on Mueller Hinton Agar

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or Sabouraud's dextrose agar medium, respectively. The turbidity of the tested strains was adjusted according to Mac-Farland (spectrophotometer VIS-723G, Rayleigh, Beijing) scale 0.5 which was used as standard inoculum. Paper discs (Whatman no. 1, England, 6 mm diameter) were placed on the agar surface and impregnated with 20 µL of stock solutions of 50 and 100 µg×cm^{-3}. The solvent of dimethylsulfoxide (10% DMSO) was used as a negative control. In addition, filter discs impregnated with 30 µg/mL tetracycline (Sigma-Aldrich) or Aphotericin B (Gibco, Life Technologies) were used as positive reference control for bacteria and fungi, respectively. The plates, after remaining at 4 °C for 2 h, were incubated at 35±2 °C (20 h) for bacterial strains and at 28±2 °C (48 h) for yeasts. All tests were performed in triplicate and susceptibility was assessed by measuring the zone of inhibition.

Cytostatic activity

The cytotoxicity evaluation of the lead compounds against mammalian cells on fibroblast 3T3 mouse BALB was determined by SRB method.[8] Cells were grown in the culture media recommended by cell line supplier. The cells 3T3BALB were grown at 37 °C in DMEM Medium supplemented with 10% (v/v) foetal bovine serum (FBS) and antimycotic and antibacterial solutions (Lonza) in humidified atmosphere having 5% CO_2. Before the test, adherent cells were detached with the trypsin EDTA solution, washed twice in phosphate-buffered saline (PBS), spun down, counted, stained with a 0.4 % solution of trypan blue, and inspected under a microscope for cell viability. Then, cells were plated on 96-well plastic culture plates (2×10^3 cells/well in 200 µl DMEM medium) and incubated at 37 °C in a CO_2-incubator for 24 h, afterwards, the tested compound were added in final concentration 5-100 µg×cm^{-3} and the cultures were incubated for 48 h in CO_2-incubator at 37 °C. Then, cells were harvested and intended for cell proliferation test.

Cell density/cell proliferation was estimated with the sulforhodamine B (SRB)-colorimetric assay. Briefly, cell cultures were fixed with cold TCA (final concentration 10% (w/v)) in cultures of adherent cells for 1 h at 4 °C, then washed four times with tap water and air-dried at room temperature (20-25 °C). A mildly acidic SRB solution (0.4% dye solution in 1% acetic acid) was added to each well for 30 min at 25 °C and then, unbound stain was removed by rinsing with an aqueous solution of 1% (v/v) acetic acid. Culture plates were then allowed to dry at room temperature. The SRB bound to the intracellular proteins was dissolved in 10 mM Trizma-base solution (pH 10.5) for 10 min on a gyratory shaker and absorbance of the SRB solution was estimated at 540 nm in a Victor 2 microplate reader (Perkin-Elmer, MA, USA).

Microscopic evaluation of cell culture was performed in the detection of cytopathic and cytotoxic effects. Changes in the cells (vacuolization, change of shape) testify to the toxicity of test compounds for cell culture. To assess cell viability of mouse fibroblast, was performed staining using the LIVE / DEAD Cell Imaging Kit and evaluated in the Evos microscope (FL, ThermoFisher Scientific). The dyed cells live in green (FITC, ex 488 / em 525) and dead in red (Texas Red, ex531 / em593) (manual of kit).

Synthetic procedures

***3-(4-bromophenethyl)-6-(tert-butyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine*, [1]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of 4-*tert*-buthylphenol (1.362 g, 9.07 mmol) in 20 mL of methanol, *p*-bromophenethyloamine (1.41 mL, 9.07 mmol) and formaldehyde 37% solution in water (24.2 mmol, 1.80 mL) were added. The resulting mixture was heated at reflux for 72 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol and put in freezer (-30 °C). After 2 days white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give colorless crystals. Yield: 1.629 g (4.35 mmol), 48%; mp 73 °C. HRMS(ESI): calcd for $C_{20}H_{25}BrNO$: 374.1114 [M + H]$^+$, found 374.1113. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.41 – 7.38 (m, 2H, ArC*H*), 7.15 (dd, J_{HH} = 8.6, 2.5 Hz, 1H, ArC*H*), 7.11 – 7.08 (m, 2H, ArC*H*), 6.94 (d, J_{HH} = 2.4 Hz, 1H, ArC*H*), 6.72 (d, J_{HH} = 8.6 Hz, 1H, ArC*H*), 4.84 (s, 2H, O-C*H*$_2$-N), 4.01 (s, 2H, Ar-C*H*$_2$-N-), 3.03-3.01 (m, 2H, CH_2-C*H*$_2$-N), 2.85 – 2.82 (m, 2H, C*H*$_2$-CH_2-N), 1.28 (s, 9H, C(C*H*$_3$)$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 (s), 143.5 (s), 139.0 (s), 131.6 (s), 130.6 (s), 124.8 (s), 124.2 (s), 120.1 (s), 119.4 (s), 116.0 (s), 82.4 (s), 52.9 (s), 50.9 (s), 34.4 (s), 34.2 (s), 31.7 (s). IR (cm^{-1}, nujol mull) 1229 (C-O-C), 938 (out-of-plane bending of C–H in benzoxazine).

***3-(4-bromophenethyl)-6-(2,4,4-trimethylpentan-2-yl)-3,4-dihydro-2H-benzo[e][1,3]oxazine*, [2]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of 4-(2,4,4-trimethylpentan-2-yl)phenol (1.359 g, 6.58 mmol) in 40 mL of methanol, *p*-bromophenethyloamine (1.02 mL, 6.58 mmol) and and formaldehyde 37% solution in water (17.6 mmol, 1.31 mL) were added. The resulting mixture was heated at reflux for 48 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol and put in freezer (-30 °C). After 24 h white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give colorless crystals. Yield: 1.947 g (4.52 mmol), 69%; mp 71 °C. HRMS(ESI) calcd for $C_{24}H_{3r}BrNO$: 430.1740 [M + H]$^+$, found 430.1738. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.41 – 7.38 (m, 2H, ArH), 7.11 (dd, J_{HH} = 8.6, 2.4 Hz, 1H, ArH), 7.09 – 7.06 (m, 2H, ArH), 6.91 (d, J_{HH} = 2.3 Hz, 1H, ArH), 6.69 (d, J_{HH} = 8.6 Hz, 1H, ArH), 4.85 (s, 2H, O-CH_2-N), 4.00 (s, 2H, Ar-CH_2-N), 3.02 – 2.97 (m, 2H, CH_2-C*H*$_2$-N), 2.84 – 2.81 (m, 2H, C*H*$_2$-C*H*$_2$-N), 1.67 (s, 2H, C*H*$_2$), 1.33 (s, 6H, C*H*$_3$), 0.73 (s, 9H, C*H*$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 151.8 (s), 142.4 (s), 139.1 (s), 131.6 (s), 130.6 (s), 125.7 (s), 125.1 (s), 120.1 (s), 119.0 (s), 115.7 (s), 82.5 (s), 57.2 (s), 53.0 (s), 50.9 (s), 38.1 (s), 34.4 (s), 32.5 (s), 31.9 (s), 31.7 (s). IR (cm^{-1}, nujol mull) 1234 (C-O-C), 937 (out-of-plane bending of C–H in benzoxazine).

***3-(4-bromophenethyl)-6-methyl-3,4-dihydro-2H-benzo[e][1,3]oxazine,* [3]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of *p*-cresol (1.369 g, 12.66

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mmol) in 40 mL of methanol, *p*-bromophenethyloamine (1.96 mL, 12.66 mmol) and formaldehyde 37% solution in water (26.7 mmol, 2.0 mL) were added. The resulting mixture was heated at reflux for 72 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol and put in freezer. After 2 days white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give colorless crystals. Yield: 3.085 g (9.29 mmol), 73%; mp 94 °C. HRMS(ESI) calcd for $C_{17}H_{19}BrNO$: 332.0644 $[M + H]^+$, found 332.0649. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.39 (d, J_{HH} = 8.3 Hz, 2H, Ar*H*), 7.08 (d, J_{HH} = 8.2 Hz, 2H, Ar*H*), 6.92 (d, J_{HH} = 7.7 Hz, 1H, Ar*H*), 6.76 (s, 1H, Ar*H*), 6.68 (d, J_{HH} = 8.3 Hz, 1H, Ar*H*), 4.84 (s, 2H, O-CH_2-N), 3.98 (s, 2H, Ar-CH_2-N), 3.00 (t, J_{HH} = 7.5 Hz, 2H, CH_2-CH_2-N), 2.82 (t, J_{HH} = 7.5 Hz, 2H, O-CH_2-N-), 2.25 (s, 3H, CH_3).^{13}C NMR (151 MHz, $CDCl_3$) δ: 152.1 (s), 139.0 (s), 131.6 (s), 130.6 (s), 130.0 (s), 128.5 (s), 127.9 (s), 120.1 (s), 119.9 (s), 116.3 (s), 82.5 (s), 52.9 (s), 50.6 (s), 34.4 (s), 20.7 (s). IR (cm^{-1}, nujol mull) 1226 (C-O-C), 936 (out-of-plane bending of C–H in benzoxazine).

***3-(4-bromophenethyl)-6,8-di-tert-butyl-3,4-dihydro-2H-benzo [e][1,3]oxazine*, [4]**. The compound obtained according to the literature procedure.[9]

***6-(tert-butyl)-3-dodecyl-3,4-dihydro-2H-benzo[e][1,3]oxazine*, [5]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of 4-tert-butylphenol (2 g, 13.31 mmol) in 10 mL of methanol, dodecan-1-amine (2.47 g,13.31 mmol) and formaldehyde 37% solution in water (29.8 mmol, 2.22 mL) were added. The resulting mixture was heated at reflux for 24 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol and put in freezer. After 2 days white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give white precipitate (at -20 °C) which was filtered off at low temperature (<0 °C). Product melted at room temperature. Yield: 3.30 g (9.18 mmol), 68%, colorless oil. HRMS(ESI) calcd for $C_{24}H_{41}NO$: 360.3261 $[M + H]^+$, found 360.3186. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.14 (d, J = 2.5 Hz, 1H, Ar*H*), 6.96 (t, J = 3.8 Hz, 1H, Ar*H*), 6.71 (d, J = 5.0 Hz, 1H, Ar*H*), 4.86 (d, J = 25.7 Hz, 2H, NCH_2O), 3.97 (d, J = 20.6 Hz, 2H, NCH_2Ar), 2.78 – 2.70 (m, 2H, N-CH_2), 1.61 - 1.53 (m, 2H, N-CH_2-CH_2), 1.29 (m, 18H, CH_2-(CH_2)$_9$-CH_3), 1.27 (m, 9H, C(CH_3)), 0.89 (t, J = 7.0 Hz, 3H, CH_2-CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 (s, 1C, Ar*C*-O), 143.2 (s, 1C, Ar*C*), 124.6 (s, 1C, Ar*C*H), 124.1 (s, 1C, Ar*C*H), 119.5 (s, 1C, Ar*C*-CH_2), 115.8 (s, 1C, Ar*C*H), 82.3 (s, 1C, OCH_2N), 51.5 (s, 1C, NCH_2), 50.6 (s, 1C, NCH_2Ar), 34.1 (s, 1C, *C*(CH_3)$_3$), 32.0 (s, 1C, C(*C*H_3)$_3$), 29.7 (s, 8C, C(*C*H_2)$_8$), 28.1 (s, 1C, N-CH_2-*C*H_2), 22.7 (s, 1C, *C*H_2-CH_3), 14.2 (s, 1C, *C*H_3). IR (cm^{-1}, nujol mull) 1232 (C-O-C), 939 (out-of-plane bending of C–H in benzoxazine).

***3-dodecyl-6-(2,4,4-trimethylpentan-2-yl)-3,4-dihydro-2H-benzo[e][1,3]oxazine*, [6]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of 4-(2,4,4-trimethylpentan-2-yl)phenol (2.0 g, 9.69 mmol) in 10 mL of methanol, dodecan-1-amine (1.79 g, 9.69 mmol) and formaldehyde 37% solution in water (21.3 mmol, 1.6 mL) were added. The resulting mixture was heated at reflux for 24 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol and put in freezer. After 2 days white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give white precipitate (at -20 °C) which was filtered off at low temperature (<0 °C). Product melted at room temperature. Yield: 3.14 g (7.55 mmol), 78%, colorless oil. HRMS(ESI) calcd for $C_{21}H_{35}NO$: 416.32 $[M + H]^+$, found 416.38. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.10 (dd, *J* = 8.6, 2.4 Hz, 1H, Ar*CH*), 6.91 (d, *J* = 2.4 Hz, 1H, Ar*CH*), 6.68 (d, *J* = 8.6 Hz, 1H, Ar*CH*), 4.84 (s, 2H, NCH_2O), 3.97 (s, 2H, NCH_2Ar), 2.81 – 2.64 (m, 2H, NCH_2), 1.67 (s, 2H, CH_2), 1.56 (dd, *J* = 14.4, 7.2 Hz, 2H, N-CH_2-CH_2), 1.32 (s, 6H, CH_3), 1.31 – 1.21 (m, 18H, CH_2-(CH_2)$_9$-CH_3), 0.88 (t, *J* = 7.0 Hz, 3H, CH_2 -CH_3), 0.72 (s, 9H, C(CH_3)$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 151.9 (s, 1C, Ar*C*-O), 142.2 (s, 1C, Ar*C*), 125.5 (s, 1C, Ar*C*H), 125.1 (s, 1C, Ar*C*H), 119.3 (s, 1C, Ar*C*-CH_2), 115.5 (s, 1C, Ar*C*H), 82.5 (s, 1C, OCH_2N), 57.2 (s, 1C, *C*H_2), 51.6 (s, 1C, NCH_2), 50.8 (s, 1C, NCH_2Ar), 38.1 (s, 1C, *C*(CH_3)$_2$), 32.5 (s, 2C, (CH_3)$_2$), 32.0 (s, 1C, *C*(CH_3)$_3$), 31.7 (s, 8C, (CH_2)$_8$), 29.8 (s, 3C, C(*C*H_3)$_3$), 29.5 (s, 1C, N-CH_2-*C*H_2), 22.8 (s, 1C, *C*H_2-CH_3), 14.3 (s, 1C, *C*H_3). IR (cm^{-1}, nujol mull) 1232 (C-O-C), 937 (out-of-plane bending of C–H in benzoxazine).

***3-dodecyl-6-methyl-3,4-dihydro-2H-benzo[e][1,3]oxazine*, [7]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of 4-methylphenol (2.0 g, 18.5 mmol) in 10 mL of methanol, dodecan-1-amine (3.42 g, 18.5 mmol) and formaldehyde 37% solution in water (40.68 mmol, 3.08 mL) were added. The resulting mixture was heated at reflux for 24 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol and put in freezer. After 2 days white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give white precipitate (at -20 °C) which was filtered off at low temperature (<0 °C). Product melted at room temperature yield: 4.20 g (13.23 mmol), 71%, colorless oil. HRMS(ESI) calcd for $C_{21}H_{35}NO$: 318.3 $[M + H]^+$, found 318.27. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.92 (dd, *J* = 8.2, 1.5 Hz, 1H, Ar*CH*), 6.77 (d, *J* = 0.7 Hz, 1H, Ar*CH*), 6.68 (d, *J* = 8.3 Hz, 1H, Ar*CH*), 4.84 (s, 2H, , NCH_2O), 3.96 (s, 2H, NCH_2Ar), 2.79 – 2.66 (m, 2H, NCH_2), 2.26 (s, 3H, CH_3), 1.59 – 1.44 (m, 2H, N-CH_2-CH_2), 1.44 – 1.15 (m, 27H, CH_2-(CH_2)$_9$-CH_3, C(CH_3)$_3$), 0.89 (t, *J* = 7.0 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 152.12 (s, 1C, Ar*C*-O), 129.74 (s, 1C, Ar*C*), 128.30 (s, 1C, Ar*C*H), 127.97 (s, 1C, Ar*C*H), 120.08 (s, 1C, Ar*C*-CH_2), 116.20 (s, 1C, Ar*C*H), 82.52 (s, 1C, OCH_2N), 51.55 (s, 1C, NCH_2), 50.43 (s, 1C, NCH_2Ar), 32.06 (s, 1C, N-CH_2-*C*H_2), 29.75 (s, 8C, C(CH_2)$_8$), 20.70 (s, 1C, *C*H_3), 14.25 (s, 1C, *C*H_3). IR (cm^{-1}, nujol mull) 1228 (C-O-C), 939 (out-of-plane bending of C–H in benzoxazine).

***6,8-di-tert-butyl-3-dodecyl-3,4-dihydro-2H-benzo[e][1,3] oxazine*, [8]**. To a round-bottom flask equipped with a stir bar and a condenser the solution of 2,4-di-tert-butylphenol (2.0 g, 9.7 mmol) in 10 mL of methanol, dodecan-1-amine (1.8 g, 9.7 mmol) and formaldehyde 37% solution in water (21.5 mmol, 1.6 mL) were added. The resulting mixture was heated at reflux for 24 h. Next the solvent was removed by rotary evaporation, oily residue was digested in 10 mL of methanol

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and put in freezer. After 2 days white solid precipitated which was filtered off and washed with 15 mL of cold methanol. The solid was recrystallized from methanol to give white precipitate (at -15 °C). Yield: 3.0 g (7.22 mmol), 74%; mp 41.7 °C. HRMS(ESI) calcd for $C_{28}H_{49}NO$: 416.3887 $[M + H]^+$, found 416.4042. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.16 (d, J = 2.5 Hz, 1H, ArC*H*), 7.81 (d, J = 2.3 Hz, 1H, ArC*H*), 4.85 (s, 2H, NCH_2O), 4.00 (s, 2H, NCH_2Ar), 2.79 - 2.68 (m, 2H, NCH_2), 1.68 – 1.54 (m, 2H, N-CH$_2$-CH_2), 1.46 - 1.38 (m, 9H, C(CH_3)$_3$), 1.36 - 1.22 (m, 27H, CH$_2$-(CH_2)$_9$-CH$_3$, C(CH_3)$_3$), 0.90 (t, J = 6.9 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 150.7 (s, 1C, Ar*C*-O), 141.9 (s, 1C, Ar*C*), 136.5 (s, 1C, Ar*C*-C), 121.9 (s, 1C, Ar*C*H), 121.8 (s, 1C, Ar*C*H), 119.2 (s, 1C, Ar*C*-CH$_2$), 81.6 (s, 1C, OCH$_2$N), 51.6 (s, 1C, NCH$_2$), 51.2 (s, 1C NCH$_2$Ar), 34.9 (s, 1C, *C*(CH$_3$)$_3$), 34.3 (s, 1C, *C*(CH$_3$)$_3$), 32.0 (s, 3C, C(*C*H$_3$)$_3$), 31.7 (s, 3C, C(*C*H$_3$), 8C, (CH$_2$)$_8$), 29.6 (s, 1C, N-CH$_2$-*C*H$_2$), 22.7 (s, 1C, *C*H$_2$-CH$_3$), 14.1 (s, 1C, *C*H$_3$). IR (cm^{-1}, nujol mull) 1224 (C-O-C), 948 (out-of-plane bending of C–H in benzoxazine).

***6-(tert-butyl)-3-(4-((trimethylsilyl)ethynyl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine,* [9]**. The solution of 1 (0.392 g, 1.05 mmol) and $[Pd(PPh_3)_2]Cl_2$ (0.037 g, 0.05 mmol) in dry triethylamine (15 ml) was degassed by freeze-pump-thaw (3 times) technique. Then ethynyl(trimethyl)silane (426 µl, 3.14 mmol) was added in one portion and the mixture was warmed up (55 °C) and mixed in the oil bath. After 24 h next portion of $[Pd(PPh_3)_2]Cl_2$ (0.037 g, 0.05 mmol), ethynyl(trimethyl)silane (426 µl, 3.14 mmol) were added and the mixture was placed back in the oil bath (55 °C) for next 48 h. During that time the reaction was monitored by ^{1}H NMR. The solvent was evaporated and the product isolated by column chromatography (silica gel, Et_2O/hexane, v/v, 1/2). Yield 72% (0.293 g, 0.75 mmol) of 9 as a yellow oil. HRMS(ESI) calcd for $C_{25}H_{33}NOSi$: 392.2404 $[M + H]^+$, found 392.2419. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.39 – 7.36 (m, 2H, C_6H_4), 7.15 (dd, J = 8.4, 1.9 Hz, 3H: 2H of C_6H_4, 1H of C_6H_3), 6.94 (d, J = 2.4 Hz, 1H, C_6H_3), 6.72 (t, J = 7.2 Hz, 1H, C_6H_3), 4.84 (s, 2H, OCH_2N), 4.02 (d, J = 10.3 Hz, 2H, NCH_2Ph), 3.05 – 3.00 (m, 2H, NCH_2CH_2), 2.91 – 2.84 (m, 2H, NCH_2CH_2), 1.28 (s, 9H, $C(CH_3)_3$), 0.24 (s, 9H, $SiMe_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 ($C_{Ph}O$), 143.5 ($C_{Ph}C(CH_3)_3$), 140.7 ($C_{Ph}CH_2CH_2$), 132.1 (CH of C_6H_4), 128.8 (CH of C_6H_4), 124.8 (CH of C_6H_3), 124.2 (CH of C_6H_3), 121.0 ($C_{Ph}CH_2N$), 119.5 ($C_{Ph}C{\equiv}C$), 116.0 (CH of C_6H_3), 105.3 ($SiC{\equiv}C$), 93.8 ($C_6H_4C{\equiv}C$), 82.4 (OCH_2N), 52.9 (NCH_2CH_2), 50.9 ($C_{Ph}CH_2N$), 35.1 (NCH_2CH_2), 34.2 ($C(CH_3)_3$), 31.6 ($C(CH_3)_3$), 0.1 ($SiMe_3$).

***6-(2,4,4-trimethylpentan-2-yl)-3-(4-((trimethylsilyl)ethynyl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine,* [10]**. Compound was synthesized according to the procedure for **9**. Used: **2** (0.128 g, 0.30 mmol), NEt_3 (7 mL), $[Pd(PPh_3)_2]Cl_2$ (0.010 g, 0.02 mmol), ethynyl(trimethyl)silane (121 µl, 0.89 mmol) were used. Reaction time: 48 h, chromatography condition: silica gel, Et_2O/hexane, v/v, 1/2. Yield 57% (0.077 g, 0.17 mmol) of **10** as a yellow powder; mp 94 °C. HRMS(ESI) calcd for $C_{29}H_{42}NOSi$: 448.3030 $[M + H]^+$, found 448.3042. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.39 – 7.35 (m, 2H, C_6H_4), 7.14 – 7.11 (m, 3H: 2H of C_6H_4, 1H of C_6H_3), 6.90 (d, *J* = 2.3 Hz, 1H, C_6H_3), 6.69 (dd, *J* = 8.6, 2.7 Hz, 1H, C_6H_3), 4.84 (s, 2H, OCH_2N), 3.99 (s, 2H, NCH_2Ph), 3.02 – 2.97 (m, 2H, NCH_2CH_2), 2.89 – 2.83 (m, 2H, NCH_2CH_2), 1.67 (s, 2H, CH_2), 1.32 (s, 6H, CH_3), 0.72 (s, 9H, $C(CH_3)_3$), 0.24 (s, 9H, $SiMe_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 151.8 ($C_{Ph}O$), 142.3 ($C_{Ph}C(CH_3)_2$), 140.7 ($C_{Ph}CH_2CH_2$), 132.1 (CH of C_6H_4), 128.7 (CH of C_6H_4), 125.6 (CH of C_6H_3), 125.0 (CH of C_6H_3), 121.0 ($C_{Ph}CH_2N$), 119.1 ($C_{Ph}C{\equiv}C$), 115.7 (CH of C_6H_3), 105.3 ($SiC{\equiv}C$), 93.7 ($C_6H_4C{\equiv}C$), 82.5 (OCH_2N), 57.2 (CH_2), 53.0 (NCH_2CH_2), 50.9 ($C_{Ph}CH_2N$), 38.1 ($C_{Ph}C(CH_3)_2$), 35.1 (NCH_2CH_2), 32.5 ($C(CH_3)_3$), 31.9 ($C(CH_3)_3$), 31.7 ($C(CH_3)_2$), 0.2 ($SiMe_3$). IR (cm^{-1}, nujol mull) 2120 (C≡C), 1249 (C–O–C), 1157 (C–N–C), 937 (out-of-plane bending of C–H in benzoxazine).

***6-methyl-3-(4-((trimethylsilyl)ethynyl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine* [11]**. Compound was synthesized according to the procedure for **9**. Used: **2** (0.797 g, 2.40 mmol), NEt_3 (20 ml), $[Pd(PPh_3)_2]Cl_2$ (0.084 g, 0.12 mmol), ethynyl(trimethyl)silane (980 µl, 7.20 mmol). Reaction time: 48 h, chromatography condition: silica gel, Et_2O/hexane, v/v, 1/2. Yield 69% (0.578 g, 1.65 mmol) of **11** as a yellow powder. HRMS(ESI) calcd for $C_{22}H_{28}NOSi$: 350.1935 $[M + H]^+$, found 350.1930. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.43–7.40 (m, 2H, C_6H_4), 7.17 (d, J_{HH} = 8.3 Hz, 2H, C_6H_4), 6.98 – 6.94 (m, 1H, C_6H_3), 6.79 (d, J_{HH} = 1.1 Hz, 1H, C_6H_3), 6.74 – 6.71 (m, 1H, C_6H_3), 4.86 (s, 2H, OCH_2N), 4.00 (s, 2H, NCH_2Ph), 3.06 – 3.01 (m, 2H, NCH_2CH_2), 2.91 – 2.87 (m, 2H, NCH_2CH_2), 2.29 (s, 3H, CH_3), 0.30 (s, 9H, $SiMe_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.1 ($C_{Ph}O$), 140.7 ($C_{Ph}CH_2CH_2$), 132.1 (CH of C_6H_4), 129.9 (CCH_3), 128.7 (CH of C_6H_4), 128.4 (CH of C_6H_3), 127.9 (CH of C_6H_3), 121.0 ($C_{Ph}CH_2N$), 119.9 ($C_{Ph}C{\equiv}C$), 116.3 (CH of C_6H_3), 105.3 ($SiC{\equiv}C$), 93.8 ($C_6H_4C{\equiv}C$), 82.5 (OCH_2N), 52.9 (NCH_2CH_2), 50.6 ($C_{Ph}CH_2N$), 35.0 (NCH_2CH_2), 20.7 (CH_3), 0.2 ($SiMe_3$). IR (cm^{-1}, nujol mull) 2116 (C≡C), 1249 (C–O–C), 1157 (C–N–C), 937 (out-of-plane bending of C–H in benzoxazine).

***6,8-di-tert-butyl-3-(4-((trimethylsilyl)ethynyl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine*, [12]**. The compound obtained according to the literature procedure.[3]

***6-(tert-butyl)-3-(4-ethynylphenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine,* [13]**. To the solution of **9** (0.033 g, 0.083 mmol) in MeOH/*i*-PrOH (v/v, 1/3, 30 mL) anhydrous K_2CO_3 (0.035 g, 0.25 mmol) was added and the suspension was mixed for 24 h. The solvent was evaporated under reduced pressure, residue was suspended in water (50 mL) and product was extracted with diethyl ether (3 x 20 mL). The organic phase was dried (anhydrous $MgSO_4$), filtered and evaporated. The product did not require additional purification. Yield 98% (0.026 g, 0.082 mmol) of **13** as a yellowish powder; mp 97 °C. HRMS(ESI) calcd for $C_{22}H_{26}NO$: 320.2009 $[M + H]^+$, found 320.2004. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.42 – 7.39 (m, 2H, C_6H_4), 7.17 (d, J_{HH} = 8.3 Hz, 2H, C_6H_4), 7.14 (dd, J_{HH} = 8.6, 2.4 Hz, 1H, C_6H_3), 6.94 (d, J_{HH} = 2.4 Hz, 1H, C_6H_3), 6.72 (d, J_{HH} = 8.6 Hz, 1H, C_6H_3), 4.85 (d, J_{HH} = 2.7 Hz, 2H, OCH_2N), 4.02 (s, 2H, NCH_2Ph), 3.05 – 3.01 (m, 3H, NCH_2CH_2, $HC{\equiv}C$), 2.90 – 2.86 (m, 2H, NCH_2CH_2), 1.28 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 151.9 ($C_{Ph}O$), 143.4 ($C_{Ph}C(CH_3)_3$), 140.1 ($C_{Ph}CH_2CH_2$), 132.2 (CH of C_6H_4), 128.8 (CH of C_6H_4), 124.7 (CH of C_6H_3), 124.1 (CH of C_6H_3), 119.9 ($C_{Ph}CH_2N$), 119.4 ($C_{Ph}C{\equiv}C$), 116.0 (CH of C_6H_3), 83.8 ($C_6H_4C{\equiv}C$), 82.3 (OCH_2N), 77.0 ($C{\equiv}CH$), 52.8 (NCH_2CH_2), 50.8 ($C_{Ph}CH_2N$), 34. 9 (NCH_2CH_2), 34.1 ($C(CH_3)_3$), 31.6 ($C(CH_3)_3$).

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IR (cm^{-1}, nujol mull) 3265 (C≡CH), 1980 (C≡C), 1231 (C–O–C), 1157 (C–N–C), 933 (out-of-plane bending of C–H in benzoxazine).

3-(4-ethynylphenethyl)-6-(2,4,4-trimethylpentan-2-yl)-3,4-dihydro-2H-benzo[e][1,3]oxazine, **[14]**. Compound was synthesized according to the procedure for **13**. Used: **10** (0.297 g, 0.67 mmol), MeOH/*i*-PrOH (v/v, 1/3, 30 mL), K_2CO_3 (0.277 g, 2.00 mmol). Reaction time 24 h. Yield 75% (0.187 g, 0.50 mmol) of **14** as an orange solid. HRMS(ESI) calcd for $C_{26}H_{35}NO$: 376.2635 $[M + H]^+$, found 376.2634. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.34 – 7.31 (m, 2H, C_6H_4), 7.08 (d, J_{HH} = 8.3 Hz, 2H, C_6H_4), 7.04 (dd, J_{HH} = 8.6, 2.4 Hz, 1H, C_6H_3), 6.83 (d, J_{HH} = 2.4 Hz, 1H, C_6H_3), 6.61 (d, J_{HH} = 8.6 Hz, 1H, C_6H_3), 4.78 (s, 2H, OCH_2N), 3.93 (s, 2H, NCH_2Ph), 2.96 – 2.91 (m, 3H, NCH$_2$CH_2, *H*C≡C), 2.82 – 2.78 (m, 2H, NCH$_2$CH_2), 1.59 (s, 2H, CH_2), 1.25 (s, 6H, CH_3), 0.65 (s, 9H, C(CH_3)$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 151.8 (C_{Ph}O), 142.4 ($C_{Ph}C(CH_3)_2$), 141.1 ($C_{Ph}CH_2CH_2$), 132.3 (CH of C_6H_4), 128.9 (CH of C_6H_4), 125.7 (CH of C_6H_3), 125.1 (CH of C_6H_3), 120.0 ($C_{Ph}CH_2N$), 119.0 (C_{Ph}C≡C), 115.7 (CH of C_6H_3), 83.8 (C_6H_4C≡C), 82.5 (OCH_2N), 57.2 (CH_2), 52.9 (NCH_2CH_2), 50.9 ($C_{Ph}CH_2N$), 38.1 ($C_{Ph}C(CH_3)_2$), 35.0 (NCH$_2$CH_2), 32.5 ($C(CH_3)_3$), 31.9 (C(CH_3)$_3$), 31.7 (C(CH_3)$_2$), 1C missing. IR (cm^{-1}, nujol mull) 3266 (C≡CH), 1980 (C≡C), 1240 (C–O–C), 1157 (C–N–C), 937 (out-of-plane bending of C–H in benzoxazine).

3-(4-ethynylphenethyl)-6-methyl-3,4-dihydro-2H-benzo[e][1,3]oxazine, **[15]**. Compound was synthesized according to the procedure for **13**. Used: **11** (0.578 g, 1.65 mmol), MeOH/*i*-PrOH (v/v, 1/3, 30 mL), K_2CO_3 (0.686 g, 4.96 mmol). Reaction time 24 h. Yield 37% (0.171 g, 0.62 mmol) of **15** as an orange solid; mp 85 °C. HRMS(ESI) calcd for $C_{19}H_{20}NO$: 278.1539 $[M + H]^+$, found 278.1539. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.35 – 7.31 (m, 2H, C_6H_4), 7.09 (d, J_{HH} = 8.3 Hz, 2H, C_6H_4), 6.84 (dt, J_{HH} = 5.6, 3.2 Hz, 1H, C_6H_3), 6.68 (d, J_{HH} = 1.0 Hz, 1H, C_6H_3), 6.60 (dd, J_{HH} = 8.2, 3.8 Hz, 1H, C_6H_3), 4.77 (s, 2H, OCH_2N), 3.91 (s, 2H, NCH_2Ph), 2.96 – 2.91 (m, 3H, NCH$_2$CH_2, *H*C≡C), 2.82 – 2.77 (m, 2H, NCH$_2$CH_2), 2.17 (s, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.1 (C_{Ph}O), 141.0 ($C_{Ph}CH_2CH_2$), 132.3 (CH of C_6H_4), 129.9 (*C*CH_3), 128.9 (CH of C_6H_4), 128.4 (CH of C_6H_3), 127.9 (CH of C_6H_3), 120.0 ($C_{Ph}CH_2N$), 119.9 (C_{Ph}C≡C), 116.3 (CH of C_6H_3), 83.8 (C_6H_4C≡C), 82.5 (OCH_2N), 52.9 (NCH_2CH_2), 50.6 ($C_{Ph}CH_2N$), 35.0 (NCH$_2$CH_2), 20.7 (CH_3), 1C missing. IR (cm^{-1}, nujol mull) 3265 (C≡CH), 1227 (C–O–C), 1160 (C–N–C), 936 (out-of-plane bending of C–H in benzoxazine).

6,8-di-tert-butyl-3-(4-((trimethylsilyl)ethynyl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine**,** **[16]**. The compound obtained according to the literature procedure.[9]

6-(tert-butyl)-3-(4-((trimethylsilyl)buta-1,3-diyn-1-yl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine, **[17]** and ***1,4-bis(4-(2-(6-(tert-butyl)-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethyl)phenyl)buta-1,3-diyne*****,** **[20]**. To the solution of **13** (0.206 g, 0.65 mmol), CuI (0.0061 g, 0.032 mmol), $[Pd(PPh_3)_2]Cl_2$ (0.023 g, 0.03 mmol) and bromoethynyl(trimethyl)silane (0.171 g, 0.97 mmol)[10] in dry oxygen-free THF under nitrogen atmosphere diisopropylamine (230 µl, 1.61 mmol) was added dropwise. The mixture was stirred in room temperature for 5 h. The solvent was evaporated and the product **17** and **20** were isolated as separated fractions by column chromatography (silica gel, Et_2O/heksan, v/v, 1/1). Yield 25% (0.067 g, 0.162 mmol) of **17** as an orange grease and 8% (0.033 g, 0.05 mmol) of **20** as a yellow solid.

17: HRMS(ESI) calcd for $C_{27}H_{34}NOSi$: 416.2404 $[M + H]^+$, found 416.2408. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.41 – 7.38 (m, 2H, C_6H_4), 7.18 – 7.13 (m, 3H: 2H of C_6H_4, 1H z C_6H_3), 6.94 (d, J_{HH} = 2.4 Hz, 1H, C_6H_3), 6.74 – 6.70 (m, 1H, C_6H_3), 4.84(s, 2H, OCH_2N), 4.01 (s, 2H, NCH_2Ph), 3.02 (dd, J_{HH} = 8.5, 6.5 Hz, 2H, NCH_2CH$_2$), 2.90 – 2.86 (m, 2H, NCH$_2$CH_2), 1.28 (s, 9H, C(CH_3)$_3$), 0.23 (s, 9H, SiMe$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 (C_{Ph}O), 143.5 ($C_{Ph}C(CH_3)_3$), 141.8 ($C_{Ph}CH_2CH_2$), 132.8 (CH of C_6H_4), 129.0 (CH of C_6H_4), 124.8 (CH of C_6H_3), 124.2 (CH of C_6H_3), 119.4 ($C_{Ph}CH_2N$), 119.2 (C_{Ph}C≡C), 116.0 (CH of C_6H_3), 90.5 (Si*C*≡C), 88.1 (*C*≡C), 82.4 (OCH_2N), 77.0 (C_6H_4C≡C), 74.0 (*C*≡C), 52.8 (NCH_2CH_2), 50.9 ($C_{Ph}CH_2N$), 35.0 (NCH$_2$CH_2), 34.2 (*C*$(CH_3)_3$), 31.6 (C(CH_3)$_3$), -0.2 (SiMe$_3$).

20: mp 151 °C. HRMS(ESI) calcd for $C_{44}H_{49}N_2O_2$: 637.3789 $[M + H]^+$, found 637.3790. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.45 – 7.41 (m, 2H, C_6H_4), 7.19 (d, J_{HH} = 8.3 Hz, 2H, C_6H_4), 7.14 (dd, J_{HH} = 8.6, 2.5 Hz, 1H, C_6H_3), 6.94 (d, J_{HH} = 2.4 Hz, 1H, C_6H_3), 6.72 (d, J_{HH} = 8.6 Hz, 1H, C_6H_3), 4.85 (s, 2H, OCH_2N), 4.02 (s, 2H, NCH_2Ph), 3.03 (dd, J_{HH} = 8.5, 6.6 Hz, 2H, NCH_2CH$_2$), 2.89 (t, J_{HH} = 7.6 Hz, 2H, NCH$_2$CH_2), 1.28 (s, 9H, C(CH_3)$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 (C_{Ph}O), 143.5 ($C_{Ph}C(CH_3)_3$), 141.6 ($C_{Ph}CH_2CH_2$), 132.6 (CH of C_6H_4), 129.0 (CH of C_6H_4), 124.9 (CH of C_6H_3), 124.2 (CH of C_6H_3), 119.7 ($C_{Ph}CH_2N$), 119.4 (C_{Ph}C≡C), 116.0 (CH of C_6H_3), 82.5 (OCH_2N), 81.6 (C_6H_4C≡C), 73.8 (*C*≡C), 52.8 (NCH_2CH_2), 50.9 ($C_{Ph}CH_2N$), 35.1 (NCH$_2$CH_2), 34.2 (*C*$(CH_3)_3$), 31.6 (C(CH_3)$_3$). IR (cm^{-1}, nujol mull) 2116 (C≡C), 1908 (C≡C), 1231 (C–O–C), 1156 (C–N–C), 937 (out-of-plane bending of C–H in benzoxazine).

6-(2,4,4-trimethylpentan-2-yl)-3-(4-((trimethylsilyl)buta-1,3-diyn-1-yl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine, **[18]**. Compound was synthesized according to the procedure for **17**. Used: **14** (0.182 g, 0.49 mmol), THF (15 mL), CuI (0.005 g, 0.02 mmol), $[Pd(PPh_3)_2]Cl_2$ (0.017 g, 0.02 mmol), bromoethynyl(trimethyl)silane (0.129 g, 0.73 mmol), diisopropylamine (172 µL, 1.21 mmol). Reaction time: 4 h, chromatography conditions: (silica gel, Et_2O/heksan, v/v, 1/1). Yield 19% (0.044 g, 0.09 mmol) of **18** as a brown grease. HRMS(ESI) calcd for $C_{31}H_{42}NOSi$: 472.3030 $[M + H]^+$, found 472.3028. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.41 – 7.38 (m, 2H, C_6H_4), 7.16 (t, J_{HH} = 7.0 Hz, 2H, C_6H_4), 7.11 (dd, J_{HH} = 8.6, 2.4 Hz, 1H, C_6H_3), 6.90 (d, J_{HH} = 2.3 Hz, 1H, C_6H_3), 6.68 (d, J_{HH} = 8.6 Hz, 1H, C_6H_3), 4.84 (s, 2H, OCH_2N), 3.99 (s, 2H, NCH_2Ph), 3.00 (dd, J_{HH} = 8.6, 6.4 Hz, 2H, NCH_2CH$_2$), 2.89 – 2.84 (m, 2H, NCH$_2$CH_2), 1.67 (s, 2H, CH_2), 1.32 (s, 6H, 2 × CH_3), 0.72 (s, 9H, C(CH_3)$_3$), 0.23 (s, 9H, SiMe$_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 151.8 (C_{Ph}O), 142.4 ($C_{Ph}C(CH_3)_3$), 141.9 ($C_{Ph}CH_2CH_2$), 132.9 (CH of C_6H_4), 129.0 (CH of C_6H_4), 125.7 (CH of C_6H_3), 125.1 (CH of C_6H_3), 119.2 ($C_{Ph}CH_2N$), 119.0 (C_{Ph}C≡C), 115.7 (CH of C_6H_3), 90.5 (Si*C*≡C), 89.1 (*C*≡C), 82.5 (OCH_2N), 77.0 (C_6H_4C≡C), 74.0 (*C*≡C), 57.2 (CH_2), 52.8 (NCH_2CH_2), 50.9 ($C_{Ph}CH_2N$), 38.1 ($C_{Ph}C(CH_3)_2$), 35.1 (NCH$_2$CH_2), 32.5 (*C*$(CH_3)_3$), 31.9 (C(CH_3)$_3$), 31.7 (C(CH_3)$_2$), -0.2 (SiMe$_3$).

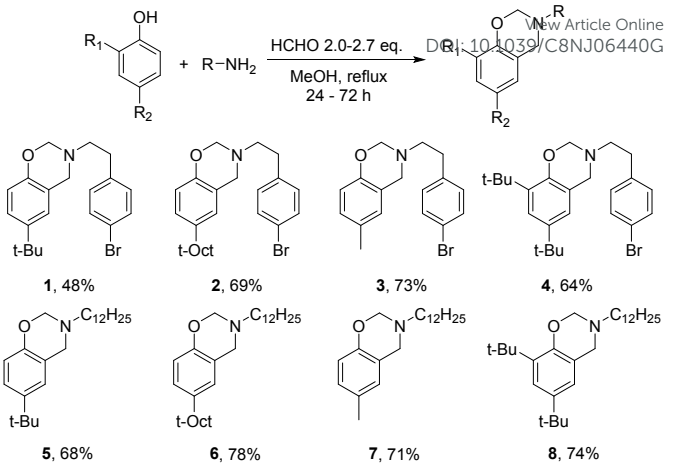
6-methyl-3-(4-((trimethylsilyl)buta-1,3-diyn-1-yl)phenethyl)-3,4-dihydro-2H-benzo[e][1,3]oxazine, **[19]** and ***1,4-bis(4-(2-(6-methyl-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethyl)phenyl)buta-1,3-diyne*** **[21].** Compounds were obtained according to the procedure for **17** and **20**. Used: **15** (0.171 g, 0.62 mmol), THF (15 mL), CuI (0.006 g, 0.032 mmol), $[Pd(PPh_3)_2]Cl_2$ (0.022 g, 0.03 mmol), bromoethynyl(trimethyl)silane (0.135 g, 0.92 mmol), diisopropylamine (217 µL, 1.54 mmol). Reaction time: 24 h, chromatography condition: (silica gel, Et_2O/heksan, v/v, 1/1). Yield 48% (0.111 g, 0.30 mmol) of **19** as a yellowish oil and 3% (0.008 g, 0.014 mmol) of **21** as an orange grease.

19: HRMS(ESI) calcd for $C_{24}H_{28}NOSi$: 374.1935 $[M + H]^+$, found 374.1940. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.40 (dq, J_{HH} = 6.2, 2.1 Hz, 2H, C_6H_4), 7.16 (d, J_{HH} = 8.3 Hz, 2H, C_6H_4), 6.92 (dd, J_{HH} = 8.3, 1.5 Hz, 1H, C_6H_3), 6.75 (d, J_{HH} = 1.3 Hz, 1H, C_6H_3), 6.68 (dd, J_{HH} = 8.3, 1.8 Hz, 1H, C_6H_3), 4.84 (s, 2H, OCH_2N), 3.97 (s, 2H, NCH_2Ph), 3.03 – 2.98 (m, 2H, NCH_2CH_2), 2.89 – 2.84 (m, 2H, NCH_2CH_2), 2.25 (s, 3H, CH_3), 0.23 (s, 9H, $SiMe_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 ($C_{Ph}O$), 141.8 ($C_{Ph}CH_2CH_2$), 132.9 (CH of C_6H_4), 130.0 (CCH_3), 129.0 (CH of C_6H_4), 128.5 (CH of C_6H_3), 127.9 (CH of C_6H_3), 119.8 ($C_{Ph}CH_2N$), 119.2 ($C_{Ph}C{\equiv}C$), 116.3 (CH of C_6H_3), 90.5 ($SiC{\equiv}C$), 88.1 ($C{\equiv}C$), 82.5 (OCH_2N), 77.0 ($C_6H_4C{\equiv}C$), 74.0 ($C{\equiv}C$), 52.8 (NCH_2CH_2), 50.6 ($C_{Ph}CH_2N$), 35.0 ($(NCH_2CH_2$), 20.7 (CH_3), -0.2 ($SiMe_3$).

21: HRMS(ESI) calcd for $C_{38}H_{37}N_2O_2$: 553.2850 $[M + H]^+$, found 553.2847. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.45 – 7.41 (m, 2H, C_6H_4), 7.17 (d, J_{HH} = 8.2 Hz, 2H, C_6H_4), 6.92 (dd, J_{HH} = 8.3, 1.6 Hz, 1H, C_6H_3), 6.76 (s, 1H, C_6H_3), 6.67 (d, J_{HH} = 8.3 Hz, 1H, C_6H_3), 4.84 (s, 2H, OCH_2N), 3.98 (s, 2H, NCH_2Ph), 3.01 (dd, J_{HH} = 8.5, 6.5 Hz, 2H, NCH_2CH_2), 2.89 – 2.85 (m, 2H, NCH_2CH_2), 2.25 (s, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 152.0 ($C_{Ph}O$), 141.6 ($C_{Ph}CH_2CH_2$), 132.6 (CH of C_6H_4), 130.0 (CCH_3), 129.0 (CH of C_6H_4), 128.5 (CH of C_6H_3), 128.0 (CH of C_6H_3), 119.9 ($C_{Ph}CH_2N$), 119.7 ($C_{Ph}C{\equiv}C$), 116.3 (CH of C_6H_3), 82.5 (OCH_2N), 81.6 ($C_6H_4C{\equiv}C$), 73.8 ($C{\equiv}C$), 52.8 (NCH_2CH_2), 50.6 ($C_{Ph}CH_2N$), 35.0 (NCH_2CH_2), 20.7 (CH_3).

Results and discussion

The new benzoxazines **1–8** (**Scheme 1**) with simple functionalization both as arms linked to a nitrogen atom and substituents in the *ortho/para* position of the aryl core have been obtained through the standard one-pot Mannich coupling while using *p*-bromophenylethylamine or dodecylamine, formaldehyde (37% solution in H_2O), and the corresponding phenols: *p*-cresol, 4-*tert*-buthylphenol, and 4-(2,4,4-trimethylpentan-2-yl)phenol. Recrystallization of the crude products **1–8** gave the corresponding benzoxazines with moderate to good yields (48–78%). Benzoxazines **6** and **7** are liquid at room temperature, nevertheless, low-temperature recrystallization from methanol proceeds smoothly. Compound **4** has previously been prepared through a similar procedure.[9]



Scheme 1. Synthesis of benzoxazines **1–8** (for the synthesis of **4**, see ref 3).

In the next step, benzoxazines **1–4** were functionalized through the Sonogashira cross-coupling using TMSC≡CH and $[Pd(PPh_3)_2]Cl_2$ or $[Pd(PPh_3)_2]Cl_2/Cu^{II}$ catalytic system in triethylamine (**Scheme 2**). The procedure typically used for such reactions gave a trace of products.[11] In all cases, the addition of two portions of ethynyl(trimethyl)silane was used to obtain products with the reported yields. Benzoxazines **9–11** were synthesized with an additional portion of the catalyst introduced after 24 h. Standard work-up procedures followed by column chromatography gave trimethylsilylacetylene benzoxazine derivatives **9–12** with good yields (57–72%). Deprotection with K_2CO_3 in an *i*-PrOH/MeOH mixture led to terminal acetylene-functionalized benzoxazines **13–16**. The elongation of acetylene to butadiynes was implemented through the Cadiot-Chodkiewicz protocol. The desired benzoxazines **17–19** were isolated by column chromatography as the main products. During the procedure, homocoupling product formation was observed as well. Small amounts of the benzoxazine-end-capped butadiynes **20** and **21** were obtained as a minor fraction (**Scheme 2**).

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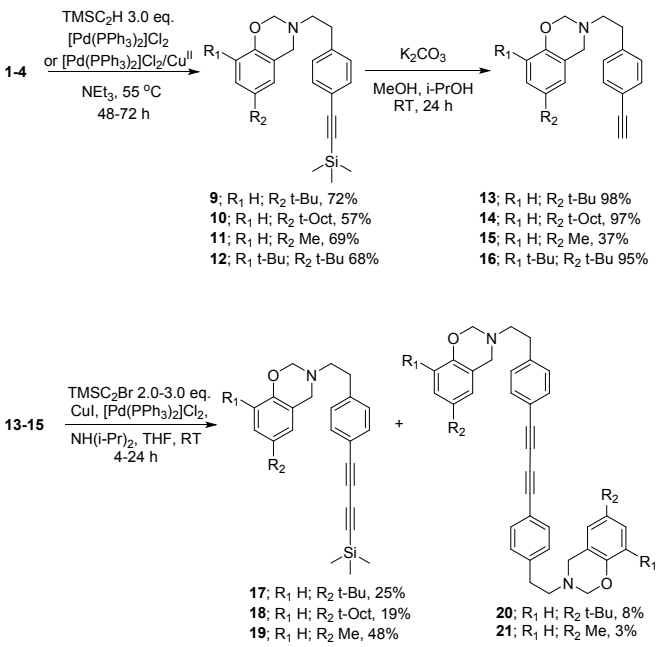

Scheme 2. Synthesis of acetylene- and butadiyne-functionalized benzoxazines **9**–**21**.

All of the obtained benzoxazines were characterized by NMR, HRMS, spectroscopic, and thermo-gravimetric methods. The diagnostic ^{1}H NMR resonances due to Ar-CH_2-N- and O-CH_2-N-, attributed to the benzoxazine motif, were observed at 3.97–4.05 and 4.84–4.88 ppm in $CDCl_3$. Chemical shifts associated with aromatic protons, trimethylsilyl, and alkyl substituents were typical for all the compounds. The signals representing the -CH_2-CH_2-N linker appeared at nearly the same positions as two triplets for **1**–**4** and **9**–**21**. The dodecyl fragment located on the nitrogen atom for **5**–**8** represented a typical set of methylene groups appearing as multiplets and triplets for the methyl group located at the end of the alkyl chain. The ^{13}CNMR spectra were used to detect the acetylene group. Chemical shifts of alkyne carbons appeared in the 72.0–105.3 ppm range. For some compounds, one of the acetylene signals overlapped with the solvent peak ($CDCl_3$, 77.2 ppm). The IR spectra were similar and exhibited bands at 936–938 and 1226–1234 cm^{-1}, characteristic benzoxazine fingerprints. Typical alkyne C≡C bands (1980–2120 cm^{-1}) also occured in the IR spectra of acetylene benzoxazine derivatives. The molecular structure in the solid state was confirmed for functionalized benzoxazines by bromobenzyl (**Fig. 1**, benzoxazine **1**-**4**), alkyl chain (**Fig. 2**, benzoxazine **8**), acetylene (**Fig. 3**, benzoxazine **13**, **15 - 16**), and butadiyne (**Fig. 4**) through X-ray analysis. X-ray experiment details and the selected bond lengths and angles are summarized in the supporting information file, Tables S1 and S2.

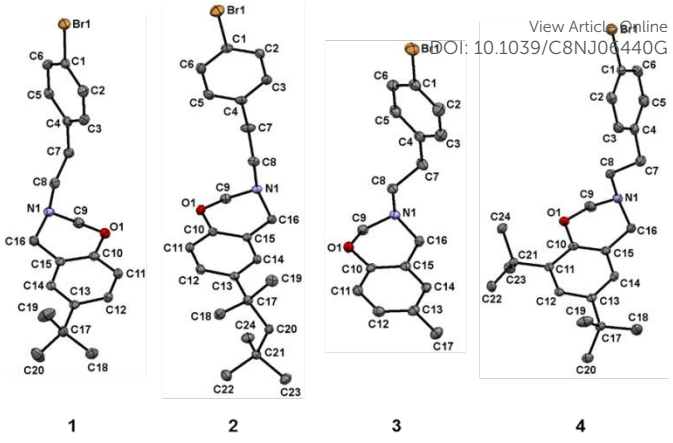

Fig. 1. Molecular structures of benzoxazines **1**-**4** with atom labeling. The thermal ellipsoids are drawn at the 50% probability level. H atoms are excluded for clarity.

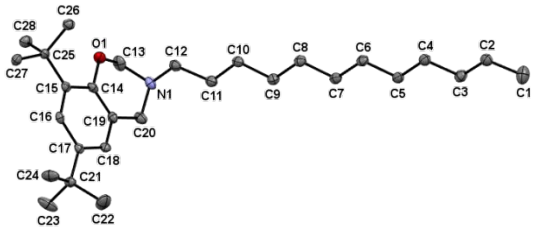

Fig. 2. Molecular structure of benzoxazine **8** with atom labeling. The thermal ellipsoids are drawn at the 50% probability level. H atoms are excluded for clarity.

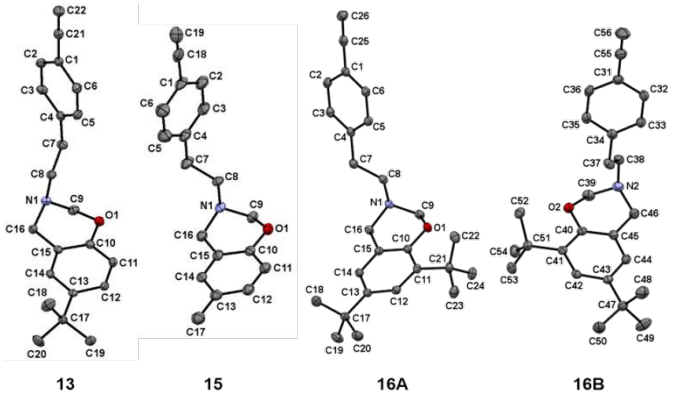

Fig. 3. Molecular structures of benzoxazines **13, 15, 16** (two molecules in asymmetric unit) with atom labeling. The thermal ellipsoids are drawn at the 50% probability level. H atoms are excluded for clarity.

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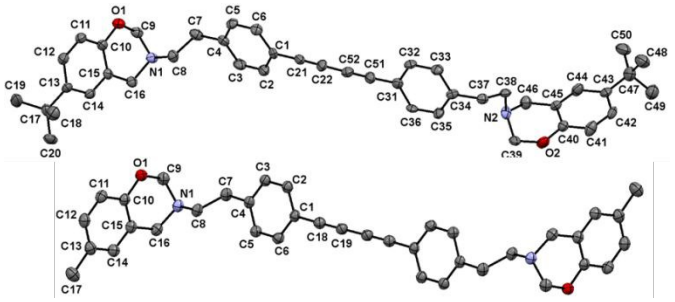


Fig. 4. Molecular structures of benzoxazines **20** (up), **21** (bottom) with atom labeling. The thermal ellipsoids are drawn at the 50% probability level. H atoms are excluded for clarity.

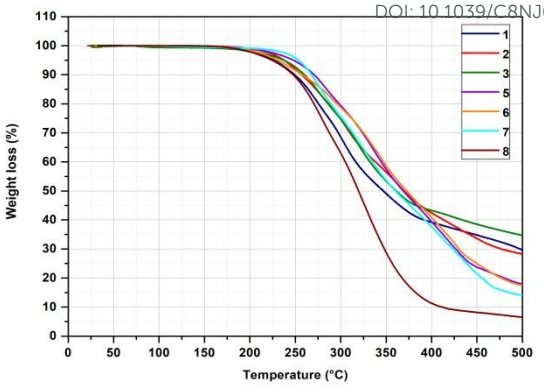
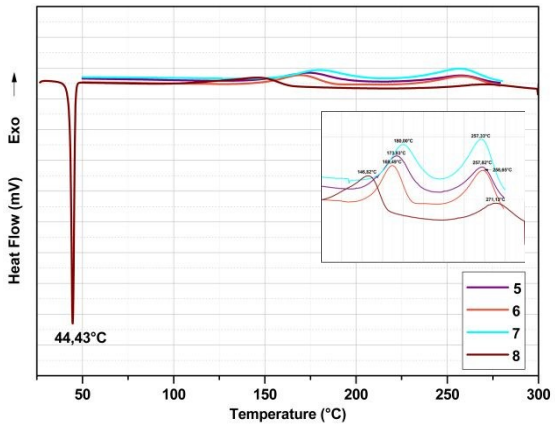


Fig. 5. TGA thermograms of uncured benzoxazine monomers.

The bond lengths and angles are comparable with those of similar benzoxazines previously described in the literature.[12] However, there are not so many X-ray data of 1,3-benzoxazines in the crystallographic database. Such information is valuable with regard to theoretical studies that usually use X-ray data as the starting point for geometry optimization. CSD accommodates only 36 structures with the 1,3-benzoxazine structural motif and only one that contains an acetylene fragment. The expected irregular chair conformation was observed for all the compounds, which is overall considered a driving force for the polymerization process.

The study of the thermal properties of benzoxazines **1–8** delivered information on their thermal stability, which is crucial for polybenzoxazine formation. Overall, the thermal polymerization of benzoxazine was carried out in the temperature range of 200–250 °C, depending on the structural motif of the monomers. The results of the TGA analysis are summarized in **Fig. 5**.

The TGA profiles for **1–8** compounds showed $T_{5\%}$ in the 227–253 °C range and $T_{10\%}$ weight loss for temperatures between 247 and 270 °C. Unfortunately, benzoxazines **1–3** do not polymerize before thermal decomposition, in analogy to compound **4**.[9] Despite the assumed design goal of functionalized benzoxazines they undergo destruction into difficult to identify products which disqualifies them in these applications. Therefore, their acetylene derivatives, which are usually less stable, were not tested in this regard. In parallel, we paid more attention to the thermal properties and the polymerization process of the long-alkyl-chain benzoxazines **5–8**. **Figure 6** presents the DSC thermogram for benzoxazines **5–8** with the same alkyl chain arm and different modifications in the aromatic ring. The sharp exotherms with the maximum temperatures at 258, 257, 256, and 271 °C correspond to the ring opening of the oxazine ring. The first exotherm at lower temperatures with the maximums at 139, 169, 173, and 179 °C is probably attributed to a cross-linking process similar to that described earlier in the literature, while multiple exothermic behavior patterns have been reported for alkyl-substituted benzoxazine motifs.[13]



monomer	first exotherm onset (°C)	first exotherm max. (°C)	heat of polymerization ΔH (J/g)	second exotherm onset (°C)	second exotherm max. (°C)	heat of polymerization ΔH (J/g)
5	147.96	173.83	64.39	231.82	257.82	46.39
6	147.77	169.49	55.14	230.92	258.65	74.30
7	157.08	180.00	60.23	230.54	257.33	85.92
8	113.01	146.82	55.31	246.91	271.13	32.83

Fig. 6. DSC profile of benzoxazines **5–8**.

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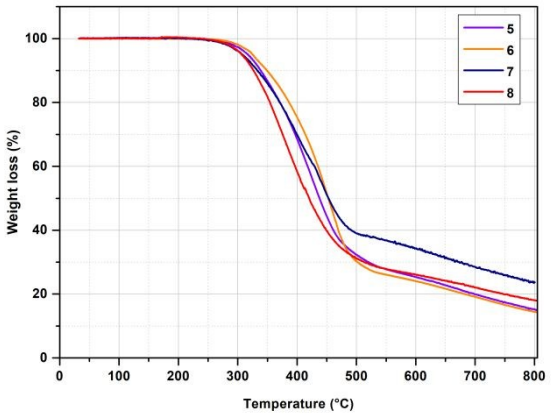
The benzoxazines were converted to polybenzoxazines by monomers melted and heated under vacuum at 180 °C for 2 h, next at 240 °C, and at 250 °C for 2 h in an air-circulating oven. The obtained polybenzoxazines were brown and transparent. The thermal stability of polybenzoxazines **5–8** are presented by the TGA profiles shown in **Fig. 7**. Benzoxazines **1–4** undergo degradation during the polymerization process, and it was impossible to obtain identifiable products. Polybenzoxazines are high-performance thermoset materials and therefore should have excellent thermal stability with high char yield. The polymers presented here showed a 10% weight loss temperature between 306 and 325 °C; the char yield was determined at 800 °C in the range of 15–22%.



sample	$T_{5\%}$[°C]	$T_{10\%}$[°C]	Y_c[%]
5	317	338	18
6	325	349	15
7	307	332	22
8	306	326	19

Fig. 7. TGA of polybenzoxazines obtained from monomers **5–8**.

The glass transition temperatures are extremely low in comparison to classical benzoxazine derivatives. The liquid nature of alkyl-chain-modified monomers and the high processing windows indicate their high processable potential.

Benzoxazines have a suitable framework for biologically active species design. The functionalization of benzoxazines by acetylenic groups resulted in poorer solubility in ordinary solvents, which led to difficulties during the study of their biological activity. Therefore their precursors (**1**-**4**) have been tested. A modification of the aryl core to include a steric obstruction located in the *para* position and the removal of the acetylenic fragment improved both the solubility and stability of the new benzoxazines. The preliminary antimicrobial tests for compounds **1–8** were performed by disc diffusion assay used for the estimation of their potential biological activity (see ESI, Table S6).[7] On the basis of diffusion tests, the potential of antimicrobial properties of tested compounds could not be unambiguously determined, due to their poor diffusion (**3**, **4**), and additionally in the case of compounds **6**, **7** very poor solubility and precipitation in the biological medium have been noticed. These preliminary screening tests enabled the selection of the most active compounds. Already at this stage, benzoxazines with a substituent located in the ortho position relative to the oxygen of the heterocyclic ring showed no activity against the tested microorganisms. Despite this fact, tests were carried out using the serial dilution method for the remaining benzoxazines. In each case, no significant activities were obtained for the highest concentrations as indicated under Table 1 as >400. For the compounds that obtained the highest activity in screening tests, detailed research was carried out, the minimal inhibitory concentration (MIC) was determined using the serial dilution method.[8] Tetracycline and amphotericin B were used as reference antimicrobial/antifungal agents at the therapeutic dose of 30 $\mu g \times cm^{-3}$. The investigated bacterial species were: Gram-positive cocci (*S. aureus, S. pseudintermedius, S. agalactiae, S. canis*), Gram-positive endospore-forming rods (*Bacillus spp.*), and Gram-negative rods (*E. coli, S. galinarum, P. vulgaris, Pectobacterium spp.*). The antifungal evaluation was determined against yeast (*C. krusei, C albicans, C. glabrata*) and filamentous fungi *(Fusarium spp., Aspergillus spp., T. harzianum*). The microbial activity data (MIC or MBC/MFC) are presented in **Table 1**.

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Table 1. Antimicrobial activity of tested benzoxazines.

	MIC μg×cm^{-3}			MBC/MFC μg×cm^{-3}		
Microorganisms	1	2	5	1	2	5
Gram-positive cocci						
S. agalactiae KP-Sag1	15.6±3^{a}	29.2±3^{b}	50.0±0^{d}	25.0±0^{a}	91.7±6^{c}	75.0±0^{bc}
S. canis KP-Sac1	15.6±3^{a}	33.3±3^{b}	47.5±6^{cd}	25.0±0^{a}	70.8±10^{b}	87.5±6^{bc}
S. pseudintermedius KP-Spi1	16.3±3^{a}	37.5±5^{b}	56.3±6^{d}	31.9±4^{a}	75.0±10^{bc}	87.5±6^{bc}
S. aureus PCM 2054	16.3±3^{a}	45.8±3^{cb}	50.0±0^{d}	28.8±3^{a}	91.7±6^{c}	81.3±6^{bc}
Gram-positive endospore forming rods						
B. cereus PCM 1948	25.0±5^{a}	45.8±3^{cd}	81.3±9^{f}	31.3±3^{a}	62.5±9^{c}	100±0^{e}
B. subtilis PCM 1949	25.0±5^{a}	40.0±6^{bc}	69.0±6^{def}	35.0±5^{a}	80.0±4^{d}	81.3±6^{d}
B. mycoides PCM 2024	25.0±5^{a}	50.0±0^{cd}	100±0^{g}	43.8±6^{b}	92.0±6de	100±0^{e}
B. megatherium PCM 1400	31.3±6^{ab}	58.3±6^{d}	100±0^{g}	56.3±6^{ab}	92.0±6de	100±0^{e}
Gram- negative rods						
E. coli PCM 2057	200±0^{c}	33.3±6^{a}	>400	400±0^{c}	50.0±0^{a}	>400
S. galinarum KP-Sg1	>400	55.0±2^{b}	>400	>400	105±10^{b}	>400
P. vulgaris PCM 542	>400	60.0±0^{b}	>400	>400	120.0±0^{b}	>400
P. aeruginosa PCM 2058	>400	>400	>400	>400	>400	>400
Gram-negative rods (plant pathogen)						
P. sbp. *carotovora* IOR 1815	46.9±3^{a}	48.3±1^{a}	187.5±10^{b}	62.5±6^{a}	96.7±2^{c}	>400
P. sbp. *carotovora* IOR 1822	46.9±3^{a}	48.3±1^{a}	187.5±10^{b}	62.5±6^{a}	88.3±5^{bc}	>400
P. atrosepticum IOR 1825	50.0±0^{a}	48.3±1^{a}	>400	75.0±9^{ab}	96.7±2^{c}	>400
P. atrosepticum IOR 1826	56.3±6^{a}	48.3±1^{a}	>400	75.0±9^{ab}	96.7±2^{c}	>400
Fungal strains						
C. albicans KP-Ca1	38.1±5^{a}	200±0^{c}	350±27^{d}	47.5±2^{a}	400±0^{c}	350±27^{b}
C. krusei KW-F117	29.1±3^{a}	200±0^{c}	350±27^{d}	50.0±0^{a}	400±0^{c}	350±27^{b}
C. globrata KP-Cg1	50.0±0^{b}	200±0^{c}	400^{e}	50.0±0^{a}	400±0^{c}	>400
F. culmorum KP-F1	200±0^{c}	>400	>400	400±0^{c}	>400	>400
T. sbp. aggressivum KP-Tha1	200±0^{c}	>400	>400	400±0^{c}	>400	>400
F. moniliforme KP-Fm1	400±0^{e}	>400	>400	>400	>400	>400
A. niger KP-An1	400±0^{e}	>400	>400	>400	>400	>400
A. flavus KP-Af1	400±0^{e}	>400	>400	>400	>400	>400

>400 – Inactivity at a dose 12.5 to 400 μg×cm^{-3}, data for **3**, **4**, **6**-**8** described are >400, and therefore they have been omitted for the clarity; ± – SD (standard deviation); Values followed by the same letter, are not significantly different ($p > 0.05$). Tukey's multiple-range test was done alone for MIC and MBC values and alone for cocci species, for Bacillus spp., for plant pathogens, for gram-negative bacteria and for fungi.

Compounds **1** and **5** did not exhibit any antibacterial activity to Gram-negative bacteria (*P. aeruginosa, P. vulgaris, S. galinarum, E. coli*) at the tested concentrations, but compound **2** exhibited strong activity against *E. coli* (MIC = 33 μg×cm^{-3} and MCB = 50 μg×cm^{-3}), *S. galinarum* (MIC = 55 μg×cm^{-3} and MCB = 105 μg×cm^{-3}), and *P. vulgaris* (MIC = 60 μg×cm^{-1} and MBC = 120 μg×cm^{-1}). Additionally, **1** and **2** were effective against Gram-negative plant pathogenic *Pectobacterium* strains (the range of MIC and MCB was 46.9–56.3 μg×cm^{-3} and 62.5–96.7 μg×cm^{-3}, respectively). The most sensitive to **1**, **2**,

and **5** were Gram-positive cocci and endospore-forming rods. Among all the tested compounds, **1** showed the strongest antibacterial activity against *Streptococcus* and *Staphylococcus* species. The applied MIC and MBC doses (15.6–25.0 μg×cm^{-3}) were up to 2- or 3-fold lower compared to **2** and **5**. The lowest degree of inhibition against endospore-forming rods was observed for **1** (MIC ranged from 25.0 to 31.3 μg×cm^{-3}), and the bactericidal effect ranged from 31.3 to 56.3 μg×cm^{-3}.

The antifungal activity of the tested compounds was determined against selected yeast and filamentous fungi. Although, none of the benzoxazines showed higher efficacy than the referenced amphotericin B against the tested fungi, **1** was found to be significantly potent against Candida species with low values of MIC and MFB (29.1–50 μg×cm^{-3}). The exchange of bromophenyl substituents in **1** for the alkyl chain as in **5** led to the reduction of antifungal activity, and **5** showed only anticandidal activity (MIC and MFB = 350 μg×cm^{-3}).

Overall, the tested compounds **1**, **2**, and **5** showed the broadest range of biological activity exhibiting the highest activity against Gram-positive, lower but interesting activity against Gram-negative *Pectobacterium spp*., and additionally antifungal effect against *C. krusei, C. albicans, F. culmorum,* and *T. aggressivum.* Benzoxazine **1** indicated effective inhibitory effect against Gram-positive bacteria strains and is equipotent to the reference standard drug (tetracycline) used against the tested bacterium. The structure-activity relationships showed that the antimicrobial activity profile of 1,3-benzoxazines depends on the substituents present in the aryl core. Benzoxazines, specifically those with the 1,3-motif, show a wide range of biological activity, which causes continuous interest in modifying and functionalizing the structure of these compounds in the context of potential bioapplications. Examples of such studies, concerning the influence of the benzoxazine aromatic ring substituents on biological activity are rare. Typically, the studies are focused on the modification of the substituents on the heterocyclic ring. Recently, the new class of anti-malarials based on benzoxazine with phytophenols core derived from natural product has been developed,[1a] where the main focus of the studies was to highlight the effect of substituents located in the benzene ring that may play an important role in the biological activity. In this context, the antitubercular agents based on 1,3-benzoxazine motifs have been evaluated. Substitution pattern of aryl ring bonded to nitrogen atoms clearly shows the differential sensitivity of Mycobacterial strains.[1b] However, the modification of aryl core of benzoxazine has not been discussed in this aspect. Whereas, the appropriate compounds have been synthesized, what more a similar relationships can be observed as presented in this work, because the analog with free ortho position was the most active compared to substituted one. The bulky obstruction in the *ortho* position in relation to the oxygen atom of the oxazine ring resulted in the complete loss of benzoxazine antimicrobial efficacy. Benzoxazine **1** with the free *ortho* position displayed significant activity and, in contrast, the analog **4**, containing *tert*-butyl substituents, is inactive against all the screened microbes. Similar behavior was observed for **8** and **5**, and the difference between them is also only in the aryl core, free or sizable substituents in the *ortho* position. These results could be a guide for further biological studies because most of the previously tested benzoxazine moieties were based on the aryl ring with free substituents or with methyl substituents bonded in this position.

Finally, benzoxazines **1**, **2**, and **5** were studied for their cytotoxic effect against BALB/3T3 mouse fibroblasts by SRB assay.[9] The morphological anomalies of cells caused by the studied benzoxazines were evidenced under a contrast microscope. The influence of the tested benzoxazines on the *in vitro* growth of mouse fibroblasts is shown in **Fig. 8**.

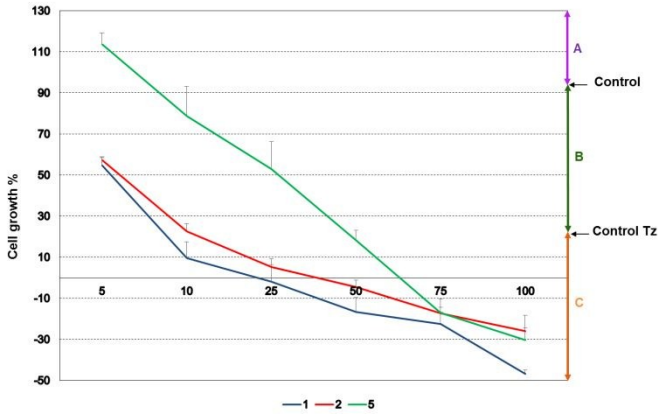

Fig. 8. Impact of **1**, **2**, and **5** on the *in vitro* growth of 3T3 BALB fibroblasts. A – cell growth stimulation, B – cell growth inhibition, C – cytotoxic effect, control Tz (start test) – time added compounds to the cell culture and the tested dose, Control (100%) – cell growth.

Benzoxazines **1** and **2** inhibit the growth of fibroblasts by about 50% at a dose of 5 μg×cm^{-3}, but compound **5** inhibited cell growth (50%) at a five-fold higher dose (~25 μg×cm^{-3}). Compounds **1** and **2** inhibit cell growth, at a similar level, in the ranges of 5–25 μg×cm^{-3} and 5–35 μg×cm^{-3}, respectively. Above these concentrations, benzoxazines **1** and **2** exhibit cytotoxic effects on cells. In contrast, **5** decreases cell growth at a higher dose, ranging from 10 to 65 μg×cm^{-3}. In addition, compound **5**, at a dose of 5 μg×cm^{-3}, causes the growth of cell proliferation, which suggests a positive effect on cells (vitality and growth increases). The photographs (ESI Photos 1–12 and Fig. S11–18) showed the effect of benzoxazines **1** and **5** on the vacuolation of fibroblast cells and cytopathic effects. Observed changes in the cells included vacuolation and the change of shape. Microscopic observations confirm the results of the SRB test.

Conclusions

The family of new N-substituted benzoxazines were synthesized and fully characterized. Their molecular structure was determined using spectroscopic methods and X-ray crystallography. The focus of our study was the experimental verification of the relation of these simple molecular motifs to potential applications as antimicrobial species. One of them, **1**, showed significant activity with MIC values in a range

equipotent and in some examples better than tetracycline against the tested bacterium. These results may be useful for the application of **1** as a new antibacterial and biocidal agent. The *ortho* position of the aryl core is crucial for the biological activity of 1,3-benzoxazines; sizable substituents caused the deactivation of these antimicrobial agents. Analysis of the literature data leads to the same conclusions. The cytotoxic assay results revealed that benzoxazines with long alkyl chains offered remarkable cell viability. The new benzoxazine monomers (**5**-**8**) have been polymerized. The glass transition temperatures are extremely low in comparison to classical benzoxazine derivatives. The liquid nature of alkyl-chain-modified monomers and the high processing windows indicate their high processable potential.

Conflicts of interest

There are no conflicts to declare.

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