C. R. Chimie 16 (2013) 665–671

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Synthesis and microbiological evaluation of several benzocaine derivatives

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ARTICLE INFO

Article history:
Received 2 February 2013
Accepted after revision 26 March 2013
Available online 6 May 2013

Keywords:
Benzocaine
Synthesis
Structure
Biological evaluation

ABSTRACT

Starting from benzocaine, a well-known anaesthetic, ten derivatives were synthesized and characterized by UV–vis, IR, NMR, and elemental analysis. Most of the compounds contain residues with recognized biological activity, like nicotinic acid (vitamin B3 or PP), biotin (vitamin B7 or H), lipoic acid (thioctic acid), adamantine, as well as other residues of crown-ether type, benzofurazane, naphtylurea, di- and tri-nitrobenzene, and a nitroxide radical. The biological evaluation of the obtained compounds included hydrophobicity (lipophobicity) assay, total antioxidant and microbiological activity tests.

1. Introduction

In the quest for new medicines, drug design and synthesis play an important part. Many medicines loose their activity due to extensive use, requesting higher doses or the replacement of the active compound. Among medicines, anaesthetics are a separate class of compounds, because many of them can induce addiction and therefore, generate drug abuse [1–4].

Design and synthesis of novel medicines is a quite long and expensive process, requesting a tremendous amount of work. Besides, the chosen chemical as a prospective medicine has to pass a huge number of tests, in which all the aspects of the possible chemical and biological factors have to be carefully addressed [5].

Benzocaine, an anaesthetic, is a simple chemical compound that induces pain relief; it is used in topical, dermal and mucous formulations, but, because its low water solubility, benzocaine cannot be used in parenteral administration. As any medicine, benzocaine has advantages and weak issues; therefore, new derivatives containing as substructure the benzocaine moiety are always of interest. Previous studies have demonstrated that benzocain-containing local anaesthetics and novel derivatives exhibited antimicrobial activity against different species, either Gram-positive, or Gram-negative, or fungal strains [6,7].

This paper deals with the synthesis, structural characterization and the microbiological activity evaluation tests of several novel benzocaine derivatives.

2. Results and discussion

2.1. Synthesis and structural characterization

Starting from benzocaine, all the compounds **1**–**10** (Fig. 1) were obtained practically in a single step, coupling benzocaine with the desired compound. Although some of the compounds (like **1**, **3**, **6**, **7**, Fig. 1) are present in the literature [7–11], we synthesized them to cover a broader

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http://dx.doi.org/10.1016/j.crci.2013.03.012

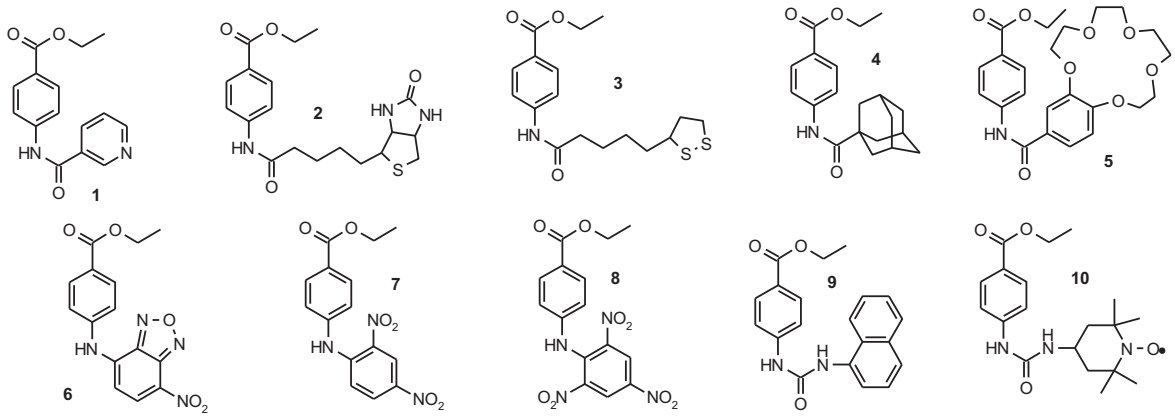

Fig. 1. Chemical structure of the synthesized compounds **1**–**10**.

range of chemicals with possible biological activity, and also to be compared with the new synthesized derivatives.

Thus, to obtain the desired compounds **1**–**5,** the coupling reactions between benzocaine and the required carboxylic acids have been tried in three different types of reactions, in order to get the highest yields:

- coupling using dicycloxexylcarbodiimide (DCC);
- coupling using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ);
- coupling using the corresponding acid chloride of the carboxylic acid.

It was shown that the higher yields were obtained by using the corresponding acid chloride of the carboxylic acid; between EEDQ and DCC, in our cases EEDQ was a better coupling agent. Compounds **6**–**8** were obtained by simple and effective nucleophilic aromatic substitution, while compounds **9** and **10** were synthesized by a coupling reaction between an amine and an isocyanate derivative. The general yields were between 40 and 95%.

Most of the obtained compounds required purification by column chromatography or preparative TLC; for analytical samples obtained in this way, structural characterization has been performed using current spectroscopic methods, like IR, UV–vis, NMR, and fluorescence and electron spin resonance (ESR), as necessarily (fluorescence for compound **6**, containing a benzofurazan moiety, and ESR for compound **10**, containing a free stable radical moiety).

All the analyses performed to characterize compounds **1**–**10** confirmed their chemical structure. Thus, in IR spectra, intense signals are noticed for the carbonyl groups, either from the ethyl ester moiety or from the amide or ureido moieties (around 1700 cm^{-1}). Amino groups appear between 3200 and 3500 cm^{-1} as broad signals. Aliphatic groups appear in IR at about 2900–3000 cm^{-1}, while the aromatic ones can be observed between 3000 and 3100 cm^{-1}. Nitro groups can easily be recognized by their values at about 1350 cm^{-1} and 1550 cm^{-1}, while the ether ones appear at about 1100–1200 cm^{-1}.

In UV–vis spectra (Table 1), the most bathochromic shift is noticed for compound **6** (472 nm, as an orange solid); this is accounted for by the nitrobenzofurazan (NBD) moiety, well known for its intense colour. Compounds **7** and **8**, containing nitro groups, are yellow solids, while all the others are white-grey solids.

In NMR spectra, amino groups are shifted under the influence of their chemical neighbours, and they appear between 7.5 and 10.5 ppm; all other ^{1}H and ^{13}C NMR values confirm the structure (see also Section 3).

Compound **6** exhibits the well-known fluorescence of the NBD moiety, with the emission value at 500 nm; compound **10**, containing a stable free radical moiety, shows the corresponding triplet in the ESR spectrum with a hyperfine coupling value of 15.5 G.

2.2. Biological evaluation

2.2.1. Lipophilicity

One of the most important properties of the medicines is their hydrophobicity or lipophilicity (usually noted log *P*, where *P* means the partitioning coefficient), which is correlated with the water or fat solubility, and therefore, with the capacity of crossing the cell membrane. The standard experimental methods used to determine the hydrophobicity values (log *P*) are the *n*-octanol/water repartition measurements [12] and the reverse-phase (RP) TLC [13], the latter being employed in this study; we

Table 1
UV–vis, lipophilicity and TAC values for compounds **1**–**10.**

Comp.	λ_{max} [a]	R_{M_0} [b]	b [b]	Log P [c]	SA [c]	TAC [d]
1	385	1.23	−2.82754	2.50	410	3.2
2	272	1.14	−2.91535	1.23	603	6.4
3	311	1.30	−2.76971	2.92	586	12
4	273	2.98	−4.31451	3.49	372	0.8
5	288	−0.36	−0.57627	1.70	485	8.8
6	472	1.51	−2.79154	3.05	515	10.4
7	341	2.42	−3.21055	3.28	549	8
8	378	2.39	−3.82341	3.23	580	3.2
9	292	2.91	−4.84119	3.74	518	43.2
10	317	1.57	−3.00695	1.69	607	22.3

[a] nm, in methanol.
[b] Experimental values from RP–TLC.
[c] Theoretical values.
[d] Experimental values.

calculated as well the theoretical value of log *P*, employing the HyperChem 8 molecular modelling package [14].

RP–TLC requires a non-polar stationary phase (i.e. silica gel impregnated with paraffin oil) and a mixture of two solvents, in which one is water (i.e. acetone–water). This method is widely used to measure the experimental lipophilicity (R_{M_0}) and the specific hydrophobic surface (b) using equations (1) and (2) [15].

$$R_M = \log\left(\frac{1}{R_F} - 1\right) \quad (1)$$

$$R_M = R_{M_0} + bC \quad (2)$$

The values of R_{M_0} and b obtained with equations (1) and (2) are the best indicators of the lipophilicity and of the specific hydrophobic surface area. The intercept R_{M_0} is the R_M value of a compound extrapolated to zero organic phase concentration in the eluent; the slope b is the change of lipophilicity caused by a unit concentration change in the organic phase (Table 1). A good linear correlation was found between R_M and C, characterized by high values of the correlation coefficient R (0.90–0.99).

The attempt to correlate the experimental lipophilicity values R_{M_0} with the theoretical ones, logs *P*, is shown in Fig. 2. It can be easily seen that there is a general trend in the evolution of the lipophilicity values, with the exception of compounds **2** and **10**, which seem to be out of range. The linear correlation found for all the compounds can be written as $\log P = 0.66R_{M_0} + 1.55$ (however, $R = 0.77$).

2.2.2. Total antioxidant capacity

The antioxidant capacity of all the synthesized compounds was measured using the well-known DPPH method [16], in which the absorbance of a mixture of 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) and the compound studied was measured initially and after 30 min at 517 nm. The total antioxidant capacity (TAC) determined using equation (3) is compiled in Table 1 [17].

$$\mathrm{TAC} = \frac{Abs_0 - Abs_{30\mathrm{min}}}{Abs_0} \times 100 \quad (3)$$

The highest TAC value was obtained for compound **9**, followed by **10**. These values can be related to the ureido moiety and/or to the naphtyl or free radical moieties.

2.2.3. Microbiological evaluation

The antimicrobial activity [18–21] of the obtained compounds was tested against bacterial and fungal strains belonging to the following genera and species: *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus*, and *Candida albicans.*

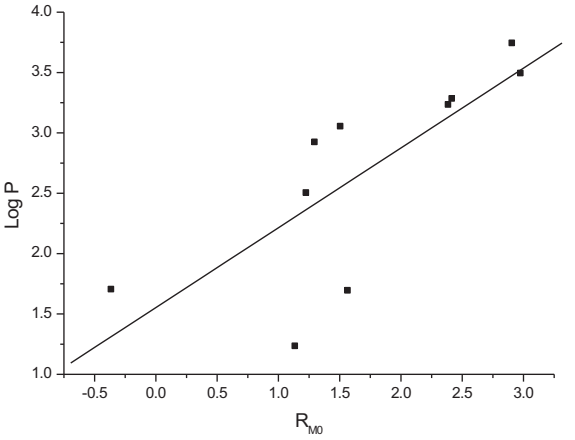

Fig. 2. Linear correlation between theoretical and experimental lipophilicity values.

Some of the tested substances exhibited moderate and good antimicrobial activity against the tested strains. A minimal inhibitory concentration (MIC) of 128 to 256 μg/mL was considered as moderate, in accordance with other studies, while a MIC value lower than 128 μg/mL was an indicator for a good inhibitory activity.

It is to be noticed that the compounds **4**, **9** and **10** exhibited a large spectrum of antibacterial activity directed against Gram-negative *P. aeruginosa* and Gram-positive *S. aureus* strains. Three other compounds, i.e. **2**, **6** and **8**, exhibited only anti-pseudomonal activity (Table 2). None of the tested compounds exhibited significant anti-fungal activity.

Concerning the anti-biofilm activity of the tested compounds, the microtitre assay allowed us to establish the concentration range that inhibited the development of microbial biofilms on the plastic walls, quantified by measuring the absorbance of the adhered and coloured cells at 490 nm. None of the tested compound inhibited the *E. coli* biofilm development (Supplementary data, Fig. S1); as well, all the tested compounds exhibited anti-*P. aeruginosa* biofilms activity at high concentations, ranging from 1024 to 64 μg/mL (Supplementary data, Fig. S2).

Table 2
Minimal inhibitory concentration values (μg/mL) of the obtained compounds.

Tested compound	*Escherichia coli*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*Candida albicans*
1	512	512	1024	1024	512
2	1024	8	1024	1024	512
3	1024	1024	1024	1024	256
4	1024	16	1024	256	512
5	512	1024	1024	512	1024
6	1024	256	1024	1024	1024
7	512	512	1024	1024	512
8	512	128	1024	1024	1024
9	1024	8	1024	256	512
10	1024	32	1024	16	512

The compound **10** inhibited *S. aureus* biofilm development on a large range of concentrations, from 1024 to 16 μg/mL, as demonstrated by the low absorbance values obtained for this compound (Supplementary data, Fig. S3); compounds **2** and **3** inhibited the *B. subtilis* biofilm development at the highest tested concentration of 1024 μg/mL (Supplementary data, Fig. S4); as well, all the tested compounds slightly inhibited *C. albicans* biofilms at higher concentrations (from 1024 to 512 μg/mL) (Supplementary data, Fig. S5).

3. Experimental

3.1. Apparatus and materials

All the chemicals, materials and solvents were purchased from Sigma–Aldrich, Alfa Aesar or Chimopar, and used as received. UV–vis spectra were recorded in methanol at ambient temperature, on an UVD-3500 double bean spectrometer, using quartz cell with 1-cm path length. IR spectra were recorded with a Bruker Vertex 70 spectrometer (as solid samples, ATR); ^{1}H- and ^{13}C NMR spectra were recorded with a Varian Inova-400 spectrometer (at selected temperatures, in deuterated solvents $CDCl_3$ and DMSO-d_6, isotopic purity 99.9%). ESR spectra were recorded with a Jeol JES-FA 100 spectrometer in DCM as a solvent and at room temperature.

3.2. Synthesis

3.2.1. Compound 1

To 2 mmol of nicotinic acid suspended in 30 mL of 1,2-dichloroethane were added two drops of DMF and 2 mL of thionyl chloride, and the mixture was refluxed for 2 h; the solvent and excess thionyl chloride were removed under vacuum, and to the residue were added 50 mL of DCM, 2 mmol of benzocaine, and 5 mL of thiethylamine; the next day, 50 mL of DCM were added, and the organic phase extracted with 100 mL of diluted aqueous hydrochloric acid (10%), followed by aqueous sodium hydrogen carbonate (3%), and then dried using anhydrous sodium sulphate. The removal of the DCM affords the crude compound, which can be purified on preparative silica TLC plates, using DCM as an eluent.

^{1}H NMR($CDCl_3$, δ ppm, *J* Hz, *T* = 303 K): 9.11(bs, 1H, H-15); 8.78(bs, 1H, HN, deuterable); 8.73(bs, 1H, H-14); 8.21(d, 1H, H-12, 7.8); 8.02(d, 2H, H-3, H-5, 8.6); 7.75(d, 2H, H-2, H-6, 8.6); 7.40(d, 1H, H-13, 7.8); 4.36(q, 2H, H-8, 7.1); 1.39(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, δ ppm, *T* = 303 K): 166.20(C-7); 164.30(C-10); 152.37(bs, C-15); 148.02(bs, C-14); 141.98(C-1); 135.87(C-12); 131.56(C-11); 130.79(C-3, C-5); 126.52(C-4); 123.86(C-13); 119.68(C-2, C-6); 61.04(C-8); 14.33(C-9). Elemental analysis: calculated for $C_{15}H_{14}N_2O_3$ (*M* = 270): C = 66.66; H = 5.22; N = 10.36; found: C = 66.62; H = 5.25; N = 10.29. IR (ATR, cm^{-1}): 3497.09; 3315.39; 3188.23; 3010.44; 2975.56; 2937.49; 2874.14; 1683.34; 1596.87; 1535.01; 1509.94; 1486.04; 1453.11; 1422.82; 1406.38; 1388.43; 1361.21; 1306.92; 1274.58; 1244.91; 1169.54; 1102.34; 1030.12; 1010.88; 856.53; 829.99; 769.22; 698.76; 499.91. R_f = 0.27 (DCM:ethyl acetate = 1:1).

3.2.2. Compound 2

This compound was prepared in a similar way as **1**, using biotin instead of nicotinic acid.

^{1}H NMR(dmso-d_6, δ ppm, *J* Hz, *T* = 303 K): 10.28(s, 1H, HN-1′, deuterable); 7.90(d, 2H, H-3, H-5, 8.8); 7.74(d, 2H, H-2, H-6, 8.8); 6.46(s, 1H, H-20 or H-21, deuterable); 6.38(s, 1H, H-21 or H-20, deuterable); 4.30(dd, 1H, H-17, $^{3}J(H^{18A}–H^{17})$ = 4.9, $^{3}J(H^{16}–H^{17})$ = 7.6); 4.28(q, 2H, H-8, 7.1); 4.15(dd, 1H, H-16, $^{3}J(H^{17}–H^{16})$ = 7.6, $^{3}J(H^{15}–H^{16})$ = 4.5); 3.13(dt, 1H, H-15, $^{3}J(H^{16}–H^{15})$ = 4.5, $^{3}J(H^{14}–H^{15})$ = 8.0); 2.83(dd, 1H, H-18A, syst AB, $^{3}J(H^{17}–H^{18A})$ = 4.9 ^{gem}J = 12.3); 2.59(d, 1H, H-18B, syst AB, ^{gem}J = 12.3); 2.37(t, 2H, H-11, 7.2); 1.54÷1.36(m, 4H, H-12, H-13); 1.31(t, 3H, H-9, 7.1). ^{13}C NMR(dmso-d_6, δ ppm, *T* = 303 K): 171.80(C-10); 165.33(C-7); 162.72(C-22); 143.65(C-13); 143.78(C-12); 141.03(C-1); 130.15(C-3, C-5); 123.91(C-4); 118.33(C-2, C-6); 61.04(C-17); 60.36(C-8); 59.21(C-16); 55.35(C-15); 39.83(C-18); 36.29(C-11); 28.19(C-12); 28.07(C-13); 24.93(C-14); 14.19(C-9). Elemental analysis: calculated for $C_{19}H_{25}N_3O_4S$ (*M* = 391): C = 58.29; H = 6.44; N = 10.73; found: C = 58.29; H = 6.39; N = 10.70. IR (ATR, cm^{-1}): 3249.80; 2928.34; 2858.72; 2361.27; 1692.16; 1596.46; 1529.39; 1462.28; 1407.37; 1366.57; 1308.09; 1275.16; 1171.99; 1103.10; 1019.92; 856.04; 767.83; 694.75; 615.00. R_f = 0.42 (DCM:methanol = 9:1).

3.2.3. Compound 3

To 1 mmol of lipoic acid, dissolved in 25 mL of DCM, were added 1 mmol of benzocaine and 1.2 mmol of EEDQ; after 2 days, 75 mL of DCM were added, and the organic phase extracted with 100 mL of diluted aqueous hydrochloric acid (10%), followed by aqueous sodium hydrogen carbonate (3%), and then dried using anhydrous sodium sulphate. The removal of the DCM affords the crude compound, which can be purified on preparative silica TLC plates, using DCM as eluent.

^{1}H NMR($CDCl_3$, δ ppm, *J* Hz, *T* = 303 K): 8.19(s, 1H, HN, deuterable); 7.98(d, 2H, H-3, H-5, 8.8); 7.64(d, 2H, H-2, H-6, 8.8); 4.35(q, 2H, H-8, 7.1); 3.53(qv, 1H, H-15, 7.1); 3.12(m, 2H, H-17); 2.45(m, 1H, H-16A, syst. AB); 2.40(t, 2H, H-11, 7.7); 1.89(m, 1H, H-16B); 1.56÷1.48(m, 4H, H-12, H-13); 1.38(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, δ ppm, *T* = 303 K): 171.77(C-10); 166.31(C-7); 142.33(C-1); 130.71(C-3, C-5); 127.94(C-4); 118.91(C-2, C-6); 60.94(C-8); 56.37(C-15); 40.23(C-17); 38.48(CH_2); 37.39(CH_2); 34.60(CH_2); 28.83(CH_2); 25.14(CH_2); 14.35(C-9). Elemental analysis: calculated for $C_{17}H_{23}NO_3S_2$ (*M* = 353): C = 57.76; H = 6.56; N = 3.96; found: C = 57.81; H = 6.56; N = 3.92. IR (ATR, cm^{-1}): 3310.45; 2975.64; 2940.72; 2925.04; 2858.51; 1704.06; 1661.41; 1608.81; 1591.95; 1520.57; 1463.04; 1404.02; 1363.57; 1334.80; 1306.00; 1275.06; 1256.19; 1170.93; 1105.86; 1039.77; 1018.42; 960.88; 852.69; 767.94; 730.37; 692.57; 579.63; 498.96. R_f = 0.53 (DCM:ethyl acetate = 9:1).

3.2.4. Compound 4

This compound was prepared in a similar way as **1**, using 1-adamatane carboxylic acid instead of nicotinic acid.

^{1}H NMR($CDCl_3$, δ ppm, *J* Hz, *T* = 303 K): 7.49(s, 1H, HN, deuterable); 8.00(d, 2H, H-3, H-5, 8.6); 7.64(d, 2H, H-2,

H-6, 8.6); 4.36(q, 2H, H-8, 7.1); 2.06(m, 3H, H-15, H-16, H-17); 1.98(m, 6H, H-12, H-13, H-14); 1.74(m, 6H, H-18, H-19, H-20); 1.39(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, δ ppm, T = 303 K): 176.29(C-7); 171.19(C-10); 142.23(C-1); 130.73(C-3, C-5); 125.79(C-4); 118.94(C-2, C-6); 60.83(C-8); 41.75(C-11); 39.23(C-12, C-14, C-18); 36.23(C-18, C-19, C-20); 27.69(C-15, C-16, C-17); 14.36(C-9). Elemental analysis: calculated for $C_{20}H_{25}NO_3$ (M = 327): C = 73.37; H = 7.70; N = 4.28; found: C = 73.32; H = 7.70; N = 4.27. IR (ATR, cm^{-1}): 3295.38; 2901.47; 2850.13; 2677.73; 1804.74; 1716.19; 1653.48; 1593.49; 1519.16; 1474.05; 1450.01; 1405.24; 1307.43; 1275.51; 1249.83; 1172.45; 1153.04; 1100.01; 995.86; 973.29; 935.36; 854.79; 766.00; 680.77; 617.42; 504.04. R_f = 0.35 (DCM).

3.2.5. Compound 5

This compound was prepared in a similar way as **1**, using 4-carboxybenzo-15-crown-5 acid instead of nicotinic acid.

^{1}H NMR($CDCl_3$, δ ppm, J Hz, T = 303 K): 8.97(bs, 1H, NH, deuterable); 8.00 (d, 2H, H-3, H-5, 8.6); 7.88(d, 2H, H-2, H-6, 8.6); 7.75(dd, 1H, H-16, 1.8, 8.4); 7.59(d, 1H, H-12, 1.8); 6.89(d, 1H, H-15, 8.4); 4.36(q, 2H, H-8, 7.1); 4.20(m, 4H, H-20, H-27, syst. A_2B_2); 3.92(m, 4H, H-21, H-26, syst. A_2B_2); 3.76(bs, 8H, H-22 ÷ H-25);1.39(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, T = 303 K, δ ppm): 166.31(C-7); 165.59(C-10); 162.40(Cq); 154.51(Cq); 142.86(C-1);121.37(C-11); 125.59(C-4); 130.61(C-3, C-5); 125.45(C-2, C-6); 119.41(C-16); 114.99(C-15); 112.02(C-12); 71.03(CH_2-CE); 70.30(CH_2-CE); 70.19(CH_2-CE); 69.21(CH_2-CE); 60.80(C-8); 14.38(C-9). Elemental analysis: calculated for $C_{24}H_{29}NO_8$ (M = 459); C = 62.73; H = 6.36; N = 3.05; found: C = 62.68; H = 6.33; N = 3.01. IR (ATR, cm^{-1}): 3504.96; 2928.12; 2870.04; 1770.45; 1708.44; 1669.36; 1596.33; 1510.90; 1451.97; 1427.62; 1347.25; 1321.39; 1267.64; 1211.39; 1174.64; 1127.40; 1029.74; 935.32; 872.12; 770.84; 755.34; 728.62; 697.93; 610.64. R_f = 0.47 (DCM:methanol = 9:1).

3.2.6. Compound 6

To 1 mmol of benzocaine dissolved in 7 mL of DMF were added 1 mmol of NBD-chloride and 2 mmol of powdered sodium hydrogencarbonate; the next day, the mixture was poured in about 100 mL of cold water, and the precipitate filtered off and dried, affording the crude compound, which can be purified on a silica column, using DCM as an eluent.

^{1}H NMR($CDCl_3$, δ ppm, J Hz, T = 303 K): 8.50(d, 1H, H-14, 8.6); 8.19(d, 2H, H-3, H-5, 8.6); 7.95(s, 1H, HN, deuterable); 7.48(d, 2H, H-2, H-6, 8.6); 6.95(d, 1H, H-15, 8.6); 4.42(q, 2H, H-8, 7.1); 1.43(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, δ ppm, T = 303 K): 165.49(C-7); 145.07(C-13); 143.78(C-12); 141.03(C-1); 139.36(C-11); 135.42(C-14); 131.69(C-3, C-5); 128.45(C-4); 127.01(Cq); 121.79(C-2, C-6); 102.20(C-15); 61.37(C-8); 14.35(C-9). Elemental analysis: calculated for $C_{15}H_{12}N_4O_5$ (M = 328): C = 54.88; H = 3.68; N = 17.07; found: C = 54.92; H = 3.68; N = 17.05. IR (ATR, cm^{-1}): 3278.64; 3097.08; 3050.33; 2985.58; 2922.98; 2853.09; 1702.40; 1626.82; 1597.56; 1554.40; 1505.53; 1446.94; 1417.18; 1397.11; 1366.16; 1308.57; 1278.61; 1184.36; 1162.47; 1091.43; 1034.79; 1016.76; 993.95; 898.26; 845.14; 809.56; 773.15; 755.76; 734.11; 699.49; 685.80; 620.58; 596.36; 511.27. R_f = 0.75 (DCM:ethyl acetate = 9:1), R_f = 0.22 (DCM).

3.2.7. Compound 7

This compound was prepared in a similar way as **6**, using 2,4-dinitro-1-fluorobenzene instead of NBD-chloride, and keeping the reaction on for 2 days. The crude compound can be purified on a silica column, using DCM as an eluent.

^{1}H NMR($CDCl_3$, δ ppm, J Hz, T = 303 K): 10.04(s, 1H, HN, deuterable); 9.19(d, 1H, H-12, 2.5); 8.24(dd, 1H, H-14, 2.5, 9.2); 8.17(d, 2H, H-3, H-5, 8.5); 7.39(d, 2H, H-2, H-6, 8.5); 7.35(d, 1H, H-15, 9.2); 4.42(q, 2H, H-8, 7.1); 1.43(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, δ ppm, T = 303 K): 165.50(C-7); 145.72(C-10); 141.01(C-1); 138.26(C-13); 132.13(C-11); 131.66(C-3, C-5); 130.06(C-14); 129.08(C-4); 124.02(C-15); 123.91(C-2, C-6); 116.30(C-12); 61.40(C-8); 14.35(C-9). Elemental analysis: calculated for $C_{15}H_{13}N_3O_6$ (M = 331): C = 54.38; H = 3.96; N = 12.68; found: C = 54.35; H = 3.95; N = 12.65. IR (ATR, cm^{-1}): 3321.81; 3113.80; 3090.06; 2991.76; 2924.50; 2854.23; 1704.04; 1621.19; 1589.76; 1514.12; 1441.72; 1422.00; 1364.52; 1336.07; 1262.69; 1221.81; 1175.14; 1127.45; 1060.21; 1017.59; 923.68; 868.81; 848.56; 828.10; 763.38; 736.91; 701.00; 686.09; 587.14; 505.14. R_f = 0.6 (DCM).

3.2.8. Compound 8

This compound was prepared in a similar way as **6**, using 2,4,6-trinitro-1-chlorobenzene instead of NBD-chloride; no purification was required.

^{1}H NMR($CDCl_3$, δ ppm, J Hz, T = 303 K): 10.26(s, 1H, HN, deuterable); 9.13(s, 2H, H-12, H-14); 8.06(d, 2H, H-3, H-5, 8.6); 7.11(d, 2H, H-2, H-6, 8.6); 4.38(q, 2H, H-8, 7.1); 1.40(t, 3H, H-9, 7.1). ^{13}C NMR($CDCl_3$, δ ppm, T = 303 K): 165.23(C-7); 141.22(C-1); 138.50(C-13); 138.06(C-11, C-15); 131.58(C-3, C-5); 130.79(C-10); 129.30(C-4); 127.24(C-12, C-14); 120.02(C-2, C-6); 61.36(C-8); 14.31(C-9). Elemental analysis: calculated for $C_{15}H_{12}N_4O_8$ (M = 376): C = 47.88; H = 3.21; N = 14.89; found: C = 47.81; H = 3.21; N = 14.83. IR (ATR, cm^{-1}): 3289.61; 3096.11; 3058.85; 2980.35; 2936.26; 1705.89; 1621.42; 1593.59; 1554.38; 1517.55; 1463.78; 1433.04; 1418.44; 1356.76; 1333.19; 1310.27; 1279.88; 1170.87; 1118.19; 1103.67; 1088.96; 1019.06; 932.82; 869.91; 825.26; 786.08; 766.68; 736.79; 722.96; 699.51; 675.01; 574.23; 485.41. R_f = 0.58 (DCM).

3.2.9. Compound 9

To 1 mmol of benzocaine dissolved in 50 mL of DCM was added 1 mmol of 1-naphtylisocyanate; after 1 week, 50 mL of DCM were added, and the organic phase was extracted with 100 mL of diluted aqueous hydrochloric acid (10%), followed by aqueous sodium hydrogen carbonate (3%), and then dried using anhydrous sodium sulphate. The removal of the DCM affords the crude compound, which can be purified on preparative silica TLC plates, using DCM as an eluent.

^{1}H NMR(dmso-d_6, δ ppm, J Hz, T = 303 K): 10.21(s, 1H, HN, deuterable); 9.33(s, 1H, HN, deuterable); 8.34(d, 1H, H-19, 8.0); 8.04(dd, 1H, H-12, 1.4, 8.0); 7.92(m, 1H, H-16); 7.91(d, 2H, H-3, H-5, 8.8); 7.68(d, 2H, H-2, H-6, 8.8);

7.66(dd, 1H, H-14, 1.4, 8.0); 7.50÷7.63(m, 2H, H-17, H-18); 7.49(t, 1H, H-13, 8.0); 4.29(q, 2H, H-8, 7.1); 1.32(t, 3H, H-9, 7.1). ^{13}C NMR(dmso-d_6, δ ppm, T = 303 K): 165.42(C-7); 152.75(C-10); 144.52(C-11); 134.01(C-1); 133.63(C-15); 130.32(C-3, C-5); 128.24(CH-13); 125.92(C-4); 125.84(CH-naft); 125.72(CH-naft); 125.66(CH-naft); 123.08(CH-naft); 122.52(C-20); 121.69(CH-19); 117.43(CH-12); 117.02(C-2, C-6); 60.19(C-8); 14.16(C-9). Elemental analysis: calculated for $C_{20}H_{18}N_2O_3$ (M = 334): C = 71.84; H = 5.43; N = 8.38; found: C = 71.86; H = 5.43; N = 8.39. IR (ATR, cm^{-1}): 3338.90; 3266.42; 2983.45; 1703.76; 1646.35; 1595.33; 1546.53; 1490.58; 1442.94; 1406.26; 1364.23; 1343.54; 1321.97; 1271.08; 1239.46; 1215.79; 1174.10; 1101.12; 856.57; 786.03; 763.81; 729.40; 695.14; 554.51. R_f = 0.45 (DCM:ethyl acetate = 9:1).

3.2.10. Compound **10**

To 0.5 mmol of benzocaine dissolved in THF was added 0.51 mmol of 4-isocyanto-TEMPO dissolved in a small amount of THF, and then, the mixture was stirred for about 1 day. A cold solution of aqueous hydrochloric acid (1 M) was added and after 15 min, the mixture was extracted with DCM, the organic layer separated, dried over anhydrous sodium sulphate, and the solvent removed under vacuum, affording the crude product, which was purified by preparative TLC.

Elemental analysis: Calculated for $C_{19}H_{28}N_3O_4$ (M = 362): C = 62.96; H = 7.79; N = 11.59; found: C = 62.81; H = 7.77; N = 11.33. IR (ATR, cm^{-1}): 3387.39; 3291.98; 3083.97; 2960.59; 2928.49; 1623.34; 1592.31; 1502.03; 1420.68; 1354.88; 1271.08; 1172.59; 1115.29; 1072.42; 935.52; 905.70; 870.15; 814.40; 729.78; 657.64; 514.68. R_f = 0.25 (DCM:ethyl acetate = 9:1).

3.3. Lipophilicity measurements

Analytical silica gel plates, impregnated overnight with 5% paraffin oil in heptane, were allowed to dry at ambient temperature and then used. The eluent was a mixture of acetone with water in different proportions (40–80% acetone). The R_M values necessary for the determination of the hydrophobicity are obtained as shown in equation (1). The specific hydrophobic surface area b was obtained using the linear correlation between the R_M values and the concentration of organic solvent (C) in the eluent. The calculated log P values were obtained using the HyperChem 8 molecular modelling package.

3.4. TAC measurements

These measurements were performed in methanol at ambient temperature, the final mixture containing DPPH at a 1.5×10^{-4} M concentration and one of the compounds **1–10** at a 0.2 mg/mL concentration. Each mixture was kept for 30 min, followed by absorbance measurement at 517 nm. The TAC values were obtained using equation (3).

3.5. Microbiological activity testing

In our experiments, we used bacterial suspensions of 1.5×10^8 CFU/mL density, corresponding to 0.5 McFarland nephelometric standard, obtained from 15–18 h bacterial cultures developed on solid media and stock solutions of the new compounds of 1024 μg/mL concentration. For the MIC assay, serial binary dilutions were performed in nutrient broth distributed in 96-multiwell plates, further inoculated with a standard inoculum of the microbial strains. The plates were incubated at 37 °C for 24 h.

The MIC was read by wells' observation: in the first wells, containing high concentrations of compounds, the culture growth was not visible, the microbial cells being killed or inhibited by the tested compound. At lower concentrations of the tested compounds, the microbial culture became visible. The lowest concentration, which inhibited the visible microbial growth, was considered the MIC value for the tested compound. In the next wells, including the standard culture growth control wells, the medium become muddy as a result of the microbial growth. In the sterility control wells series, the medium had to remain clear. From the last well without any visible microbial growth and from the first one with a microbial growth, Gram stained smears were performed for the results confirmation. For the evaluation of the influence of different complex concentrations on the ability of the tested bacterial strains to colonize the inert substratum, a very simple microtitre plate method was used. In this purpose, the microplates used for the MIC assay were emptied, washed three times by PBS (phosphate buffered saline). The biofilm formed on the plastic wells wall was fixed for 5 min with cold methanol, coloured for 15 min by violet crystal solution and resuspended by a 33% acetic acid solution. Cell density was measured by reading the optical density of the coloured solution at 490 nm using an ELISA reader (Apollo LB 911).

4. Conclusion

Ten compounds derived from benzocaine were synthesized and their corresponding structure characterized by specific means. The biological assays have demonstrated that the substances **9** and **10** have the potential to be used as large spectrum antimicrobial agents, being active both on Gram-negative *P. aeruginosa* and the Gram-positive *S. aureus*. Four of the tested compounds, **2**, **4**, **9**, and **10**, proved to be very active, exhibiting low MIC values on *P. aeruginosa* strains. The tested substances also exhibited an inhibitory activity on the ability of microbial strains to develop biofilms, the intensity of this effect being dependent on the tested microbial strains and the concentration of the tested substances.

Acknowledgements

This work was partially supported by a Grant of the Romanian National Authority for Scientific Research, CNCS–UEFISCDI, project number PN-II-ID-PCE-2011-3-0408.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crci.2013.03.012.

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