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Short communication

Synthesis, antimicrobial data and correlation analysis in a set of 2-alkyl-5-amidobenzotriazoles

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Summary — A set of 2-alkyl-5-amidobenzotriazoles has been prepared and characterized. Shake-flask partition coefficients (log P) and capacity factors (log *k'*) have been experimentally determined. The *in vitro* antimicrobial activity against Gram–positive, Gram–negative and *Candida albicans* have been correlated, through regression analysis, with the corresponding partition coefficients.

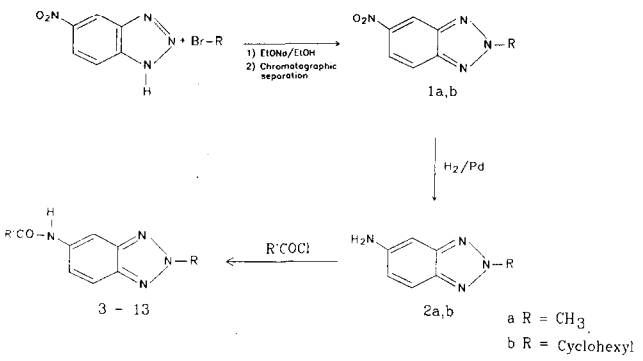
benzotriazoles / lipophilicity / antimicrobial activity / QSAR

Introduction

Some time ago [1] a number of 2-cyclopentyl-5-amidobenzotriazole derivatives were synthesized in our laboratory to explore the antimicrobial properties of these molecules. The results of this study showed that modifications of the lipophilic character of the considered compounds could offer opportunities for further modulation of their activity against Gram–positive and Gram–negative bacteria as well as against fungi. Following these observations, we have planned the preparation of other structurally-related benzotriazole derivatives, whose substituents allow variations of hydrophobic properties in a wider range than hitherto studied.

Chemistry

Compounds of general formula reported in table I were synthesized according to scheme 1, by reaction of the corresponding 2-alkyl-5-aminobenzotriazole (**2a, b**) with the appropriate acyl chloride



Scheme 1.

The 5-aminobenzotriazole derivatives were prepared by catalytic reduction of the corresponding 5-nitroderivatives obtained by reaction of 5-nitrobenzotriazole with iodomethane or cyclohexylbromide respectively. This reaction, carried out in absolute ethanol and sodium ethoxide, gave a mixture of products corresponding to 1,5-, 1,6- and 2,5-substituted isomers, respectively, whose separation was performed by chromatography on silica gel column using CH_2Cl_2/hexane 9:1 v/v as eluent. The

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reaction gave a mixture of 3 isomers in 75% overall yield; the compound bearing the substituent in position 2- was obtained in the highest yield. All compounds listed in table I were characterized by PMR, IR and UV spectroscopy.

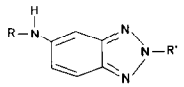
Examination of IR spectra shows the presence of characteristic absorption frequencies reflecting the nature of the functional group and the substitution on position 2 of the benzotriazole ring. The nature of the functional group was evidenced by the presence of stretching at 3220–3350 cm^{-1} (NH) and 1680–1650 cm^{-1} (C=O) for the amides; while the compounds bearing the NO_2 group were characterized by a sharp stretching at 1530 cm^{-1}.

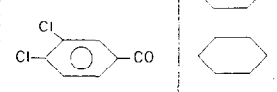
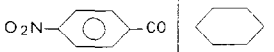
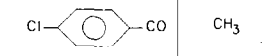
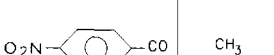
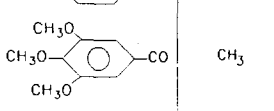
The ultraviolet spectra show the presence of an aromatic chromophoric system for all compounds. Particularly the aliphatic amides show a single absorption maximum ranging between 280–300 nm; the UV spectra of arylamido–derivatives were characterized by 2 absorption maxima ranging between 258–263 nm and 300–306 nm; while the chloroaliphatic amides show absorption maxima ranging between 233–246 nm and 295–303 nm.

In addition to IR and UV spectra all compounds reported in this paper were characterized by PMR spectroscopy whose data were fully consistent with the described structures.

Finally, partition coefficients (log P) were determined in the octanol/water system and the capacity factors (log k') of all substances considered were correlated, through regression analysis, with the corresponding partition coefficients.

Table I. Benzotriazole derivatives and their hydrophobic and chromatographic characteristics.



N	R	R'	M.W.	m.p.	yield %	log P[a]	log k'
3	Cl_2CHCO		327.21	137–139	47	3.87 (±0.04)	0.93
4	Ph–CO		320.40	211–213	49	4.45 (±0.05)	1.05
5			388.28	190–192	38	5.85[b]	1.80
6			410.47	130–131	39	4.45[b]	1.13
7			365.39	236–237	47	4.57[b]	1.19
8	$ClCH_2CO$	CH_3	244.65	176–177	24	1.22 (±0.02)	-0.43
9	C_2H_5OCO	CH_3	220.23	147–149	51	1.83 (±0.02)	-0.10
10	Ph–CO	CH_3	252.28	165–166	25	2.44 (±0.03)	0.10
11		CH_3	286.72	228–230	47	3.13 (±0.03)	0.50
12		CH_3	297.13	279–281	48	2.53 (±0.03)	0.26
13		CH_3	342.35	112–114	34	2.38 (±0.02)	0.15

[a]Nos in parentheses indicates the 95% confidence interval.
[b]These values are calculated from equation 1.

Results and discussion

Partition coefficients (table I) were determined by the 'shake-flask' method according to Leo *et al* [2]. The details are described in the experimental section. Owing to the limited aqueous solubility of some compounds, HPLC chromatographic indices were determined as an alternative measure of partitioning. As already shown [1], mixtures of water and methanol allowed good separations with reasonable retention times. Data reported in table I are relative to a methanol/water mixture in the ratio 3:2 (v/v). The measured octanol/water partition coefficients (log P) (compounds **3**, **4**, **8–13**) were correlated with the corresponding chromatographic indices (log k') and equation [1] was used to estimate the remaining values (compounds **5–7**).

$$\log P = 2.09\ (\pm 0.22) \log k' + 2.09\ (\pm 0.13) \qquad (1)$$
$$n = 8\ r = 0.994\ s = 0.122\ F = 689.07$$

In this equation n is the number of compounds considered, r is the correlation coefficient, s the standard deviation of the regression and the figures in parentheses are the 95% confidence limits; F represents the statistical significance of the equation. The good correlation coefficient and the low standard deviation of equation (1) based on data covering a wide range of log P values clearly demonstrate the excellent HPLC modeling of the 'shake-flask' procedure.

Table II summarizes the *in vitro* antimicrobial activities of this set of compounds against Gram–positive *(Staphylococcus aureus)* and Gram–negative bacteria *(Salmonella Wiener, Serratia marcescens* and *Pseudomonas aeruginosa)* as well as their fungistatic activity against *Candida albicans*; moreover, table II includes antimicrobial activities of some reference antibiotics tested under the same conditions.

In a previous paper [1] we reported that the introduction of the amide group in position 5- of the 2-cyclopentylbenzotriazole resulted in increased activity

Table II. Antimicrobial activity of compounds **3–13** and some reference antibiotics tested under the same conditions (MIC μg/ml, in parentheses log 1/*C*).

Compound	*S aureus*	*S wiener*	*S marcescens*	*P aeruginosa*	*C albicans*
3	100 (3.52)	50 (3.82)	100 (3.52)	100 (3.52)	25 (4.12)
4	50 (3.81)	100 (3.51)	> 200 (> 3.21)	100 (3.51)	> 200 (> 3.21)
5	200 (3.29)	> 200 (> 3.29)	> 200 (> 3.29)	> 200 (> 3.29)	> 200 (> 3.29)
6	100 (3.61)	100 (3.61)	100 (3.61)	200 (3.31)	50 (3.91)
7	50 (3.86)	100 (3.56)	100 (3.56)	200 (3.26)	100 (3.56)
8	> 200 (> 3.09)	> 200 (> 3.09)	200 (3.09)	200 (3.09)	200 (3.09)
9	200 (3.04)	100 (3.34)	100 (3.34)	100 (3.34)	100 (3.34)
10	200 (3.10)	100 (3.40)	100 (3.40)	100 (3.40)	100 (3.40)
11	50 (3.76)	50 (3.76)	50 (3.76)	50 (3.76)	100 (3.46)
12	100 (3.47)	50 (3.77)	100 (3.47)	100 (3.47)	100 (3.47)
13	200 (3.23)	100 (3.53)	100 (3.53)	100 (3.53)	100 (3.53)
Ampicillin	12.5 (4.45)	–	25 (4.15)	–	–
Cephalotin	12.5 (4.50)	–	100 (3.60)	–	–
Erythromycin*	12.5 (4.77)	–	–	–	–
Gentamicin**	25 (4.43)	12.5 (4.73)	12.5 (4.73)	12.5 (4.73)	–
Amphotericin B	–	–	–	–	12.5 (4.87)

*Calculated as erythromycin A ($C_{37}H_{67}NO_{13}$, mw 733.9). **Calculated as gentamicin C_1 ($C_{21}H_{43}N_5O_7 \cdot 2H_2SO_4$, mw 673.6).

in several instances. In the present study we have carried out structural modifications introducing adequate substituents on the aromatic moiety and on the nitrogen atom in position 2- of the benzotriazole nucleus, in an attempt to study the modulation of antimicrobial activity resulting from variation of lipophilic properties of the compounds. The data reported in table II were expressed as log 1/*C*, where *C* represents the lowest concentration of antimicrobial agent that results in the complete inhibition of visible growth of microrganisms (MIC). Examination of these data indicates that several of the newly synthesized compounds have the same order of activity as the reference antibiotics. As a general trend when the compound lipophilicity is increased, an increase of activity is observed; in the extremely lipophilic compound **5**, the activity on *S aureus* is drastically reduced, and it is completely lost on Gram–negative bacteria as well as on *Candida albicans*.

Note that the ranges spanned by the log 1/*C* values are very small for all organisms considered; in such cases regression analysis is of limited validity. However, in an attempt to place in a quantitative context the results summarized in table II, equations

(2–6) in table III were derived. The regression coefficients (table III) for each equation are listed under the variable headings; standard deviations and correlation coefficients are listed under *s* and *r*. Of course, these equations are based only on derivatives for which antimicrobial activities were quantitatively defined. The value of the overall *F* statistic for each equation is shown under $F_{n,k}$.

Antibacterial activity on Gram–positive cells *(S aureus)* has been correlated through equation [2]. Although this equation is based on rather few data points, it has been possible to define the optimum hydrophobicity (log P)$_0$ for maximum activity, which turned out to be 4.15.

The congeners already mentioned tested against Gram–negative cells showed the QSARs formulated by equations (3–5).

Equation (3) correlates the effects on *Salmonella Wiener* and shows the optimum hydrophobicity at the (log P)$_0$ value of 3.45. This equation is not as solid as one would like; nevertheless, it is significant at the 95% level ($F_{2,6,0.05} = 5.14$). As shown in equation (4), a parabolic dependence is also observed for the activity on *Pseudomonas aeruginosa*, with a lipophilicity optimum of 3.19. Equation (5) describes the activity on *Serratia marcescens*; this last equation shows a reliable value of correlation coefficient, associated with a low standard deviation, and an optimum of lipophilicity which increases to 3.66.

The above analysis eqs (2–5) provides another illustration that the maximal antibacterial activity is exerted by drugs having an optimal (log P)$_0$ value. Moreover, as reported for other classes of compounds the different values of (log P)$_0$ observed for Gram–positive and Gram–negative bacteria can be attributed to the different morphology of these microorganisms; owing to their lipid-rich protective layer of Gram–negative organisms, they are protected from hydrophilic as well as from highly lipophilic compounds. Therefore the lower (log P)$_0$ observed for the Gram–negative bacteria eqs (3–5) may be attributed to the higher lipid content of the cell wall compared to that of Gram–positive species (eq 2).

The conclusions drawn from eqs (2–5) might be a good guide for the design of new antibacterial derivatives; however, one should move with some caution along a given parameter. Gross structural changes may lead to results which are extremely difficult to incorporate into the developed correlation equations.

The inhibitory action of this set of benzotriazole derivatives against *Candida albicans* correlated by equation (6); adding a term in (log P)^2 to eq (6) makes a slight improvement in the correlation ($r = 0.814$). However, confidence limits cannot be placed on (log P)$_0$; hence, this higher order equation is of little value. In equation (6) one can see an increase in antifungal activity as log P increases but the low value of the slope suggests a poor sensitivity of the system to the hydrophobic effect; that is, the interaction is not very responsive to each additional unity of hydrophobicity. Concerning the influence of chemical modification on the antifungal activity, the 2-cyclohexyl-5-dichloroacetamido derivative (compound **3**) is the most interesting in all congener sets listed in table II.

Figure 1 shows observed and predicted activity values *versus* lipophilicity (log *P*) of tested compounds. Although equations (2–6) cannot be regarded as completely definitive in view of the total data variance accounted for and the limited number of

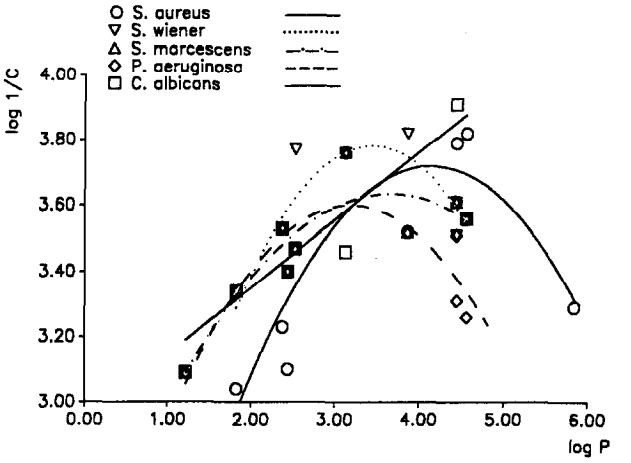


Fig 1. Observed and predicted (eqs 2–6) antimicrobial data *versus* log P.

Table III. Correlation equations for the antimicrobial activities of compounds **3–13**. log $1/C = a \log P + b (\log P)^2 + c$.

a	*b*	*c*	*n*	*r*	*s*	*log P$_0$*	$F_{n,k}$	*Eq No*
1.18 ± 0.65	−0.14 ± 0.09	1.28 ± 1.14	10	0.875	0.162	4.15	11.45	2
1.30 ± 0.96	−0.19 ± 0.14	1.53 ± 1.47	9	0.817	0.111	3.45	6.08	3
0.89 ± 0.46	−0.14 ± 0.07	2.17 ± 0.64	10	0.868	0.103	3.19	10.73	4
0.67 ± 0.43	−0.09 ± 0.07	2.41 ± 0.59	9	0.907	0.092	3.66	14.04	5
0.21 ± 0.15		2.94 ± 0.46	9	0.782	0.024		10.96	6

data points, they nevertheless suggest a relatively coherent view of the mechanism of action of the compounds under study, showing that any general hypothesis regarding their action must include the effects of hydrophobic–lipophilic interactions.

Experimental protocols

All compounds gave satisfactory elemental analyses (C, H, N and S) within ± 0.3% of the theoretical values and were characterized by IR, UV and PMR spectroscopy. Melting points, determined with a Kofler apparatus, are uncorrected. Nuclear magnetic resonance (PMR) spectra where recorded on a Bruker WM 250 spectrometer. Chemical shifts are reported in parts per million (δ) from an internal tetramethylsilane standard. Splitting patterns are designed as follows: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad.

Infrared IR spectra were obtained on a Perkin–Elmer Model 177 spectrophotometer.

Although the IR and PMR spectral data of compounds **3–13** are not included because of the lack of unusual features, they were obtained for all compounds reported and were consistent with the assigned structures.

UV spectra were taken on a Beckman DU-40 spectrophotometer.

Chromatographic separations were performed on a silica gel column (Kieselgel 40, 0.063–0.200 nm, Merck). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel-60F-254 glass backed plates and visualized by UV.

The partition coefficients (P) were determined by the classic 'shake-flask' procedure at room temperature using octanol as lipophilic phase and water as hydrophilic phase. Octanol and water phases were adjusted in volume so that satisfactory amounts of sample were present in each phase. The analysis of the concentrations of the partitioned substance were made using a Beckman DU-40 spectrophotometer. Four determinations were made on each tested compound.

High performance liquid chromatography was performed using a μ-Bondapack C_{18} stainless steel column (3.9 mm x 100 mm) from Waters Associates. Methanol/water 3:2 (v/v), at a constant flow rate of 0.5 ml/min, was employed as eluent. Elution was continued until a stable base line was obtained. Solutions were first filtered (Millipore) and degassed to reduce contamination or column clogging. Compound retentions were expressed by the logarithm of the capacity factor (k'), defined as $\log k' = \log[(t_r - t_0)/t_0]$ where t_r and t_0 are the elution times of the retained and unretained compound, respectively. Acetone served as a suitable non retained compound to define the dead volume t_0.

Preparation of 2-methyl-5-nitrobenzotriazole ***1a***

In a general typical procedure clean sodium (2.3 g, 0.1 mol) was added to anhydrous ice cooled ethanol (100 ml), and vigorously stirred until complete dissolution. To this solution was successively added 5-nitrobenzotriazole (11.9 g, 0.1 mol) and then iodomethane (14.2 g, 0.1 mol).

The reaction mixture was kept under reflux for 24 h and monitored by TLC (CH_2Cl_2/hexane 9:1 v/v). After cooling, the ethanol was removed, under reduced pressure, and the residue was treated with diethyl ether (100 ml) separated from the sodium iodide and washed with 2 N sodium hydroxide and water to remove the unreacted 5-nitrobenzotriazole. The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to dryness. TLC examination of the reaction mixture showed the formation of 3 UV absorbing products, one of which was preponderant. Fractionation, performed on a silica gel column (5 x 80 cm) using as eluent CH_2Cl_2/hexane 9:1 v/v, led to isolation of the faster moving compound which, on the basis of UV and PMR spectral analysis, proved to be 2-methyl-5-nitrobenzotriazole. Further elution led to isolation of the 2 remaining isomers.

Analytical purification of each product was obtained by crystallization from the appropriate solvent. Relevant data are: $C_7H_6N_4O_2$, mw = 178.15; mp = 190–191°C, yield 59%; IR ($CHCl_3$) ν_{max} 1530 cm^{-1}; UV (EtOH) λ_{max} 290 and 247 nm (log ε = 3.98 and 4.23). PMR ($CDCl_3$) δ: 8.85 (1H, d, J = 1.1 Hz, H-4); 8.24 (1H, dd, J = 8.5 and 1.1 Hz, H-6); 7.98 (1H, d, J = 8.5 Hz, H-7); 4.62 (3H, s, CH_3).

Preparation of 2-cyclohexyl-5-nitrobenzotriazole ***1b***

Following the procedure previously described, clean sodium (2.3 g, 0.1 mol) was added to anhydrous ice cooled ethanol (100 ml), and vigorously stirred until complete dissolution. To this solution was successively added 5-nitrobenzotriazole (11.9 g, 0.1 mol) and then cyclohexylbromide (16.3 g, 0.1 mol). The reaction mixture was kept under reflux for 72 h and monitored by TLC (CH_2Cl_2/hexane 9:1 v/v). Work-up of the reaction mixture and chromatographic separation of obtained products was performed according to the above-described procedure. Analytical purification of 2-cyclohexyl-5-nitrobenzotriazole was effected by crystallization from diethyl ether. Relevant data are: $C_{12}H_{14}N_4O_2$, mw = 246.27; mp = 84–85°C, yield 38%; IR ($CHCl_3$) ν_{max} 1535 cm^{-1}; UV (EtOH) λ_{max} 292 and 248 (log ε = 4.09 and 4.29). PMR ($CDCl_3$) δ: 8.85 (1H, d, J = 1.1 Hz, H-4); 8.22 (1H, dd, J = 8.5 and 1.1 Hz, H-6); 7.98 (1H, d, J = 8.5 Hz, H-7); 4.85 (1H, tt, J = 11.0 and 4.5 Hz, H-1'); 2.4–1.3 (10H, cm, remaining aliphatic protons).

Preparation of 5-amino-2-alkylbenzotriazoles ***2a, b***

To 0.25 mol of 5-nitro-2-methylbenzotriazole or 5-nitro-2-cyclohexylbenzotriazole in dry methanol (50 ml) 1.5 g of 10% palladium on activated carbon was added and the resulting mixture was stirred at room temperature, under hydrogen pressure until the theoretical amount of hydrogen was absorbed. TLC examination (diethyl ether) showed the quantitative formation of the 2-methyl-5-aminobenzotriazole and 5-cyclohexyl-5-aminobenzotriazole respectively. The suspension was then filtered and the solid phase washed twice with dry methanol. The filtrate when evaporated to dryness *in vacuo*, gave the expected 5-aminobenzotriazole derivative, successively characterized by IR and PMR spectroscopy.

2-methyl-5-aminobenzotriazole ***2a***. $C_7H_8N_4$, mw = 148.17; oil; IR ($CHCl_3$) ν_{max} 3500 cm^{-1}; PMR ($CDCl_3$) δ: 7.98 (1H, d, J = 8.5 Hz, H-7); 7.80 (1H, d, J = 1.1 Hz, H-4); 7.35 (1H, dd, J = 8.5 and 1.1 Hz, H-6); 4.51 (3H, s, CH_3).

2-cyclohexyl-5-aminobenzotriazole ***2b***. $C_{12}H_{16}N_4$, mw = 216.29; oil; IR ($CHCl_3$) ν_{max} 3500 cm^{-1}; PMR ($CDCl_3$) δ = 7.98 (1H, d, J = 8.5 Hz, H-7); 7.80 (1H, d, J = 1.1 Hz, H-4); 7.37 (1H, dd, J = 8.5 and 1.1 Hz, H-6); 4.72 (1H, tt, J = 11.0 and 4.5 Hz, H-1'); 2.4–1.3 (10H, cm, remaining aliphatic protons).

Preparation of 5-amido-2-alkylbenzotriazoles ***3–13***

0.01 mol of 5-amino-2-alkylbenzotriazole (**2a** or **2b**) was dissolved in 50 ml of anhydrous benzene. To this solution was successively added 0.01 mol of the appropriate acid chloride and 0.01 mol of anhydrous pyridine. The reaction mixture was vigorously stirred and refluxed for 4 h then cooled and filtered; the solid salt formed was washed twice with dry ether. The combined ether solution was evaporated *in vacuo* to give compounds **3–13**, which were further purified by crystallization from diethyl ether or chloroform.

Antimicrobial activity

Antimicrobial activity of the prepared compounds was tested against Gram-positive and Gram-negative bacteria as well as against fungi.

Minimum inhibitory concentrations (MICs) were determined by the broth dilution technique [3]. Tested microrganisms were *Staphylococcus aureus* (ATCC 6538); *Serratia marcescens* and *Salmonella Wiener* (clinical isolates); *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231).

The substances, dissolved in 10% acetone-water solution at 2 mg/ml concentration were diluted in broth in the range 200–12.5 μg/ml.

In the antibacterial testing procedure Mueller-Hintor broth (BBL) was used as a growth medium [4]; cultures, grown at 37°C overnight, were diluted in the same broth approximately 10^7 CFU/ml and used as inoculum. Test cultures were incubated at 37°C for 24 h.

In the antifungal testing procedure Sabouraud dextrose broth (BBL) was employed and the inoculum resulted from 48 h growth of the organism at 25°C; test tubes were also incubated at 25°C for 48 h.

All the results are presented as μg/ml or as log $1/C$, where C represents the molar concentration; the lowest concentration of antimicrobial agent that results in the complete inhibition of visible growth of microrganism represents the minimal inhibitory concentration (MIC) (table II).

Some very active known antimicrobial substances were employed during the test procedures as references. They were gentamicin sulfate (Gram–); erythromycin (Gram+); ampicillin and cephalotin (Gram+ and Gram–); amphotericin B (fungi). The obtained MICs are listed in table II.

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