Journal of Thermal Analysis and Calorimetry, Vol. 95 (2009) 3, 929–935

THE EFFECT OF *m*-ALKOXYPHENOL COMPOUNDS ON THE *CHROMOBACTERIUM VIOLACEUM* RESPIRATION METABOLIC RATE
Microcalorimetric and theoretical investigations

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A structure-activity relationship study (SAR) was applied to correlate the biological activities of *m*-alkoxyphenol compounds on *Chromobacterium violaceum* respiration with chemical structure properties. The biological activities of these compounds on metabolism rates were obtained through microcalorimetry. The calculations to estimate several physicochemical properties were carried out at the semi-empirical AM1 and ab initio DFT levels using the CEP-31G basis set and were parameterized using the continuum-solvation model COSMO for solvent contribution. *m*-alkoxyphenols properties were evaluated by chemometric analyses to carry out a correlation between the physicochemical properties and their biological effects. These compound effects increase with lateral hydrocarbon chain length, volume, dipole moment, proton affinity, energies of HOMO and LUMO, partition coefficient and enthalpy of formation and decrease with solvent effects and ionization enthalpy.

Keywords: *m-alkoxyphenols, biological activity, chemometric analysis, C. violaceum, microcalorimetry, theoretical methods*

Introduction

The mechanisms of phenol action on microorganisms, specifically on mitochondrial respiratory functions, have been investigated for more than forty years [1]. Reverse electron transfer can be affected by inhibitors of the enzymatic complexes involved in the succinate dehydrogenase, NADH dehydrogenase, and ATP synthetase processes and by uncouplers of oxidative phosphorylation. It can also cause damage to the membrane [2, 3]. The action of alkoxyphenol compounds has been explored due to the fact that these compounds are frequently used in biotransformation reactions [4, 5]. Recently, some of them have been demonstrated medical significance as clinical potential as cytostatic drugs in the chemotherapy of tumors [6].

An earlier previous investigation focused on *m*-alkoxyphenol biological activity on some microorganisms, such as Gram-negative and Gram-positive bacteria and suggested that the most important factor that influences the biological activity is the lipophilic character, allowing the *m*-alkoxyphenols to act in the cytoplasm after crossing the cellular membrane or irreversively damaging the cellular membrane [7].

Taking into account the earlier results obtained for alkoxyphenol biological activities [7], the current investigation explored in more details the biological activity on the *Chromobacterium violaceum* respiration metabolic rate by using flow microcalorimetry which was then combined with theoretical methods with the objective to correlate the biological activities of these compounds with several physicochemical properties. Due to the absence of experimental data, semi-empirical and ab initio methods were employed to calculate some physicochemical properties of these compounds. This procedure was established in order to obtain a possible explanation of the biological effect of *m*-alkoxyphenols by considering chemometric analyses (HCA, hierarchical cluster analysis and PCA, principal component analysis), to obtain a correlation between the physicochemical properties and the biological activities of these compounds.

Experimental

Materials and methods

Chemicals

Glucose (Hoersht), NaCl, KCl, Na_2HPO_4 and KH_2PO_4 were reagent grade. The *m*-alkoxyphenols were purchased from Aldrich.

Microorganism

Biological tests were done on *Chromobacterium violaceum* respiration. This bacterium is a class which belongs the classification aerobic gram-negative, being able to be found in the ground and waters of tropical and subtropical regions. *C. violaceum* was discov-

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1388–6150/$20.00

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ered during the microbiological analysis in a water treatment station in Manaus (Amazon), where colonies of violet bacteria were found, however its main habitat is the Rio Negro in the north of Brazil [8, 9]. *C. violaceum* presents a low degree of pathogenicity, however, can cause serious infections, in immunopressure organisms [8].

Bacterium culture

C. violaceum was grown in a 1.5 dm^3 reactor flask (B. Braun Biotech., Biostat B2) at 310 K that contained a sterilized culture medium of composition (g dm^{-3}): 3.0 of yeast extract, 7.5 of glucose and 7.5 peptone bacteriologic in distilled water in a sterilized PBS (Phosphate Buffered Solution) whose composition (g dm^{-3}) was NaCl 8.0; KCl 0.20; Na_2HPO_4 1.15 and KH_2PO_4 0.20, at a final pH of 7.0.

The reactor flask was inoculated with 1.0 cm^3 of *Chromobacterium violaceum* and incubated at 298 K on a rotary shaker (200 rpm; Gallenkamp). After 14 h of incubation, the growth of the cells was separated from the culture medium by centrifugation at 4000 rpm for 20 min. The cells were washed three times after suspending in PBS solution and then centrifuged. After the last centrifugation, the cells were suspended again in 100 cm^3 of PBS solution containing 10% dimethylsulfoxide and conditioned in 1.0 cm^3 polypropylene ampoules (Corning). The ampoules were inserted into a thin perforated styrofoam plate which was placed 8 cm above the liquid nitrogen level in an appropriate container. When the temperature in the control ampoule reached 200 K, the ampoules were immersed in liquid nitrogen and stored in the cryogenic cylinder [10]. A viability count, which was performed periodically, gave $1.3 \cdot 10^{10}$ cells cm^{-3}. The cells were stored for six months and recovered with 95% of viability.

Culture medium

The composition of the culture media used for the respiration metabolic rate of *C. violaceum* was (g dm^{-3}): glucose 1.80; K_2HPO_4 8.75; K_2PO_4 1.88, at pH=7. Without bioactive compound addition, this media was defined as a control preparation. The inoculation of microorganisms (900 μL of the cell suspension) was always done immediately after a cellular reactivation process by defrosting an ampoule for 3 min in a water bath at 310 K, followed by manual stirring for 20 s.

Microcalorimeter

For investigations on heat output production phenomenon to follow in vitro bacterial growth, the use of microcalorimeter tools have become indispensable [7, 11], due to the sensitivity of heat flow measurements, which are on the order of microwatts, and the fact that the samples are not destroyed at the end of the process [7, 12, 13]. In the present case the flow microcalorimeter LKB-2277 that was used has been described previously [3].

The calorimeter is fit with two flow-through vessels, where the reaction mixture involving microorganisms plus drug were continuously pumped by a peristaltic pump from a reaction flask outside the calorimeter, in a closed loop system, with a flow rate of 30 cm^3 h^{-1} at 298.15±0.20 K. The heat output from the metabolic rate of the microorganisms is detected a pair of thermocouples placed around the flow-through cell [10]. The detection limit is typically 4 μW; and, for the culture samples studied, ca. 100 μW were detected. The power output is amplified and recorded as a biological response (BR) or as a potency/time (*p*–*t*) curve.

Theoretical data

Physicochemical properties for *m*-alkoxyphenol compounds were analyzed using quantum chemical methods at the ab initio level of computation using the Hartree–Fock and B3LYP levels of theory employing CEP-31G and CEP-31+G basis sets. To minimize the computational time, the calculations were conducted using Steven–Basch–Krauss compact effective pseudopotential (CEP) [15]. Thus, the experimental data presented are complimented by semiempirical and ab initio calculations, such as solvent effect (SE), proton affinity (PA), dipole moment (dm) ionization enthalpy (IE), enthalpy of formation ($\Delta_f H^0$), volume, energies of HOMO and LUMO and hydrocarbon chain length.

Proton affinity (PA) was calculated as the differences in absolute enthalpies of reactions that characterize these processes.

$$\mathrm{PA:\ HA_{(g)} \rightarrow A^-_{(g)} + H^+_{(g)}\ ;}$$

$$\mathrm{PA = \Delta H = H(HA_{(g)}) - H(A^-_{(g)}) - H(H^+_{(g)})} \qquad (1)$$

In the last expression: $H(X)=E_{\mathrm{eletr}}+E_{\mathrm{vib}}+E_{\mathrm{rot}}+E_{\mathrm{trans}}+nRT$, where E_{eletr} is the electronic energy computed by structural optimization for both neutral and anionic species via density functional theory levels (DFT) employing the CEP-31G and CEP-31+G basis set, respectively, E_{vib}, E_{rot} and E_{trans} are the vibrational, rotational and translational energies, respectively, which were computed at the Hartree–Fock level employing the CEP-31G basis set.

Ionization enthalpy (IE) values were obtained directly as the differences in electronic energies $E(A^n)$ and $E(A^+)$ for both neutral and cationic species respectively.

$$\text{IE: } A_{(g)} \rightarrow A^{+}_{(g)} + e^{-};\ IE = E(A^{+}) - E(A^{n}) \qquad (2)$$

IE was computed via structural optimization employing the B3LYP/CEP-31G basis.

The enthalpy of formation ($\Delta_f H^0$) was calculated directly by the quantum chemical method AM1. Solvent effect (SE) was obtained through the COSMO method [16], involving the B3LYP level and also employing the CEP-31G basis set, while the values of volume, dipole moment, energies of HOMO and LUMO and hydrocarbon chain length were obtained directly from B3LYP/CEP-31G calculations. All calculations were carried out with the Gaussian/98 program [17].

Chemometric analysis

Calculated properties of these compounds were assessed through chemometric analysis (HCA, hierarchical cluster analysis and PCA, the principal component analysis) [18] in order to classify an *m*-alkoxyphenol series in relation to their biological activities. The chemometric analysis used in this investigation was identical to the one used before [3].

Results and discussion

Biological activity of m-alkoxyphenol compounds

In biological microcalorimetry, the biological response, BR, is defined by comparing the maximum height of the calorimetric power *vs.* time curve for each compound, with the maximum height of control curve (100% CR) [19]. The addition of the homologous alkoxyphenols, such as *m*-methoxyphenol, *m*-ethoxyphenol, *m*-propoxyphenol, *m*-butoxyphenol and *m*-pentoxyphenol on *C. violaceum* cultures was examined and the results are listed in Table 1. The data indicate that the biological activities of *m*-alkoxyphenols increase as the hydrocarbon chain length increases, as a result of increasing lipophilic properties of these compounds.

Based on these results, a concentration of 14.00 mmol dm^{-3} for the *m*-alkoxyphenol compounds showed that *m*-propoxyphenol was drastically more toxic than the *m*-methoxyphenol and *m*-ethoxyphenol (Table 1). On the other hand, the biological effects of *m*-butoxyphenol and *m*-pentoxyphenol on the *C. violaceum* respiration were not involved at this concentration, due to their higher toxicity than *m*-propoxyphenol, as also shown in Fig. 1.

For this reason, the concentration of 3.00 mmol dm^{-3} was chosen for comparison of the biological effects of these compounds on *C. violaceum* respiration metabolic rate, as shown in Table 1 and Fig. 2. The biological response (BR) values obtained using concentrations of 3.00 mmol dm^{-3} of

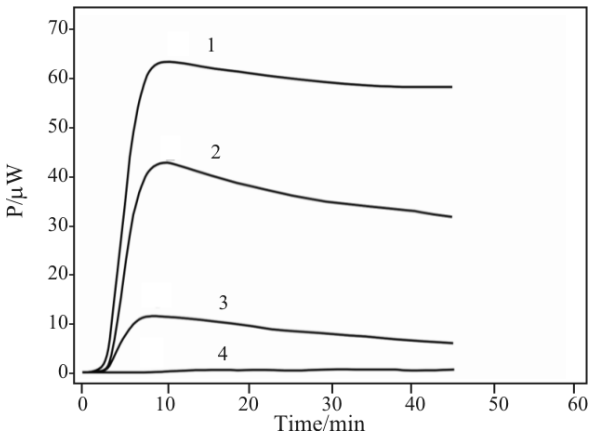

Fig. 1 Biological response curves *vs.* time for effects of *m*-alkoxyphenol compounds on *C. violaceum* respiration. The sequence presented in a 14.00 mmol dm^{-3} concentration in comparison with the control 1 – without drug, 2 – *m*-methoxyphenol, 3 – *m*-ethoxyphenol and 4 – *m*-propoxyphenol

Table 1 Biological response values (BR) of *m*-alkoxyphenols in two concentrations (3.00 and 14.00 mmol dm^{-3}) on *C. violaceum* respiration, compared with control in which the maximum value of (BR) represents 100% of the microcalorimeter baseline deflection

Molecule	% BR**	
	3.00 mmol dm^{-3}	14.00 mmol dm^{-3}
Control*	100	100
m-methoxyphenol	98	71
m-ethoxyphenol	82	17
m-propoxyphenol	24	0.4
m-butoxyphenol	11	–
m-pentoxyphenol	2	–

*Control is the *C. violaceum* culture without drug
**Standard deviation of BR value is near 2%

m-methoxyphenol, *m*-ethoxyphenol, *m*-propoxyphenol, *m*-butoxyphenol and *m*-pentoxyphenol were 98, 82, 24, 11 and 2%, respectively. *m*-pentoxyphenol presented a high biological effect on *C. violaceum* respiration. The biological responses decrease as the *m*-alkoxyphenol volume (–CH_2–) increases: *m*-methoxyphenol>*m*-ethoxyphenol>*m*-propoxyphenol>*m*-butoxyphenol>*m*-pentoxyphenol, as shown in Fig. 2 and Table 1.

The biological response, BR, values showed that the biological effects on the respiration process of *C. violaceum* increase with the increasing hydrophobic character of the *m*-alkoxyphenol compounds, as a result of increasing the lateral hydrocarbon chain length, as illustrated in Fig. 2.

Several mechanisms of chemical substance action on microorganisms, which may influence their

Table 2 Calculated physicochemical property values for *m*-alkoxyphenols. Proton affinities (PA-g and PA-liq), ionization enthalpies (IE-g and IE-liq), enthalpy of formation ($\Delta_f H^0$), solvent effect on neutral compound forms (SE), dipole moment in debye (dm-liq and dm-g), HOMO and LUMO energies, volume in nm (*V*), lateral hydrocarbon chain length in nm (C–L), *n*-octanol/water partition coefficient (log*P*) [21] and other properties are in kJ mol^{-1}, with g and liq being the gas and aqueous phases, respectively

m-alkoxyphenols	BR/%	log*P*	SE	PA-liq	IE-liq	dm-liq	HOMO-liq	LUMO-liq	C–L/nm	*V*/nm^3	$\Delta_f H^0$-g	PA-g	IE-g	dm-g	HOMO-g	LUMO-g
m-methoxyphenol	98	3.80	–58.28	1196.42	561.35	3.83	–585.62	–30.40	0.204	0.426	–252.55	1448.14	761.27	2.88	–568.79	–19.01
m-ethoxyphenol	82	4.75	–58.91	1198.42	558.36	4.10	–584.83	–32.48	0.375	0.456	–276.19	1449.11	751.52	2.99	–564.82	–15.83
m-propoxyphenol	24	6.14	–55.40	1199.13	558.17	4.03	–582.78	–28.57	0.502	0.464	–304.80	1448.87	750.46	3.05	–564.01	–15.12
m-butoxyphenol	11	7.17	–54.39	1199.64	557.78	4.08	–582.21	–28.01	0.633	0.489	–333.26	1448.77	749.21	3.11	–563.35	–14.52
m-pentoxyphenol	2	8.56	–53.72	1199.85	557.40	4.08	–581.50	–27.25	0.762	0.494	–362.00	1448.78	748.24	3.14	–562.86	–14.10

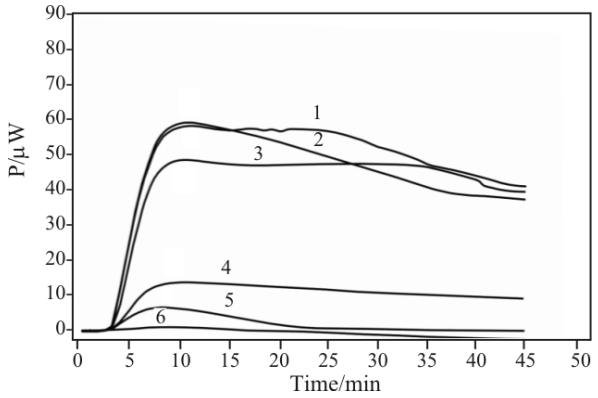

Fig. 2 Biological response curves *vs.* time for *m*-alkoxyphenol compounds on *C. violaceum* respiration. The sequence presented in a 3.00 mmol dm^{-3} concentration in comparison with the control 1 – without drug, 2 – *m*-methoxyphenol, 3 – *m*-ethoxyphenol, 4 – *m*-propoxyphenol, 5 – *m*-butoxyphenol and 6 – pentoxyphenol

mitochondrial respiratory functions, have been suggested. In general, as bacterial respiration is related to bacterial membrane function, if the permeability of *m*-alkoxyphenols increases due to its hydrophobic character, the compound will cause a higher biological effect on the microorganism [20].

The results obtained confirm the hypothesis of the influence of *m*-alkoxyphenols on the microorganism respiratory process. This effect was observed previously in microcalorimetry studies for the homologous series of *m*-alkoxyphenols on *E. coli* [20] and on *Saccharomyces cerevisiae* [2]. A comparison, considering the previous results [20], with the current investigation, shows that *C. violaceum* inhibition was about three times more sensitive than with *E. coli*, when using the same compounds.

Theoretical data of m-alkoxyphenols

An investigation of the structure activity-relationship of *m*-alkoxyphenols on *C. violaceum* cultures was carried out based on the physicochemical properties listed in Table 3. A total of fifteen descriptors, such as PA, IE, enthalpy of formation, $\Delta_f H^0$, SE on the neutral structures of *m*-alkoxyphenol compounds, on the proton affinity AP-liq and on the ionization enthalpy IE-liq, energies of HOMO and LUMO, dipole moment (dm), volume (*V*), lateral hydrocarbon chain length (C–L) and *n*-octanol/water partition coefficient (log*P*) [21] was obtained. The carbon length (C–L) is geometrically optimized for *m*-alkoxyphenol compounds as shown in Fig. 3.

The calculated enthalpy of formation values, proton affinity and ionization enthalpies for *m*-alkoxyphenol compounds are presented in Table 2. The sequence: *m*-methoxyphenol<*m*-ethoxyphenol< *m*-butoxyphenol<*m*-propoxyphenol<*m*-pentoxyphen-ol

Table 3 Coefficients of the variables obtained through principal component analysis of *m*-alkoxyphenols, Part 4, Fig. 4

Variables	PC1 coefficients	PC2 coefficients	PC3 coefficients
Log*P*	0.27	0.19	–0.33
SE	0.24	0.34	0.28
PA-liq	0.28	–0.07	0.18
IE-liq	–0.27	0.20	–0.07
dm-liq	0.32	–0.38	–0.21
HOMO-liq	0.27	0.19	0.22
LUMO-liq	0.20	0.48	0.41
C–L	0.27	0.12	–0.31
V	0.28	0.03	–0.36
$\Delta_f H^0$-g	0.27	–0.18	0.38
PA-g	0.17	–0.53	0.27
IE-g	–0.27	0.19	–0.15
dm-g	0.28	0.04	–0.07
HOMO-g	0.27	–0.14	0.15
LUMO-g	0.28	–0.12	0.13

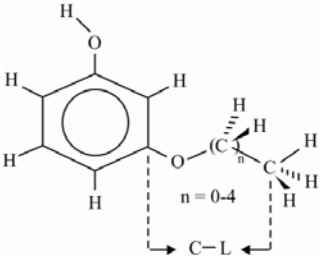

Fig. 3 Hydrocarbon chain length (C–L) obtained after the *m*-alkoxyphenol compounds geometrically optimized

was observed. This sequence can be explained on the basis of the nature of the volume of each molecule in the neutral form, which indicates that the stability of these compounds increases with the respective sizes. The neutral molecules *m*-methoxyphenol, *m*-ethoxyphenol, *m*-butoxyphenol, *m*-propoxyphenol and *m*-pentoxyphenol gave the solvation values –58.28, –58.91, –55.40, –54.39 and –53.72 kJ mol^{-1}, respectively. By considering the listed data, a decrease from solvation values of *m*-methoxyphenol to *m*-pentoxyphenol was decreased and, in this sequence, the only difference is a CH_2 group. Consequently, the change in lipophilic character must be considered, as shown in Table 2. The properties of HOMO and LUMO energies, volume, dipole moment and the lateral hydrocarbon chain length (C–L) were obtained after the optimization of each geometry of the studied compounds. These properties follow the sequences:

HOMO and LUMO (gas phase): *m*-methoxyphenol<*m*-ethoxyphenol<*m*-propoxyphenol<*m*-butoxyphenol<*m*-pentoxyphenol

HOMO (aqueous phase): *m*-methoxyphenol<*m*-ethoxyphenol<*m*-propoxyphenol<*m*-butoxyphenol<*m*-pentoxyphenol

LUMO (aqueous phase): *m*-ethoxyphenol<*m*-methoxyphenol<*m*-propoxyphenol<*m*-butoxyphenol<*m*-pentoxyphenol

Volume: *m*-methoxyphenol<*m*-ethoxyphenol<*m*-propoxyphenol<*m*-butoxyphenol<*m*-pentoxyphenol

C–L:
m-methoxyphenol<*m*-ethoxyphenol<*m*-propoxyphenol<*m*-butoxyphenol<*m*-pentoxyphenol

dm: (gas phase) *m*-pentoxyphenol<m-propoxyphenol<*m*-butoxyphenol<*m*-ethoxyphenol<*m*-methoxyphenol

log *P*: *m*-methoxyphenol<*m*-ethoxyphenol<*m*-propoxyphenol<*m*-butoxyphenol<*m*-pentoxyphenol

Structure-activity relationship (SAR)

The principal component analysis (PCA) method was applied to obtain a correlation between the physicochemical properties of *m*-alkoxyphenols and the respective biological activity, since each of the variables presented in Table 2 was autoscaled. The first two principal components explained 99.78% of the total variance in the data obtained, where PC1=84.55%, PC2=14.32% and PC3=0.90% as seen in Part 2, Fig. 4.

Figure 4, Part 1 shows three distinct groups A, B and C, observed for the *m*-alkoxyphenol compounds studied. The first group contains the highly active compounds (*m*-propoxyphenol, *m*-butoxyphenol and *m*-pentoxyphenol), the intermediately active compound (*m*-ethoxyphenol) is located in group B and the least active compound (*m*-methoxyphenol) formed group C. From Part 3, it can be seen that PC1 alone is responsible for the separation between the high, intermediate and least active compounds.

The first principal component, PC1, has variants with similar coefficients: PC1= –0.28 AP-liq+0.17 AP-g+0.27 EI-liq–0.27 $\Delta_f H^0$+0.24 SE+0.27log*P*+0.28 Volume+0.32 dm-liq+0.27 C–L+0.27 HOMO-liq+0.20 LUMO-liq–0.27 EI-g+0.28 md–g+0.27 HOMO-g+0.28 LUMO-g, is dominated by the +0.32 dm-liq. For PC2, the total data variance also is represented by the variants, PC2= –0.07 AP-liq–0.53 AP-g+0.20 EI-liq–0.18 $\Delta_f H^0$+0.34 SE+0.19log*P*+0.03 Volume–0.38 dm-liq+0.12 C–L+0.19 HOMO-liq+0.48 LUMO-liq+0.19 EI-g+0.04 md-g–0.14 HOMO-g–0.12 LUMO-g. However, PC2 is dominated by the –0.53 AP-g+0.48 LUMO–0.38 dm-liq+0.34 SE. The third principal component, PC3, also presented total variance

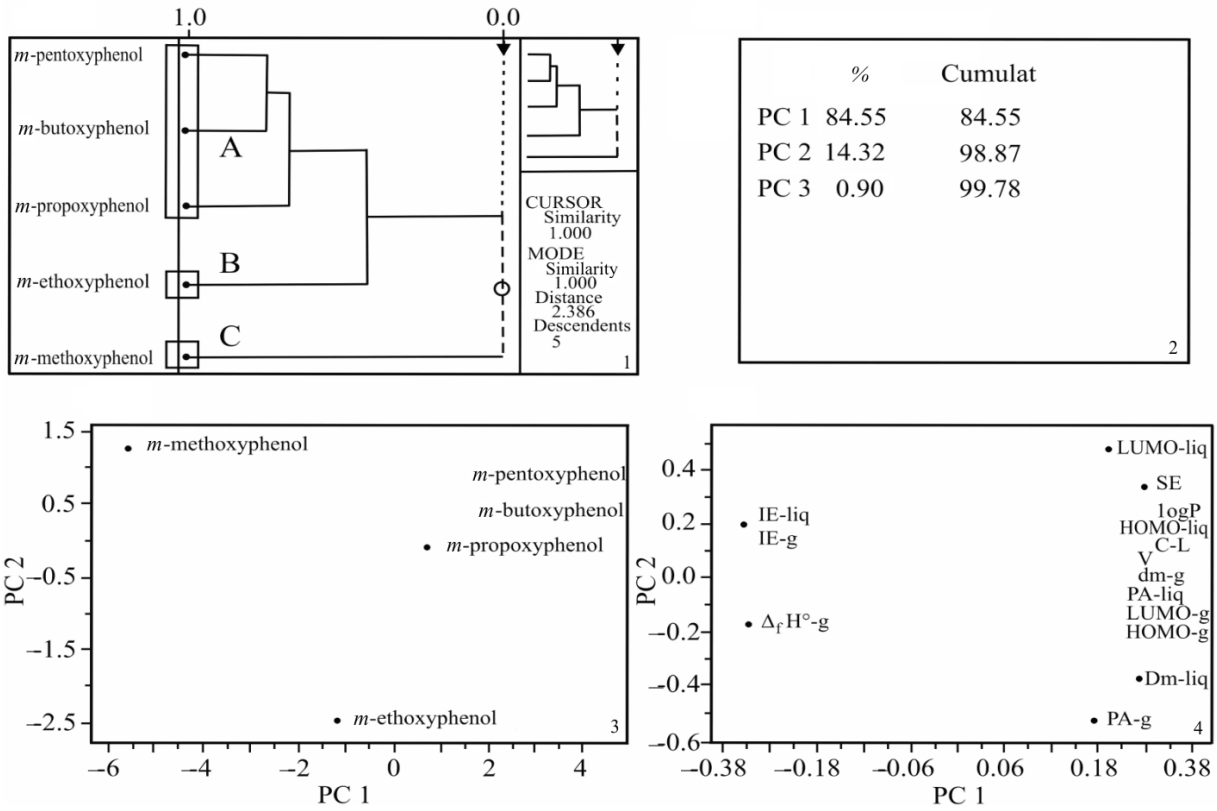

Fig. 4 HCA and PCA plots for *m*-alkoxyphenols. 1 – Dendrogram plot, 2 – PC results, 3 – scores plot and 4 – loadings plot

data having the same variants, PC3=+0.18 AP-liq+0.27 AP-g–0.07 EI-liq+0.38 $\Delta_f H^0$+0.28 SE–0.33log*P*–0.36 Volume–0.21 dm-liq–0.31 C–L+0.22 HOMO-liq+0.41 LUMO–0.15 EI-g–0.07 md-g+0.15 HOMO-g+0.13 LUMO-g. Note that PC3 is dominated by the +0.41 LUMO+0.38 $\Delta_f H^0$–0.36 Volume–0.33log*P*–0.31 C–L, as shown in Fig. 4, Part 4, and Table 3.

The results of the principal component analysis, PCA, shown in Fig. 4 and Tables 2 and 3, reveal in a convincing way how the highly active molecule *m*-pentoxyphenol depends on high values of AP-liq, HOMO, LUMO, dm, log*P*, C–L, volume and $\Delta_f H^0$ variables, which combine with small SE and EI values.

Conclusions

Microcalorimetry, ab initio and semi-empirical, PCA and HCA methods were useful to classify the *m*-alkoxyphenol compounds with regard to their biological activities on *Chromobacterium violaceum* respiration. The biological activity of *m*-alkoxyphenols and their physicochemical properties show that the lipophilic character of *m*-alkoxyphenol compounds is one of the most important factors related to the inhibition mode on *C. violaceum*. However, it is not a sufficient prerequisite for their biological activity, due to the fact that the activities of these compounds also depend on other properties such as, AP, EI, $\Delta_f H^0$, SE, log*P*, volume, dm, C–L, HOMO and LUMO. The results also indicate that the biological activities of *m*-alkoxyphenolic compounds increase as the lateral hydrocarbon chain length, volume, dipole moment, proton affinity, energies of HOMO and LUMO, log*P* and exothermic enthalpies of formation, $\Delta_f H^0$, increase and decrease with the solvent effects and ionization enthalpies.

Acknowledgements

The authors are indebted to FAPESP for financial support. FAPESP is also acknowledged for fellowships to MMB and DAO and CNPq for a fellowship to CA.

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Received: December 11, 2007
Accepted: June 19, 2008
OnlineFirst: September 20, 2008

DOI: 10.1007/s10973-007-8706-3