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Synthesis and Evaluation of Pseudopeptidic Fluorescence pH Probes for Acidic Cellular Organelles: In Vivo Monitoring of Bacterial Phagocytosis by Multiparametric Flow Cytometry

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A new family of fluorescent anthracenic pH probes has been synthesized, chemically characterized, and their photophysical properties have been investigated by steady-state and time-resolved fluorescence spectroscopy. The ability of these compounds to monitor pH has been investigated in solution and it was found that molecules **1**–**12** can act as fluorescent sensors for pH in a range between 4.6 and 6.5. This range corresponds to the pH of acidic organelles in the cell (pH 4.5–6.0) for which a limited number of probes are described. The acid–base behavior of each sensor varies slightly depending on the nature of substituents close to the amines present in the molecules. Thus, the pK_a of this family of compounds can be finely tuned by the appropriate selection of the synthetic building blocks at the design stage. To test the potential diagnostic applications of this family of probes, macrocyclic pseudopeptide **2** was used to monitor the phagocytosis of a culture of GFP-labelled bacteria by human monocytic cells U937 using flow cytometry as the analytical tool. It was found that the occurrence of bacterial killing was concomitant with the production of reactive oxygen species and a drop in pH, the latter monitored indirectly by macrocyclic sensor **2**, which suggests its potential use for diagnostic purposes.

Introduction

In vivo imaging of cells or tissues is an area that has seen considerable developments since its conception and has evolved from academic research to practical applications in only a few decades.[1] Presently, the study of live cells, either as individual entities or as populations, is carried out by using advanced optical technologies for data acquisition and molecular or macromolecular chemicals (probes) specifically designed for analyzing certain objectives within the cell.[2] Fluorescence imaging is one of the most popular techniques for the study of living cells due to its high analytical sensitivity.[3] Among the probes used to monitor the complex biochemical processes taking place in living cells, the green fluorescent protein (GFP) and its family have emerged as paradigmatic examples of protein-based fluorescent sensors.[4] Low-molecular-weight probes have also been developed for cell imaging. Thus, rationally designed molecules have been synthesized to monitor in vivo concentrations of chemical species like Zn^{II},[5] Mg^{II},[6] Cu^{II},[7] Cu^{I},[8] chloride,[9] nitric oxide,[10] singlet oxygen,[11] and hydroxyl radicals[12] or to stain certain cellular structures such as mitochondria,[13] nucleus,[14] nucleolus,[15] or membranes[16] to mention a few. The number of molecular sensors developed so far is very high and specific reviews can be found in the literature.[3,17]

One of the most important targets in the context of biochemical analysis is pH and, most importantly, intracellular pH. It is well known that cytosolic pH is a key parameter determining the proper functioning of cellular machinery.[18] It is held at about 7.4 by means of a complex series of buffering mechanisms. Its measurement by fluorescent probes is carried out with a number of synthetic molecules, new examples of which are continuously emerging in the literature.[19–24] Very recently, an excellent review on this topic was published.[25] At the cellular level not all chemical processes take place at neutral pH and some of them need the presence of high concentrations of protons, like the degradation of intracellular waste materials by specific enzymes or the defensive action of cells as a response to the presence of pathogens (phagocytosis). The specialized vesicles responsible for such processes (phagosomes, endosomes, and lysosomes) can have a pH about three units more acidic than that of cytosol. The pH reported for these acidic vesicles varies within the range 4.0–6.5 depending on the biochemical processes taking place and, in comparison with the vast collection of available pH sensors for the neutral regions,[20,24,26] the number of useful acidic probes is much reduced.[21,23]

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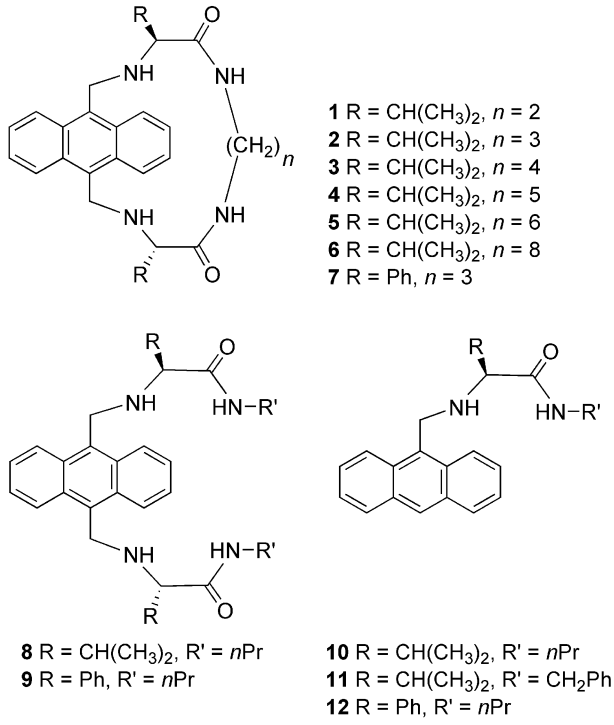
The synthesis of new families of fluorescent probes for acidic intracellular environments is important not only for basic biochemical research but also for the potential applicability of such probes as diagnostic tools for disorders associated with unbalanced acidity at the cellular level, such as chromic granulomatous disease,[27] mucolipidosis type IV,[28] Batten disease,[29] and several oncological processes.[30] In fact, the unbalanced acidity of cancerous tissues was first recognized by Warburg decades ago[31] and such knowledge has led to new approaches for cancer treatment. To continue developing new diagnostic protocols to approach such diseased states, new pH probes are needed.

A few years ago we reported four new synthetic macrocyclic pseudopeptidic compounds as fluorescent probes for acidic organelles in live cells and compared them with commercial reference sensors of the acidic vesicles.[32] With the objective of expanding the pH range of sensitivity, herein we report the synthesis of eight new fluorescent sensors based on this pseudopeptidic scaffold. We describe the synthetic procedure, the chemical characterization, and the photophysical parameters of the full family of probes. The main conclusion that can be drawn from this study is that a biochemically important range of pH (ca. 5–6) can be covered by this new family of fluorescent sensors and also that it is possible to finely tune the pK_a of the fluorescent sensors by the appropriate selection of the synthetic building blocks. To test the potential biomedical utility of the acidity sensors reported herein, we followed the phagocytosis process of a culture of fluorescent bacteria by human leukocytes. The intracellular acidity was taken as a reporter parameter, measured by one of the synthetic probes and using flow cytometry as the bioanalytical tool. Probes for flow cytometric analysis of pH are limited to a few examples and, to the best of our knowledge, this is the first time that a pseudopeptidic probe has been used to monitor bacterial phagocytosis using flow cytometry.

Results and Discussion

Synthesis

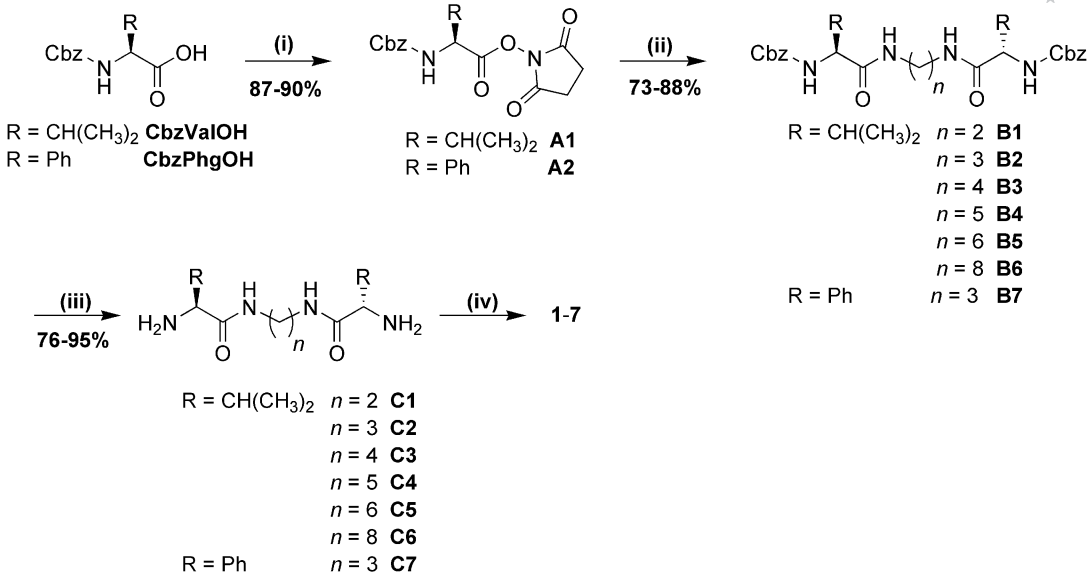
A series of molecules with pH-responsive fluorescence have been synthesized and their chemical structures are shown in Scheme 1. All these compounds have at least a secondary amine connected to a fluorescent anthracene group separated by a methylene bridge. This feature enables an intramolecular photoinduced electron-transfer (PET) process,[33,34] which quenches the emission from the anthracene moiety. Such quenching is avoided by protonation of the amines at acidic pH, which leads to the restoration of the fluorescence. This conceptual scheme was pioneered and studied in detail by de Silva and co-workers[35–39] and has been recently applied to the development of systems of extended sensitivity over a very wide linear range of pH.[39] Details about the intrinsic properties of this class of PET sensors with the "fluorophore-spacer-amine" scaffold have been reported extensively[35,37,39] and will not be discussed here.



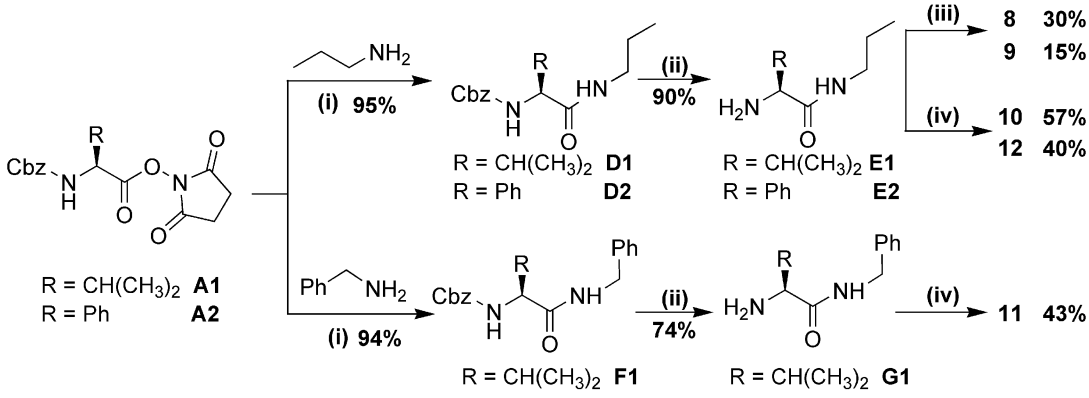
Scheme 1.

All the molecules in Scheme 1 were synthesized following a methodology previously developed by our group that allows the synthesis of pseudopeptidic compounds with high yields starting from simple amino acids.[40–45] The probes are of three types: Macrocyclic bis-aminoamides **1**–**7**, open-chain bis-aminoamides **8** and **9**, and mono-aminoamides **10**–**12**. As can be seen in Scheme 1, the differences between these compounds comprise slight variations in the chemical nature of the building blocks employed for their synthesis, such that either valine or phenylglycine have been used as well as different polymethylene bridges with n = 2, 3, 4, 5, 6 or 8 units. The objective of such minimal changes was to demonstrate that the properties of the probes, and especially the values of pK_a, can be finely modulated. Thus, smooth pK_a changes from molecule to molecule by the appropriate selection of the building blocks have been accomplished.

Macrocycles **1**–**7** were synthesized by activation of the corresponding N-protected amino acid (Cbz-L-Val or Cbz-L-Phg) with *N*-hydroxysuccinimide/DCC in dry THF followed by coupling with the corresponding 1,*n*-alkanediamine in DME to afford the Cbz-protected intermediates **B1**–**B7** depicted in Scheme 2. After deprotection with HBr/AcOH, the free amine intermediates **C1**–**C7** were obtained, which were then coupled to 9,10-bis(bromomethyl)-anthracene in anhydrous acetonitrile. The macrocycles were obtained in moderate-to-good yields (20–35% after column purification), as has been described in previous reports for similar compounds bearing benzenic or naphthalenic units instead of anthracenic groups.[42,43,46] The main feature of this macrocyclization reaction is that it takes place without the need for high-dilution conditions or the involvement of



Scheme 2. General methodology for the preparation of macrocycles **1**–**7**. Reagents and conditions: (i) *N*-hydroxysuccinimide, DCC, THF; (ii) 1,*n*-alkanediamine, DME, RT; (iii) HBr/AcOH 33% followed by NaOH treatment; (iv) 9,10-bis(bromomethyl)anthracene, dry K_2CO_3, anhydrous CH_3CN, reflux, chromatographic purification.



Scheme 3. General methodology for the preparation of open-chain pseudopeptides **8**–**12**. Reagents and conditions: (i) THF, reflux; (ii) HBr/AcOH 33% followed by NaOH treatment; (iii) 9,10-bis(bromomethyl)anthracene, anhydrous CH_3CN, dry K_2CO_3, reflux; (iv) 9-(chloromethyl)anthracene, anhydrous CH_3CN, dry K_2CO_3, reflux.

templates, as typically described in the synthesis of large macrocycles.[45,47] It has been demonstrated that a combination of solvophobic effects and the occurrence of preorganization of the intermediates, assisted by intramolecular hydrogen-bonding, explain such good yields.[40]

The open-chain derivatives were synthesized in a similar way but by using the corresponding alkaneamines (Scheme 3). Overall, several hundreds of milligrams were obtained for compounds **1**–**12**, which were characterized unambiguously by ^1H and ^{13}C NMR, FTIR, ESI-MS, HRMS (EI^+), UV/Vis, and fluorescence spectroscopy, as shown in the Exp. Sect. and Supporting Information (ESI).

Compound **1** yielded crystals suitable for X-ray diffraction after slow evaporation from acetonitrile at room temperature (Figure 1). The macrocycle crystallizes in the orthorhombic system and displays an orientation of the macrocyclic ring perpendicular to the anthracenic moiety. It is worth mentioning the existence of an intramolecular hydrogen bond (2.10 Å) between the NH of one amide and the carbonyl of the other, which supports the aforementioned participation of this kind of interaction in the preor-

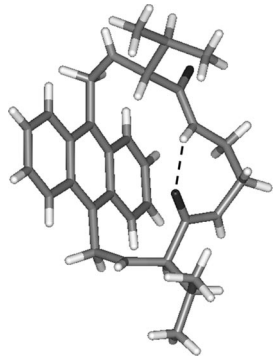


Figure 1. Molecular structure of macrocycle **1** obtained by X-ray diffraction showing the intramolecular hydrogen bond.

ganization of the chain for the macrocyclization step. More details about the packing of **1** in the solid state can be found in the Exp. Sect.

Photophysical Evaluation

The electronic absorption spectra of 2 μM solutions of **1**–**12** were recorded in water at pH 3 (see the Supporting Information). As expected, compounds **1**–**9**, substituted at the 9- and 10-positions of the anthracene moiety, display absorption wavelength maxima redshifted by about 10 nm (ca. 400 nm) as compared with **10**–**12**, substituted only at the 9-position. However, little difference can be appreciated when comparing the series of macrocycles **1**–**7** or any macrocycle bearing valine (**1**–**6**) with the open-chain analogue **8** (see the Supporting Information). Steady-state fluorescence emission spectra were recorded for **1**–**12** in water at pH 3 (Figure 2). The shape and position of the spectra are similar for the 9,10-disubstituted probes **1**–**9** and only small differences could be found when compared with the spectra of the probes **10**–**12** substituted only at the 9-position. The results parallel the observations made in the UV/Vis absorption spectra.

The emission quantum yield for each pseudopeptidic compound was quantified at pH 3 and 8 (ϕ_F^{pH3}, ϕ_F^{pH8}) using anthracene in deoxygenated ethanol as reference.[48] The emission quantum yields at acidic pH are in the range 0.74–0.87, in agreement with the emission efficiency reported for analogous derivatives.[37,49] However, at basic pH the emission quantum yields are much lower, between 0.007 and 0.149. As expected, the drop in emission quantum yield in basic pH is compatible with the PET mechanism inducing internal quenching of the emission.[37]

The fluorescence lifetimes of compounds **1**–**12** were also measured by using the time-correlated single-photon counting (TCSPC) technique, exciting with light of 372 nm (pulsewidth 1.3 ns) from a pulsed LED (light-emitting diode). The recorded lifetimes range from 12.0 to 13.9 ns in the case of disubstituted derivatives **1**–**9**, and from 12.3 to 14.2 ns in the case of monosubstituted anthracenes **10**–**12**. From the fluorescence lifetimes of probes **1**–**12** and the emission quantum yields of the free (ϕ_F^{pH8}) and protonated (ϕ_F^{pH3}) species it was possible to determine the corresponding rate constants for the deactivation of the first singlet state (S_1) of molecules **1**–**12** from Equations (1), (2) and (3) in which k_F is the radiative rate constant from the S_1, k_D is the nonradiative rate constant, and k_{PET} is the rate constant for the PET process from the free amine to the S_1 of the anthracene moiety. These equations imply that k_{PET} in acidic conditions is negligible due to the high oxidation potential of the ammonium ion, as has been discussed elsewhere.[37]

$$\phi_F(\text{pH 3}) = \frac{k_F}{k_F + k_D} \quad (1)$$

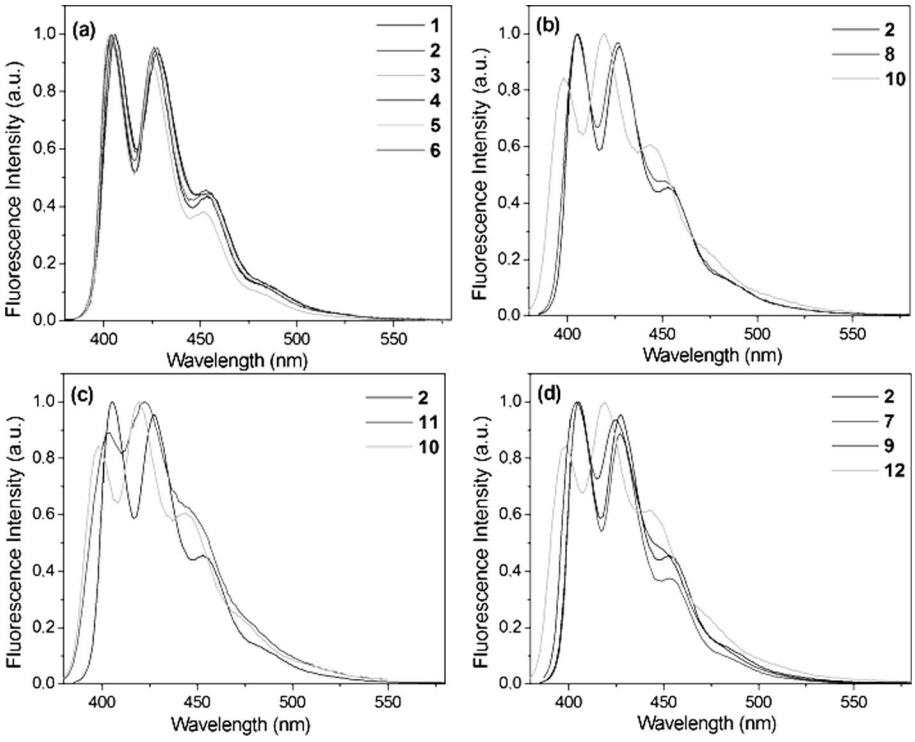


Figure 2. Comparative normalized emission spectra of synthesized anthracene derivatives in H_2O (0.2% DMSO at pH = 3.00). (a) Anthracenophanes **1**–**6** with different numbers of methylenic groups in the aliphatic bridge. (b) Anthracene derivatives **2**, **8**, and **10** with different numbers of pseudopeptidic chains in the structure. (c) Anthracenic derivatives **2**, **10**, and **11** with different substituents in the chemical structure. (d) Anthracene derivatives **2**, **7**, **9**, and **12** with different side-chains in the amino acid unit and different numbers of pseudopeptidic chains in the structure.

$$\tau = \frac{1}{k_F + k_D} \quad (2)$$

$$\phi_F(\text{pH } 8) = \frac{k_F}{k_F + k_D + k_{PET}} \quad (3)$$

The values reported in Table 1 constitute the complete photophysical characterization of probes **1**–**12**. Note the high uniformity for almost all the calculated parameters except for the PET rate constant, which is somewhat higher for the open-chain disubstituted compounds **8** and **9** [$(6.5–9.0)\times10^9$ s^{-1}] than for the cyclic disubstituted anthracenes **1**–**7** [$(1.7–3.8)\times10^9$ s^{-1}] or the monosubstituted probes **10**–**12** [$(1.1–3.6)\times10^9$ s^{-1}]. The differences between **8**, **9**, and **10**–**12** can be explained by taking into account the presence of the two amino groups with high conformational freedom near the anthracene acceptor, which would lead to higher electron-transfer efficiency than in the most constrained macrocyclic molecules.[37,49]

Table 1. Photophysical properties of compounds **1**–**12**.

	λ_a [nm]	$\log(\varepsilon_m$ [$M^{-1}cm^{-1}$])	λ_e [nm]	ϕ_e[a]	ϕ_{min}[b]	τ[a] [ns]	k_F [10^7 s^{-1}]	k_D [10^7 s^{-1}]	k_{PET} [10^9 s^{-1}]
1	400	3.9	404 426 455	0.87	0.016	13.9	6.3	0.9	3.8
2	400	3.9	405 427 453	0.85	0.033	13.0	6.5	1.2	1.9
3	399	4.0	403 425 451	0.87	0.019	13.0	6.7	1.0	3.5
4	399	3.9	406 428 453	0.83	0.021	12.7	6.5	1.3	3.0
5	399	3.9	405 427 453	0.80	0.035	13.0	6.1	1.5	1.7
6	398	3.9	404 426 452	0.78	0.022	12.8	6.1	1.7	2.7
7	400	4.0	405 427 453	0.74	0.149	12.2	6.1	2.1	3.3
8	397	3.9	405 427 452	0.77	0.009	13.0	5.9	1.8	6.5
9	397	4.0	404 425 453	0.74	0.007	12.0	6.2	2.2	9.0
10	370	3.7	398 419 444	0.84	0.044	13.6	6.2	1.2	1.3
11	370	3.7	404 422 449	0.83	0.016	14.2	5.8	1.2	3.6
12	369	3.7	399 419 443	0.79	0.053	12.3	6.4	1.7	1.1

[a] Measured at pH 3. [b] Measured at pH 8.

Notwithstanding, all the calculated PET rates are one or two orders of magnitude lower than those reported by de Silva for analogous anthracenic pH sensors.[37] The reason for this can be found in the different nature of the amines participating in the photoinduced electron-transfer process: Molecules described by de Silva[37] contain tertiary amino groups as photo-oxidizable moieties, those reported herein bear secondary amino groups that are characterized by higher oxidation potentials than the previous ones.[50]

Acid–Base Behavior

The practical applicability of **1**–**12** as biomedical pH sensors rely on the existence of an on–off switching process of the emission that takes place when the pH changes from acid to basic. This change is characterized by the pK_a of the ammonium ion in the fully protonated form of the probe and can be calculated by fluorimetric titration. Thus, the fluorescence intensity (I_F) versus pH profiles were determined and the experimental data fitted to the Henderson–Hasselbalch-type mass action equation [Eq. (4)]. This methodology assumes that the pK_a of the ground-state ammonium ion is the same as the pK_a* of the same molecule in its excited state, which has already been proved for analogous sensors.[37] An example of this kind of measurement is shown in Figure 3 for compound **1**.

$$\log \frac{(I_{max} - I)}{(I - I_{min})} = \text{pH} - pK_a \quad (4)$$

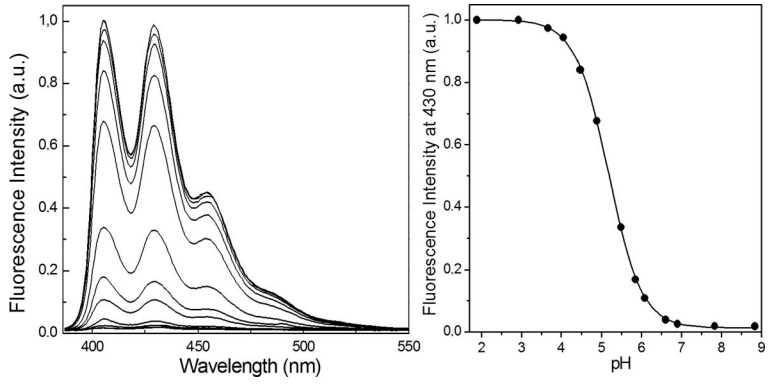


Figure 3. (a) Emission spectra of **1** as a function of the pH. (b) Intensity at the maximum emission of **1** as a function of pH. Experimental conditions: 2 μM of **1**, H_2O (0.2% DMSO), universal buffer (0.04 M), NaCl (0.1 M); λ_{exc} = 374 nm.

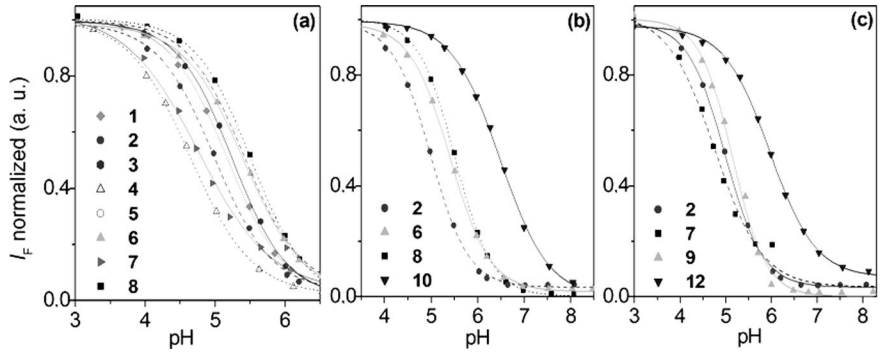


Figure 4. Comparative graphs of normalized maximum emission intensity as a function of pH for different pseudopeptidic fluorescent probes. Sample preparation: 2 μM, H_2O, 0.2% DMSO, universal buffer (0.04 M), NaCl (0.1 M): (a) compounds **1**–**8**; (b) compounds **2**, **6**, **8**, and **10**; (c) compounds **2**, **7**, **9**, and **12**.

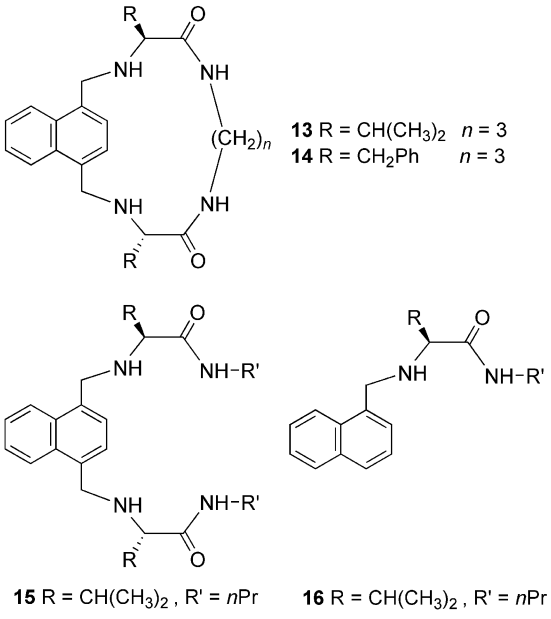
From the pK_a values obtained by using this methodology (Figure 4 and Table 2), compounds **1**–**12** can be seen as a family of pH probes with potential applications over a broad pH range (two units) not covered satisfactorily by commercial sensors: From quasineutral (pK_a of **10** is 6.5) to very acidic (pK_a of **7** is 4.6). A detailed analysis of the data presented in Table 2 affords several interesting conclusions. First, the macrocyclic compounds **1**–**6** bearing valine have pK_a values in the acidic region (4.6–5.4). There is an apparent tendency for the value of pK_a to diminish with decreasing size of the macrocycle, although this trend is not linear. The reason for this nonlinear behavior could be the existence of preferred conformations imposed by the number of units in the polymethylenic chain. In previous studies by our group we have shown that larger macrocyclic rings are not necessarily more flexible because intramolecular hydrogen bonds can play an important role.[51] This topic is beyond the scope of this work, but deserves further investigation in the future.

Table 2. Acidity parameters of the anthracenic probes **1**–**12**.

Compound	pK_a	$\phi_F^{pH3}/\phi_F^{pH8}$
1	5.2	54
2	5.0	26
3	5.2	46
4	4.6	39
5	5.4	23
6	5.4	35
7	4.6	5
8	5.5	86
9	5.1	106
10	6.5	19
11	6.1	52
12	6.0	15

Pseudopeptidic compounds **10** and **11**, with only one substituent in the anthracenic ring and also a valine, are characterized by values of pK_a above 6. The lower pK_a values for the diamines can be ascribed to the occurrence of a first protonation of one of the basic sites, which makes more difficult the protonation of the second amine (observable by fluorescence change) due to electrostatic repulsion between positive charges. This repulsion does not take place in the case of the monoamine probes and hence the observed pK_a is around one unit higher. This explanation has been given in the past to rationalize the behavior of similar compounds.[37,38,52] Moreover, the steric hindrance of the bulky anthracenic moiety should also contribute to the low basicity of this family of sensors, which makes them useful for the measurement of acidic pH values.[34] From our previous research on photoactive pseudopeptidic macrocycles we had access to compounds **13**–**16** presented in Scheme 4 and Table 3.[43,44] These molecules are not useful as biological pH sensors because the electronic absorption maxima are located in the UV-B region of the electromagnetic spectrum (typical λ_{max} = 250–300 nm). However, they share with molecules **1**–**12** the occurrence of PET quenching of the fluorescence at basic pH and restoration of the emission at acidic pH. Thus, we decided to measure the pK_a of such naphthalenic compounds in order to compare their acid–base behavior with that of the anthracenic probes. The comparison shows lower pK_a values for compounds with larger organic residues [pK_a(**13**) = 5.2 > pK_a(**2**) = 5.0; pK_a(**15**) = 5.9 > pK_a(**8**) = 5.5], which suggests the hydrophobicity around the protonation site to be a key factor in understanding the more acidic pK_a values of the anthracene-based probes. Nevertheless, a complete explanation of the basicities displayed by the family of sensors presented herein cannot be straightforward because many factors must be taken into account. Comprehensive studies have been reported in the literature dealing with this topic.[53]

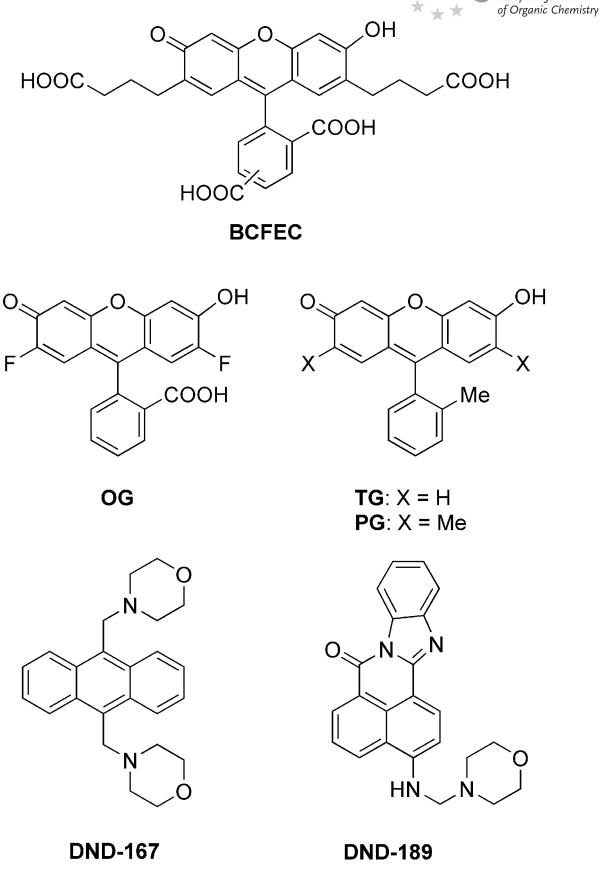
Overall, the results obtained highlight the importance of the appropriate selection of the building blocks for the synthesis of this type of PET pH sensor and, more importantly, the possibility of tailoring the basicity of the amines in the probes by modulating the solvation through careful control of the hydrophilic/hydrophobic balance of the residues in close proximity to the protonation site. Most of the fluorescent pH probes currently used for cell or tissue imaging in biological studies do not have the fine-tuning capability shown by the sensors presented herein. Paradigmatic examples of pH sensors used for biological studies are fluorescein and its derivatives. Of these, 2′,7′-bis(2-carboxy)-fluorescein (BCFEC) has been used extensively because its pK_a of 7.0 allows for numerous applications in the neutral pH range.[26,54] However, synthetically modifying the xanth-



Scheme 4.

Table 3. Acidity parameters of the naphthalene derivatives **13**–**16**.

Compound	pK_a
13	5.2
14	4.9
15	5.9
16	6.7



Scheme 5.

enic scaffold of fluoresceins to shift the pK_a towards more acidic values is difficult. Such approaches have led to the development of other probes like Tokyo Green (TG),[55] Oregon Green (OG),[56] Pennsylvania Green (PG),[57] and others.[58] However, the shifts in the pK_a values are large and often unpredictable. For instance, the pK_a for TG is 6.2, whereas the pK_a for OG is 4.8 and identical to that of PG (Scheme 5). The same can be said for the family of BODIPY[24] and cyanine dyes[21,22,59] also used for bioimaging. The pseudopeptidic probes presented herein offer a palette of pK_a values that differ only by 0.1–0.2 units. Moreover, the synthetic protocol is easier than that used for the synthesis of fluorescein derivatives because it starts from readily available commercial products (the chiral pool of amino acids), it is short (activation, amine coupling, deprotection, and fluorophore attachment), and yields pure compounds in amounts ranging from 200 to 500 mg (easily scalable to multigram scale).

For an appropriate comparison of the PET sensors described herein with other families of probes for acidity, not only should the pK_a values at which the on–off change takes place be taken into account, but also the ratio of emission intensities between such states. This ratio reflects to some extent the visual contrast that potentially could be achieved in a bioimaging application. For the fluorescein derivatives, typical quantum yields for the off state (in this case it occurs at acidic pH) are ϕ_{min} = 0.3–0.4, whereas the on state typically displays ϕ_{max} = 0.8–0.9, that is, $\phi_{max}/\phi_{min} \approx$ 2–3. The PET probes reported herein, as well as those previously described by de Silva and others[35–39], show a much higher contrast with maximum values of ϕ_{max} = 0.7–0.9 but minimum values for the off state being much lower with ϕ_{min} = 0.007–0.149 (see Table 2 for the ratios ϕ_{max}/ϕ_{min}), which leads to ϕ_{max}/ϕ_{min} ratios of up to 106, as shown for compound **9**. However, as a major disadvantage of the probes based on the anthracene chromophore, the need to excite at 370 nm with a UV-A laser should be noted; in contrast, fluorescein can be used in combination with the widely available argon-ion laser (λ_{exc} = 488 nm). Nevertheless, the need to have probes that are excitable selectively in the UV-A range is also important, as will be discussed later in the section dealing with the biochemical assays of living cells by flow cytometry.

Another characteristic that has to be taken into account in order to choose a suitable fluorescence pH probe is the range of response to the concentration of protons. Figure 5 shows the pH ranges of potential utility for the synthesized probes along with those of two commercially available pH sensors (DND-167, pK_a = 5.6, and DND-189, pK_a = 6.1). The range for which the fluorescence intensity changes from 20 to 80% of its maximum intensity (I_{max} = 100% is attained at pH 3) is depicted for each compound. This has been done to visualize the range in which the pH probe changes by a factor of four in response to a pH change and

hence to illustrate their potential utility in imaging applications. Although this is an arbitrary criterion, it is useful for internal comparison between sensors.

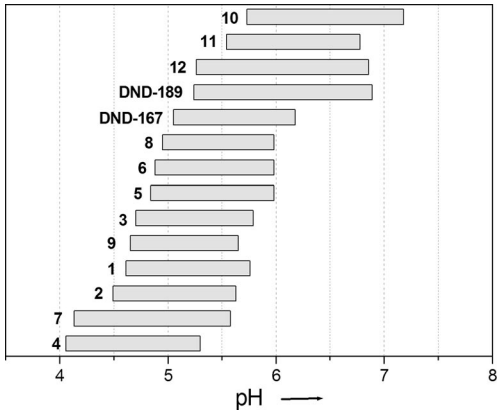


Figure 5. pH range for which a change in fluorescence emission from 20 to 80% takes place for the different sensors (2 μM, 0.2% DMSO in H_2O).

Figure 5 highlights an important feature of the pH sensors through the different amplitudes of such pH intervals (ΔpH), that is, the sharpness of the off–on transition. Depending on the application of the sensor, such changes should be sharper or smoother and this can also be tuned by appropriate selection of the building blocks.

Phagocytosis Assays

It has been widely established that macrophages are key players of the innate immune response.[60] In their bactericidal role, intracellular lysosomes are reported to reach significant acidic values (4.0–5.5).[61] The complex function of protons within such organelles is still a matter of research owing to the complex relationships that exist between many chemical species. However, it seems clear that during phagocytosis or the active ingest of microorganisms, there is a drop in lysosomal pH accompanied by an increase in reactive oxygen species (ROS).[62] Current fluorescent tests for monitoring pH changes during phagocytosis are scarce.[63,64] There is a lack of many available pH probes for two reasons. On the one hand, the technique of choice for the high-throughput analysis of human samples (typically blood) is flow cytometry (FC) and some probes routinely used for microscopic applications are not useful for FC. On the other hand, although there are a very large variety of commercial pH probes for the neutral pH range, the number of markers for the acidic regions is much more limited, as stated above. Consequently, any new pH sensor able to monitor indirectly such bacterial killing would be very useful for diagnostic purposes. Note that defective phagocytosis is involved in the development of a large number of disorders like chronic granulomatous disease.[27]

Previously, we have shown that compound **2** internalizes easily into living RAW 264.7 mouse macrophages and accumulates in the lysosomes, as characterized by an acidic pH.[32] This was demonstrated by confocal laser scanning microscopy (CLSM) using for comparison two commercial lysosome markers such as DND-189 and DND-26. The excellent ability of **2** for translocating the cellular membrane without special conditioning of the cells or derivatization of the probe is very important, taking into account the great effort that is currently directed towards the development of complex carriers capable of penetrating the intracellular medium, like cell-penetrating peptides.[65] This feature, combined with the high contrast (lysosomes vs. cytoplasm and other organules) of the images obtained in CLSM, encouraged us to extend the studies with this probe towards biomedical developments. The following assay was developed to test the potential utility of the pseudopeptidic compounds for the high-throughput analysis of cells in fluid samples. A culture of murine macrophages RAW 264.7 was incubated with macrocycle **2** and with the superoxide-sensitive fluorogenic substrate hydroethidine (Invitrogen, 2.5 μg/mL) for 30 min at 37 °C in the dark and in the presence of the GFP-expressing *E. coli* strain IC103 (bacteria/cell ratio 5:1). Hydroethidine is sensitive to reactive oxygen species (ROS)[66] and the object of this test was to assess if during bacterial phagocytosis the production of ROS occurred with a simultaneous drop in pH in cells loaded with both probes. As can be seen in Figure 6, microscopic analysis shows that bacteria (green fluorescence due to the expressed GFP) are phagocyted by the macrophages, which concomitantly display the action of ROS (red) and some level of acidity (blue). Such levels of ROS and pH are not measurable only by analysis of some dozens of cells using optical microscopy. However the excitation of thousands of them passing through a flow cytometer allows the extraction of quantifiable information corresponding to the three fluorescences arising from each single cell.

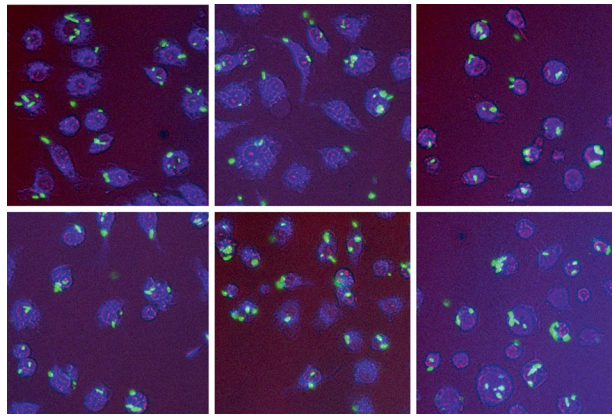


Figure 6. High content analysis (HCA) images of the murine macrophages RAW 264.7 incubated simultaneously with two probes (hydroethidine for reactive oxygen species in red and probe **2** for pH in blue) and allowed to phagocyte green fluorescent protein (GFP)-expressing bacteria *E. coli* (small rods in green).

For flow cytometric analysis the cell line U937, that is, human tumoral monocytic cells, is routinely utilized, which displays phagocytic features analogous to RAW 264.7 but shows higher mobility, an important prerequisite for flow cytometry. A culture of U937 was thus incubated with

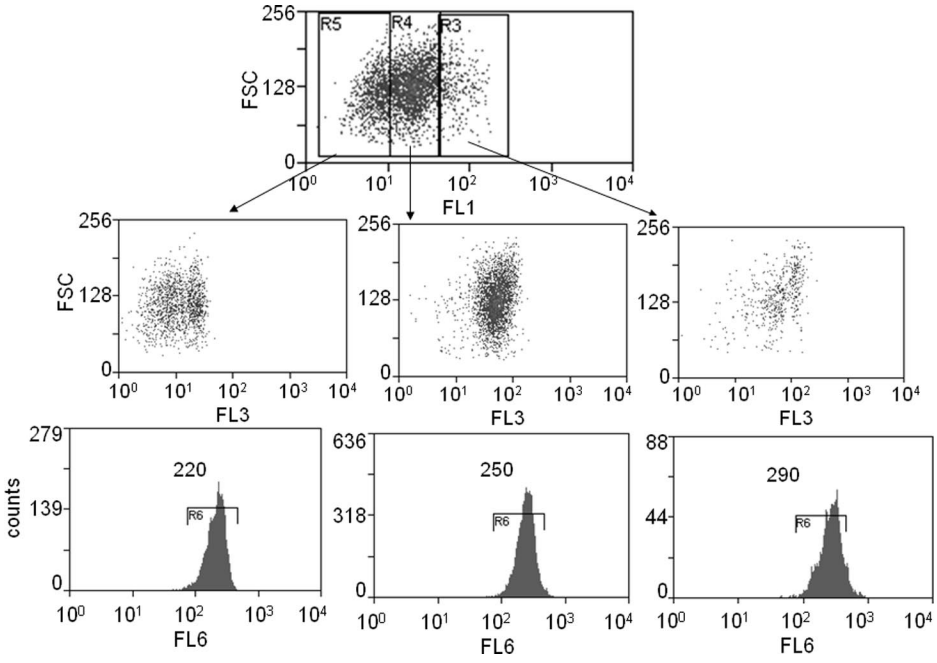


Figure 7. Flow cytometry results. FSC is the forward-side scattering parameter (each dot represents one cell displaying the targeted fluorescence, FL1 or FL3). FL1 is the channel for the fluorescence emission of GFP (showing all the macrophages engulfing GFP-expressing bacteria), FL3 is the channel for the fluorescence emission of hydroethidine (showing macrophages stained with this superoxide probe), and FL6 is the channel for visualizing the fluorescence emission of macrophages stained with the pH probe **2**.

hydroethidine and **2** and exposed to fluorescent IC103 bacteria, as in the experiment with RAW 264.7. Flow cytometric analysis (Figure 7) showed that the complete population of U937 could be divided into three different subpopulations according to their degree of phagocytosis (monitored by the emission of GFP of the bacteria in the FL1 channel). Each population showed a different degree of ROS generation, according to the hydroethidine-derived fluorescence (FL3 channel). Importantly, the fluorescence of **2** (FL6 channel) was found to also increase in parallel with the production of ROS, in accordance with the acidification of the lysosomes during microbial destruction. This result indicates that **2** can be used effectively to monitor acidity changes during phagocytosis and hence it can provide an indirect measurement of the bacterial killing.

Some years ago, a phagocytosis assay based on the fluorescence of the cyanine dye CypHer5E was reported.[63] However, such an assay involved chemical attachment of the fluorochrome to latex particles and zymosan and its utility was constrained by the low fluorescence quantum yields of the protonated cyanines[22,67] ($\phi_{max} < 0.1$) and a sensitivity range limited by the pK_a of the dye (7.3), which is far from the pH ranges of the lysosomes (4.0–5.5). Our probe has several advantages, such as the lack of any derivatization prior to internalization in cells, high quantum yields in the on state ($\phi_{max} = 0.85$), and, most importantly, exact matching of the pK_a (5.0) with the acidity values when the lysosomes are functional. An advantage of the CypHer5E-based assay is the fact that the excitation wavelength used in the reported procedure is 650 nm, whereas that for **2** is much shorter ($\lambda_{exc} = 350$ nm). Nevertheless, the potential problem of cell damage seems to be minimal in FC applications, given the short time that each cell is excited during the operation of a flow cytometer. In addition, multiparametric monitoring of biological samples requires the use of multiple laser excitation sources and hence it is also advantageous to use a light source far away from the conventional long wavelength lasers, which could be useful for other probes.

The phagocytosis assay presented herein using **2** as pH sensor demonstrates that this kind of PET probe can be used not only in static imaging measurements of several live cells (CLSM) but also in the dynamic characterization of thousands of cells from a sample of biological fluid (FC).

Conclusions

We have synthesized a new family of fluorescent pseudopeptidic probes and their ability to monitor pH has been determined in solution and in cells using flow cytometry. The successful results indicate the potential utility of these probes in diagnostic assays, which present a number of advantages with respect to commonly used cellular pH sensors, such as not requiring derivatization prior to internalization and high quantum yields at acidic pH. Most importantly, the biochemically important pH range between 5 and 6 can be covered by this new family of synthetic probes and it is possible to finely tune the pK_a of the synthetic probes by appropriate selection of the synthetic building blocks. The synthetic modular scheme proposed is an excellent approach to the development of new tailor-made probes for

other analytes. Their potential applications in diagnostic assays have been investigated with macrocycle **2**, which was used to monitor bacterial phagocytosis using flow cytometry. Current efforts involve the development of this family of compounds with other fluorophores and amino acids to expand the application of these molecules.

Experimental Section

Materials and Methods: All commercially available reagents (Aldrich or Fluka) were used without further purification. 9,10-Bis(bromomethyl)anthracene was prepared as described previously.[68] For spectrophotometric measurements, concentrated stocks were prepared in DMSO (HPLC grade, Scharlab) and diluted with ultrapure Milli-Q®. For aqueous measurements, appropriate amounts of HCl or NaOH were added to adjust the pH. Buffers used for titrations were composed of universal buffer (0.04 M) and NaCl (0.1 M). Melting points were measured with a Büchi 510 melting point apparatus. NMR spectra were recorded with a Varian INOVA 500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR). Chemical shifts are reported in ppm using residual undeuteriated solvent peaks as internal standards. Mass spectra (ESI) were recorded with a Micromass Quattro LC spectrometer equipped with an electrospray ionization source and a triple-quadrupole analyzer. HRMS were recorded with a VG Autospect at the Universitat de Valencia. Infrared spectra were recorded with a Perkin–Elmer 2000 FT-IR spectrometer. UV/Vis absorption spectra were recorded with a Hewlett–Packard 8453 apparatus. Steady-state fluorescence spectra were acquired with a Spex Fluorolog 3–11 instrument equipped with a 450 W xenon lamp. The spectra were processed with the appropriate correction files. Excitation spectra were also recorded to assure that no impurities were responsible for the emission at longer wavelengths Time-resolved fluorescence measurements were performed by the time-correlated single-photon counting (TCSPC) technique with an IBH-5000U instrument. Samples were excited with a pulsed light-emitting diode at 372 nm (pulsewidth 1.3 ns). Data were fitted to the appropriate exponential model after deconvolution of the instrument response function by an iterative deconvolution technique using the IBH DAS6 fluorescence decay analysis software in which reduced χ^2 (values between 0.90–1.19) and weighted residuals serve as parameters for goodness-of-fit measurements. All measurements were performed at 295 K (SCIC-UJI).

A high content assay (HCA) was performed on murine macrophages RAW 264.7. Macrophages were seeded in a 12-well plate (250000 cells/mL) and incubated for 2 h with GFP-expressing *E. coli* strain IC103 (in-house collection at the Centro de Investigación Principe Felipe, Valencia, Spain) at a 5:1 bacteria/macrophage ratio. The HCA was performed in the automated cell imager IN Cell Analyzer 1000 (GE Healthcare) using the automated high-content image analysis software. Flow cytometry experiments were performed with a High-Speed cell sorter MoFlo (Beckman-Coulter, CA, USA) equipped with 488 and 351 nm laser light.

Suitable crystals of **1** for single-crystal X-ray diffraction were obtained by slow evaporation in acetonitrile. $C_{30}H_{39}N_5O_2$, M_r = 507.16, orthorhombic, space group $P2_12_12_1$, a = 13.386(2), b = 15.187(3), c = 27.803(5) Å, V = 5652.2 (16) Å^3, Z = 8; color yellow, $D_{calcd.}$ = 1.192 g cm^{-3}, $F(000)$ = 2180, Siemens Smart CCD 1K diffractometer, T = 273(2) K, Mo-K_α radiation, λ = 0.71073 Å, 2θ range = 3–38–61.04°, 46328 reflections collected, 9296 independent reflections (R_{int} = 0.0742). The final R factor was 0.0620 with $wR2$ = 0.1695 for 4368 observed reflections with $I > 2\sigma(I)$ (R = 0.1534, $wR2$ = 0.2265 for all data), GOF = 0.937. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were generated according to the stereochemistry and refined by using a riding model.

CCDC-764555 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Quantum Yield Calculations: The emission quantum yield (ϕ_F) for each pseudopeptidic compound was determined from Equation (5) in which ϕ is the quantum yield, I_F is the integrated intensity, OD is the optical density, and n is the refractive index. The subscript ref refers to the reference fluorophore of known quantum yield, that is, anthracene in deoxygenated ethanol.[48] In this expression it is assumed that the sample and reference are excited at the same wavelength so that it is not necessary to correct for the different excitation intensities of different wavelengths.

$$\frac{\phi_F}{\phi_{Fref}} = \frac{n^2}{n_{ref}^2} \times \frac{I_F}{I_{Fref}} \times \frac{OD_{ref}}{OD} \quad (5)$$

Synthesis: General: The open-chain pseudopeptidic precursors **B1**–**B7** and **C1**–**C7** were obtained on the multigram scale following the experimental procedure described previously.[40] Characterization of the new compounds **B4** and **C4** is included in the Supporting Information whereas the spectral properties of the remaining compounds matched those reported.[40] The reaction of such precursors with 9,10-bis(bromomethyl)anthracene is described in detail for the synthesis of macrocycle **1**. Macrocyclic anthracenophanes **2**, **3**, **5**, and **6** were synthesized following this general method and their spectral properties matched those described. For the remaining macrocycles **1**, **4**, **7**, an identical procedure was followed. The open-chain compounds **8**–**12** were synthesized by using *n*-propylamine or benzylamine instead of 1,*n*-alkanediamine. In the case of the monosubstituted anthracenophanes **10**–**12**, the precursors were allowed to react with 9-chloromethylanthracene under the same experimental conditions to obtain the corresponding open-chain derivatives.

Compound 1: Compound **C1** (0.79 g, 3.06 mmol), anhydrous K_2CO_3 dried in an oven at 110 °C (4.23 g, 30.60 mmol), and 9,10-bis(bromomethyl)anthracene (1.11 g, 3.06 mmol) were placed in a flask containing anhydrous CH_3CN (400 mL) and the mixture was heated at reflux for 8 h under nitrogen. The reaction mixture was filtered (hot) and the solvent evaporated under reduced pressure. The crude product was purified by silica gel flash chromatography using MeOH/CH_2Cl_2 (1:40) as eluent to give 492 mg of **1** as a yellow solid; yield 35%; m.p. 206–207 °C. $[\alpha]_D^{20}$ = 157.9 (c = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.75 (d, J = 7.2 Hz, 6 H, CH_3 *i*Pr), 1.12 (d, J = 7.2 Hz, 6 H, CH_3 *i*Pr), 2.12 (s, 2 H, HN), 2.30 (m, 2 H, CH *i*Pr), 2.49 (m, 4 H), 3.14 (d, J = 3.8 Hz, 2 H, CH *i*Pr), 4.81 (d, J = 14.8 Hz, 2 H), 5.12 (d, J = 14.8 Hz, 2 H), 5.93 (s, 2 H, HN), 7.56 (m, 4 H, HAnt), 8.44 (m, 4 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.9, 19.9, 31.3, 38.8, 45.2, 68.6, 125.0, 124.5, 125.2, 125.8, 129.4, 130.3, 132.3, 174.7 ppm. IR (KBr): $\tilde{\nu}$ = 3289, 2960, 1649, 1546 cm^{-1}. MS (ESI): m/z = 461.6 [M + H]$^+$, 483.6 [M + Na]$^+$, 500.5 [M + K]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 460.2838; found 460.2835.

Compound 2: Yield 24%.[32]

Compound 3: Yield 22%.[32]

Compound 4: This compound was obtained as described above starting from 9,10-bis(bromomethyl)anthracene and **C4**. Yellow solid; yield 20%; m.p. 228–230 °C. $[\alpha]_D^{20}$ = –82.3 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.41 (m, 2 H), 0.81 (d, *J* = 6.9 Hz, 6 H, CH_3 *i*Pr), 0.90 (m, 4 H), 1.25 (d, *J* = 7.0 Hz, 6 H, CH_3 *i*Pr), 2.48 (m, 2 H, CH *i*Pr), 2.70 (m, 2 H), 3.10 (d, *J* = 3.4 Hz, 2 H, CH *i*Pr), 3.16 (m, 2 H), 4.84 (d, *J* = 13.5 Hz, 2 H), 5.00 (d, *J* = 13.5 Hz, 2 H), 7.35 (s, 2 H, HN), 7.58 (m, 4 H, HAnt), 8.39 (d, *J* = 8.8 Hz, 2 H, HAnt), 8.46 (d, *J* = 8.7 Hz, 2 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 17.0, 20.4, 25.5, 29.2, 30.9, 39.1, 45.1, 66.2, 125.2, 126.1, 130.2, 131.9, 173.9 ppm. IR (KBr): $\tilde{\nu}$ = 3447, 3337, 2928, 1655, 1523 cm^{-1}. MS (ESI): *m*/*z* = 503.6 [M + H]$^+$, 525.5 [M + Na]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 502.3308; found 502.3308.

Compound 5: Yield 24%.[32]

Compound 6: Yield 25%.[32]

Compound 7: This compound was obtained as described above starting from 9,10-bis(bromomethyl)anthracene and **C7**. Yellow solid; yield 20%; m.p. 145–147 °C. $[\alpha]_D^{20}$ = 152.8 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.11 (m, 2 H), 1.80 (br. s, 1 H, HN), 2.05 (m, 2 H), 2.27 (m, 2 H), 2.69 (br. s, 1 H, HN), 4.67 (s, 2 H), 4.96 (d, *J* = 7.6 Hz, 2 H), 5.09 (d, *J* = 7.6 Hz, 2 H), 6.67 (s, 2 H, HN), 7.28–7.35 (m, 6 H, HPh), 7.42 (m, 4 H, HPh), 7.60–7.67 (m, 4 H, HAnt), 8.50 (d, *J* = 4.36 Hz, 2 H, HAnt), 8.57 (d, *J* = 4.36 Hz, 2 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 28.0, 35.6, 47.0, 71.3, 124.9, 125.5, 126.4, 127.3, 128.5, 129.1, 130.2, 130.8, 132.4, 140.3, 172.2 ppm. IR (KBr): $\tilde{\nu}$ = 3330, 2929, 1666, 1524 cm^{-1}. MS (ESI): *m*/*z* = 543.2 [M + H]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 542.2682; found 542.2687.

Compound 8: The deprotected monoamide **E1** (1.06 g, 6.73 mmol), 9,10-bis(bromomethyl)anthracene (1.22 g, 3.36 mmol), and dry K_2CO_3 (4.64 g, 33.6 mmol) were dissolved in anhydrous CH_3CN (250 mL). The reaction was heated at reflux under nitrogen for 8 h. The K_2CO_3 was separated by hot filtration. The product precipitated in the resulting solution upon cooling. The solid was washed with cold CH_3CN and dried in a vacuum oven for 20 h at 65 °C to yield 544 mg of **8** as a yellow solid; yield 30%; m.p. 228–232 °C. $[\alpha]_D^{20}$ = –59.5 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.83 (d, *J* = 2.93 Hz, 3 H), 0.84 (d, *J* = 3.0 Hz, 3 H), 0.98 (m, 6 H), 1.04 (m, 6 H), 1.60 (m, 4 H), 1.71 (br. s, 2 H, HN), 2.20 (br. s, 2 H), 3.25 (s, 2 H), 3.33 (br. s, 2 H), 4.65 (d, *J* = 11.2 Hz, 2 H), 4.74 (d, *J* = 11.0 Hz, 2 H), 7.38 (s, 2 H, HN), 7.58 (m, 4 H, HAnt), 8.35 (m, 4 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 11.6, 17.6, 19.9, 23.1, 31.4, 40.8, 45.8, 69.4, 124.7, 126.1, 130.1, 131.5, 173.4 ppm. IR (KBr): $\tilde{\nu}$ = 3312, 2970, 1633, 1549 cm^{-1}. MS (ESI): *m*/*z* = 519.5 [M + H]$^+$, 541.4 [M + Na]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 518.3633; found 518.3621.

Compound 9: This compound was obtained as described above for **8** starting from 9,10-bis(bromomethyl)anthracene and **E2**. Yellow solid; yield 15%; m.p. 208–210 °C. $[\alpha]_D^{20}$ = –26.4 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.81 (t, *J* = 6.8 Hz, 6 H, CH_3 Pr), 1.41 (m, 4 H, CH_2 Pr), 2.70 (m, 2 H, HN), 3.08 (m, 4 H, CH_2 Pr), 4.49 (m, 4 H), 4.57 (m, 2 H), 7.28 (t, *J* = 6.8 Hz, 2 H, HPh), 7.34 (t, *J* = 7.5 Hz, 4 H, HPh), 7.44 (d, *J* = 7.8 Hz, 4 H, HPh), 7.53 (dd, *J* = 2.6, *J*′ = 6.7 Hz, 4 H, HAnt), 8.11 (t, *J* = 5.5 Hz, 2 H, HN), 8.36 (dd, *J* = 2.9, *J*′ = 6.5 Hz, 4 H, HPh) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 11.3, 22.3, 40.3, 43.5, 65.7, 125.0, 125.4, 127.2, 128.1, 129.8, 131.6, 140.3, 171.8 ppm. IR (KBr): $\tilde{\nu}$ = 3314, 2961, 1650, 1541 cm^{-1}. MS (ESI): *m*/*z* = 587.4 [M + H$^+$], 609.3 [M + Na]$^+$, 625.3 [M + K]$^+$. HRMS (FAB): calcd. for [M + H]$^+$ 587.3386; found 587.3364.

Compound 10: The deprotected monoamide **E1** (0.28 g, 1.12 mmol), 9-(chloromethyl)anthracene (0.25 g, 1.12 mmol) and dry K_2CO_3 (1.55 g, 11.2 mmol) were dissolved in anhydrous CH_3CN (75 mL). The reaction was heated at reflux under nitrogen for 8 h. The K_2CO_3 was separated by hot filtration. The solution was concentrated under reduced pressure. The crude product was purified by silica gel flash chromatography using MeOH/CH_2Cl_2 (1:40) as the eluent to give 222 mg of an orange solid; yield 57%; m.p. 166–167 °C. $[\alpha]_D^{20}$ = –45 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.81 (d, *J* = 6.9 Hz, 3 H, CH_3 *i*Pr), 0.97 (t, *J* = 7.4 Hz, 3 H, CH_3 Pr), 1.01 (d, *J* = 7.0 Hz, 3 H, CH_3 *i*Pr), 1.58 (m, 2 H, CH_2 Pr), 2.20 (m, 1 H), 3.27 (m, 1 H, CH *i*Pr), 3.31 (m, 2 H, CH_2 Pr), 4.63 (d, *J* = 12.3 Hz, 1 H), 4.71 (d, *J* = 12.4 Hz, 1 H), 7.48 (m, 3 H), 7.55 (m, 2 H), 8.03 (d, *J* = 8.4 Hz, 2 H, HAnt), 8.26 (d, *J* = 8.9 Hz, 2 H, HAnt), 8.44 (s, 1 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 11.7, 17.6, 19.9, 23.2, 31.4, 40.9, 45.8, 69.3, 123.7, 125.2, 126.6, 127.3, 127.8, 129.5, 130.3, 131.7, 134.2, 173.5 ppm. IR (KBr): $\tilde{\nu}$ = 3309, 2961, 1630, 1546 cm^{-1}. MS (ESI): *m*/*z* = 349.2 [M + H]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 348.2201; found 348.2205.

Compound 11: This compound was obtained as described above for **10** starting from 9-chloromethylanthracene and **G1**. Yellow solid; yield 43%; m.p. 143–144 °C. $[\alpha]_D^{20}$ = +5.6 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.84 (d, *J* = 6.9 Hz, 3 H, CH_3 *i*Pr), 1.03 (d, *J* = 6.9 Hz, 3 H, CH_3 *i*Pr), 1.80 (br. s, 1 H, HN), 2.25 (m, 1 H, CH *i*Pr), 3.38 (s, 1 H, CH *i*Pr), 4.38 (dd, *J* = 4.9, *J*′ = 14.5 Hz, 1 H), 4.60–4.71 (m, 3 H), 7.32 (m, 2 H), 7.37 (m, 5 H), 7.44 (m, 2 H), 7.80 (m, 1 H), 8.00 (d, *J* = 8.4 Hz, 2 H, HAnt), 8.10 (d, *J* = 8.7 Hz, 2 H, HAnt), 8.41 (s, 1 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 17.5, 19.9, 31.4, 43.2, 45.7, 69.4, 123.5, 125.0, 126.4, 127.2, 127.5, 127.6, 128.1, 128.8, 129.3, 130.1, 131.5, 134.1, 138.7, 146.9, 173.5 ppm. IR (KBr): $\tilde{\nu}$ = 3297, 2869, 1637, 1535 cm^{-1}. MS (ESI): *m*/*z* = 397.2 [M + H]$^+$, 419.2 [M + Na]$^+$, 435.2 [M + K]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 396.2202; found 396.2201.

Compound 12: This compound was obtained as described above for **10** starting from 9-(chloromethyl)anthracene and **E2**. Yellow solid; yield 40%; m.p. 158–160 °C. $[\alpha]_D^{20}$ = –15.3 (*c* = 0.01, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.80 (m, 3 H, CH_3 Pr), 1.41 (m, 2 H, CH_2 Pr), 2.73 (br. s, 1 H, HN), 3.08 (m, 2 H, CH_2 Pr), 4.48 (s, 1 H), 4.51 (d, *J* = 12.1 Hz, 1 H), 4.58 (d, *J* = 10.6 Hz, 1 H), 7.29 (m, 1 H, HN), 7.33 (m, 2 H), 7.44 (m, 2 H), 7.52 (m, 4 H), 8.08 (m, 3 H, HAnt), 8.33 (d, *J* = 7.7 Hz, 2 H, HAnt), 8.55 (s, 1 H, HAnt) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 11.2, 22.2, 40.3, 43.3, 65.7, 124.4, 124.9, 125.8, 126.7, 127.2, 128.0, 128.7, 129.9, 130.9, 131.3, 140.2, 171.7 ppm. IR (KBr): $\tilde{\nu}$ = 3332, 2872, 1638, 1540 cm^{-1}. MS (ESI): *m*/*z* = 383.0 [M + H]$^+$. HRMS (EI$^+$): calcd. for [M]$^+$ 382.2045; found 382.2041.

Supporting Information (see also the footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra of new compounds.

Acknowledgments

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