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Towards calixarene-based prodrugs: Drug release and antibacterial behaviour of a water-soluble nalidixic acid/calix[4]arene ester adduct

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ABSTRACT

A water-soluble calixarene-based heterocyclic podand incorporating a quinolone antibiotic subunit, the nalidixic acid, was synthesised and fully characterised. Its prodrug behaviour was assessed in vitro by HPLC, demonstrating the release of the tethered quinolone in model biological conditions. Microbiological studies performed on various Gram-positive and Gram-negative reference strains showed very interesting antibacterial activities.

The highly interesting organisational and carrier properties of the calixarenes have been modestly investigated in the medicinal field; only very few reports, essentially patents, have been devoted to their use as therapeutical agents.[1–7] In order to develop calixarene-based podands shaped as potent drug carriers and dispensers, we have recently reported the synthesis and the characterisation of drug-containing calix[4]arene species displaying two carboxy-protected 6-aminopenicillanic acids or two nalidixic acid residues, tethered on distal phenol rings via amide or ester linkages, respectively.[8–10]

As these compounds were designed as hydrophobic structures, the lack of solubility in biological media made them uncompliant for in vitro standard evaluation as antimicrobial agents. Gaining hydrosolubility thus appeared the only way to access the biological activities, notably to verify the prodrug behaviour of such structures, main aim of the project. Thus, we developed a synthetic strategy leading to a water-soluble analogue of the previous compounds, by means of introduction of hydrophilic groups at the upper rim of the calixarene platform.

We present here our preliminary synthetic, analytical and biological results, concerning a prodrug-like structure that involves one nalidixic acid moiety tethered via a propyl linker to the lower rim of the tetra-*para*-(aminoethyl)calix[4]arene.

The synthetic strategy involves the grafting of the bromopropyl ester of nalidixic acid at the lower rim of the calix[4]arene platform. The latter was chosen in order to allow the main reaction and purification processes in organic solvent, prior to releasing the water-soluble form by a simple and non-destructive step. Among the various water-soluble calixarene species available in the literature and in our laboratory, the tetra-*para*-(aminoethyl)calix[4]arene **1** was first chosen. Its Boc-protected form allows in fact functionalisation processes at the lower rim in classical solvents, and it is easily deprotected in TFA/CH_2Cl_2 medium, to give the corresponding water-soluble tetra ammonium species.

The tetra-*para*-(aminoethyl)calix[4]arene **1** was prepared according to Gutsche's procedure.[11] The four amino functions were protected as Boc-derivatives, affording the tetra-*para*-(*N*-*tert*-butoxycarbonyl-aminoethyl)calix[4]arene **2**, with a yield of ca 70% after chromatography. The bromopropylnalidixate was reacted with an excess of **2** in refluxing MeCN, in the presence of K_2CO_3 as base, and of a catalytic amount of KI, to give the mononalidixic podand **3** with a yield of 60%. The four Boc-protective groups of **3** were discarded by treatment with trifluoroacetic acid in CH_2Cl_2, at room temperature, to give the tetra-ammonium salt **4** in a quantitative yield after lyophilisation.

The expected biological degradation process should give the hydroxypropyl calixarene **6** as by-product of nalidixic acid. In order to evaluate its own antibacterial activity, **6** was synthesised by a two-step process involving basic degradation of **3** into the tetra-

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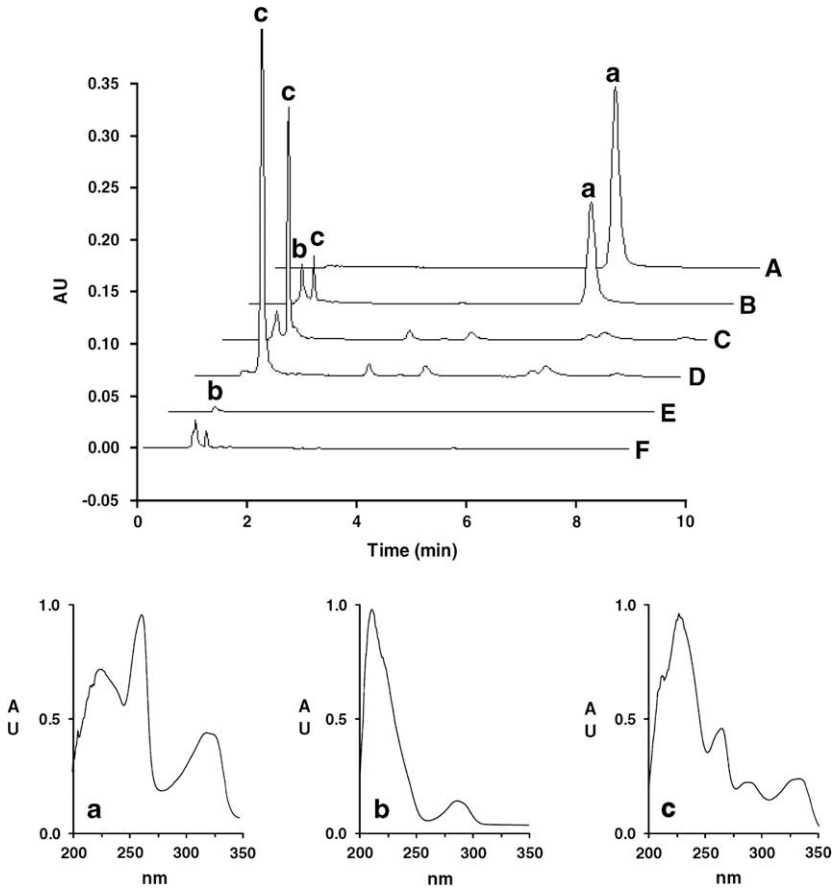


Figure 1. Top: Typical chromatograms (λ = 254 nm) obtained for nalidixic acid (**Nal**) standard (**A**), compound **4** in plasma medium at t = 22 h (**B**), compound **4** in plasma medium at t = 0 h (**C**), compound **4** in mobile phase (**D**), alcohol **6** (**E**) and blank plasma (**F**). Bottom: UV spectrum of **Nal** (**a**), alcohol **6** (**b**) and compound **4** (**c**) extracted from chromatograms **A**, **E** and **D**, respectively.

BOC alcohol **5**, followed by deprotection of the amino groups in the TFA/CH_2Cl_2 medium (Scheme 1).

All compounds were characterised by IR, UV–vis, ^{1}H and ^{13}C NMR, elemental analysis and mass spectrometry. The two water-soluble species **4** and **6**[12] are in the cone conformation, as assessed with ^{1}H NMR by the presence of two AB-shaped Ar-C*H*$_2$-Ar resonance signals, and with ^{13}C NMR, by their location at 30.9 ppm for ester **4** and 30.96 and 31.12 ppm for alcohol **6**. Elemental analyses were consistent with the presence of the four expected trifluoroacetate groups for both compounds **4** and **6**, four H_2O associated to ester **4**, and three H_2O associated to alcohol **6**.

The expected prodrug behaviour of **4** was evaluated by HPLC analysis of its decomposition into hydroxypropylcalixarene **6** and nalidixic acid **Nal** in biological medium, that is, rat plasma. The chromatographic conditions were optimised in order to focus on **Nal** formation while allowing relatively short retention times for all the components involved in the hydrolysis, that is, **Nal**, **4** and alcohol **6**. The chromatographic profile of lyophilised **4** remained unchanged after a one-year storage period at 4 °C, revealing its high stability in the solid form.

The chromatographic study was carried out with an RP18 stationary phase (125 × 4 mm, 100 Å, 3 μm), using a 50 mM ammonium acetate buffer pH 2.0/MeCN/MeOH 65/30/5 (v/v/v) mobile phase with a flow rate of 1.0 mL/min at 25 °C. A 20 μL injection loop was used with diode array detection from 200 to 350 nm.

The hydrolyses were carried out on samples consisting in 1.0 of **4** mixed to 1.0 mL of plasma incubated at 37 °C. Aliquots of the reaction medium (50 μL) were mixed to an equivalent volume of MeCN to allow a sufficient protein precipitation. After centrifugation (14,000 rpm, 5 min), 50 μL of supernatant were diluted five-fold with the mobile phase before injection. As shown on Figure 1, the simultaneous release of nalidixic acid and the disappearance of **4** were effectively observed (curve **B** vs **C**). All compounds under study were identified with the help of the Diode Array Detector and specific UV spectra were obtained for **Nal**, compounds **4** and **6** (Fig. 1, bottom). As illustrated by curve **F**, no interferences in the chromatographic zone of nalidixic acid were observed, and the major part of the void volume could be explained by residual proteins from the rat plasma used.

The reaction kinetic was evaluated from 0 to 90 h as illustrated in Figure 2. The nalidixic acid quantity (release percentage) was

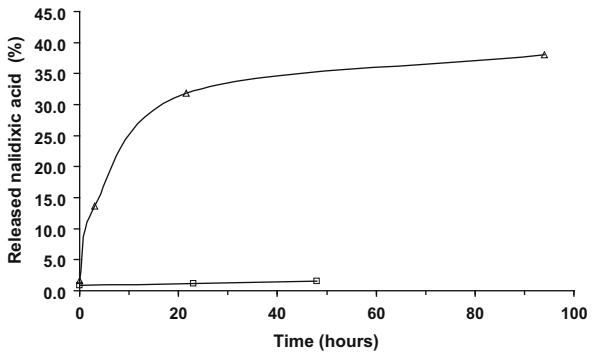
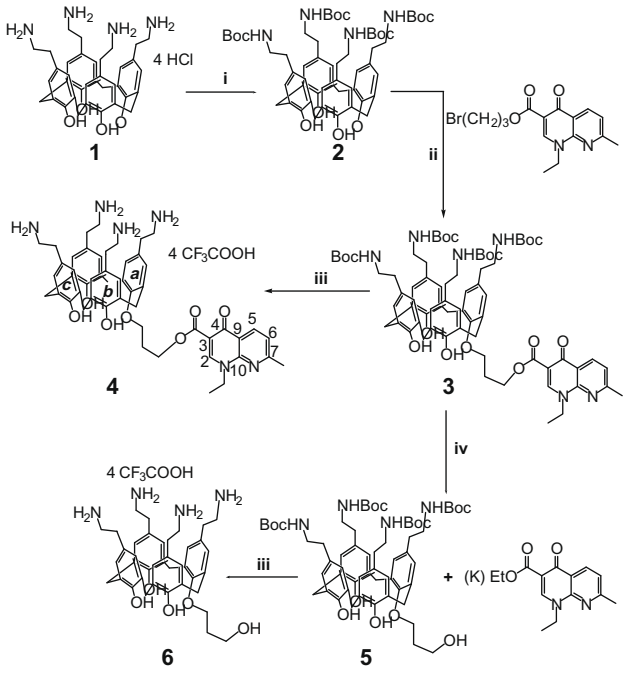


Figure 2. Kinetic of nalidixic acid release at 37 °C in rat plasma (Δ) and in Mueller–Hinton broth (□).



Scheme 1. Synthetic pathway to podands **4** and **6**. (i) NaOH, $(Boc)_2O$, 1,4-dioxane, H_2O, 70%; (ii) MeCN, K_2CO_3, KI, rflx, 60%; (iii) CH_2Cl_2, TFA, 95%; (iv) EtOH, K_2CO_3, 61%. (atom numbering refers to NMR analysis[12]).

calculated versus the maximum that could be obtained from compound **4**. A plateau was reached around 24 h indicating that the reaction was terminated. The maximum percentage reached 40%; this result was balanced as the sample treatment (protein defecation) could wash down significant quantities of active substances. Analysis of blank assays (t_0) showed that nalidixic acid was not sequestered by the precipitate. The survey of residual concentration of **4** in solution showed, in the limits of the method, that the latter was present in proportions lower than expected. The analysis of the precipitate treated in acidic conditions (0.1 M HCl) showed the release of residual **4** in an approximately complementary amount. Thus **4** appears retained by seric proteins, but in a way that probably limits its hydrolysis, explaining the plateau observed for the formation of nalidixic acid.

The Mueller–Hinthon broth employed in the in vitro antibacterial assays should not display any enzymatic activities, due to sterilisation process. Nevertheless, a chemical hydrolysis of the ester junction could occur, and edge off biological results. Its hydrolytic properties were thus evaluated according to the above-mentioned procedure. In order to keep present all possible hydrolytic potency, the protein precipitation step was discarded. No release of nalidixic acid was observed at 24 h (approved standard time for MIC determination), and 48 h, confirming thus the neutrality of Mueller–Hinthon broth in these evaluations (Fig. 2).

The antibacterial activities (minimum inhibitory concentration; MIC) of prodrug **4**, of its two expected metabolites nalidixic acid (**Nal**) and 3-hydroxypropyl calix[4]arene **6**, and of sodium trifluoroacetate were evaluated in liquid phase against various Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and Gram-positive (*Staphylococcus aureus* ATCC 25923, and ATCC 29213, *Enterococcus faecalis* ATCC 29212) bacteria, according to CA-SFM[13] and NCCLS.[14] Experiments were carried out according to microdilution protocols performed in 96-well U shape microtiter plates described by NCCLS.[14]

Sodium trifluoroacetate did not exhibit any activity at concentrations inferior to 256 μg mL^{-1}).

The results presented in Table 1 show first that the activity of nalidixic acid **Nal** is conform to the literature,[15] close to the 32 μg mL^{-1} recommended by NCCLS.

The calixarene species **4** displays MIC values of 35 μg mL^{-1} for *E. coli*, 70 μg mL^{-1} for the two *S. aureus* strains, and 140 μg mL^{-1} for *E. faecalis* and *P. aeruginosa*, while the mono-alcohol **6** needs from 2 (*E. faecalis*, *P. aeruginosa*) to 4 (*E. coli*, *S. aureus*) times higher concentrations, in ranges considered as non-active.

Considering in these conditions a 100% hydrolysis (best case), the quantities of theoretically released nalidixic acid **Nal** and monoalcohol **6** were calculated and compared to their MIC values. The results given in Table 2 show that for the most susceptible strains, that is, *E. coli* and *S. aureus*, the theoretical concentration of released substance is 4 times less than the MIC for **Nal**, and ca 4 times less for mono-alcohol **6**. For the less susceptible strains (*E. faecalis*, *P. aeruginosa*), this ratio rises to 2 and 2.4, respectively. In all cases, and even with the ideal hypothesis of 100% hydrolysis, the MIC values obtained for the podand **4** are not consistent with a simple release of nalidixic acid. Two hypotheses can be made at this stage of the work, involving either a specific activity for compound **4**, or an additive or a multiplicative effect between **4** or **6** and nalidixic acid.

These two important points are currently under investigation.

We have prepared a new water-soluble calixarene species incorporating, via a labile bound, a quinolone antibiotic subunit. This compound, designed as a nalidixic acid prodrug, effectively displays this behaviour, that is, the release of quinolone in biological medium, as assessed by analytical studies. In vitro antibacterial evaluation showed that the title compound was efficient on one Gram negative (*E. coli*) and two Gram positive (*S. aureus*) reference strains, with a clear gain of activity when compared to its two hydrolysis by-products. This observation calls for much deeper

Table 1
MIC values in μmole.L^{-1} (μg mL^{-1}) obtained by broth microdilution method, according to CLSI guideline, of compound **4** and its two hydrolysis by-products **Nal** and alcool **6** over two Gram negative (*E. coli* and *P. aeruginosa*) and three Gram positive (two *S. aureus* and *E. faecalis*) reference strains

	4[a]	**Nal**[a]	**6**[a]
E. coli ATCC 25922	25 (35)[b]	100 (23)	110 (128)
S. aureus ATCC 25923	50 (70)	200 (46)	220 (256)
S. aureus ATCC 29213	50 (70)	200 (46)	220 (256)
E. faecalis ATCC 29212	100 (140)	200 (46)	>220 (>256)
P. aeruginosa ATCC 27853	100 (140)	200 (46)	220 (256)

[a] Molecular weights obtained from elemental analysis: **4**: 1396.47; **Nal**: 232. 24; **6**: 1164.98.
[b] Values in brackets in μg/mL^{-1}.

Table 2
Comparison of MIC values (in μg mL^{-1}) and theoretical amounts of **Nal** and **6** with 100% release

	4	Theoretical released **Nal** in μg mL^{-1}	**Nal**	Theoretical released **6** in μg mL^{-1}	**6**
E. coli ATCC 25922	35	5.8	23	29.2	128
S. aureus ATCC 25923	70	11.6	46	58.4	256
S. aureus ATCC 29213	70	11.6	46	58.4	256
E. faecalis ATCC 29212	140	23.2	46	116.8	>256
P. aeruginosa ATCC 27853	140	23.2	46	116.8	256

investigation, related to its proper antibacterial behaviour, as well as to additive and/or multiplicative effects between the two released by-products or between title compound and nalidixic acid. This investigation is already under way, in parallel to a wider antibacterial screening involving multiresistant bacteria,[16] bacterial membrane targeting studies,[10,17] and hydrolysis studies with bacterial esterases.

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