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Fliposomes: new amphiphiles based on *trans*-3,4-bis(acyloxy)-piperidine able to perform a pH-triggered conformational flip and cause an instant cargo release from liposomes

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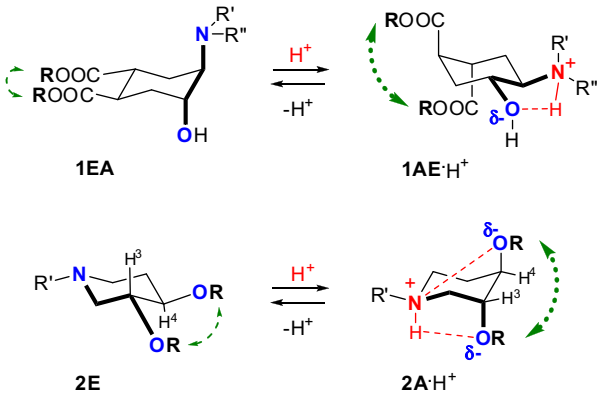
ABSTRACT

Amphiphilic *trans*-3,4-bis(acyloxy)-1-benzylpiperidines able to perform a pH-triggered conformational flip (flipids) have been suggested as components of a new type of pH-sensitive liposomes (fliposomes). According to ^{1}H NMR, their acid-induced conformational flip occurs in methanol-d_4 when the apparent pD decreases from 6 to 3. The protonation-generated intramolecular hydrogen bond and electrostatic interactions make the conformer with axial acyloxy-groups predominant, which drastically increases the separation of hydrocarbon chains. The power of this trigger was estimated as $\geqslant$10 kJ/mol. This flip perturbs the liposome membrane causing rapid release of the liposome cargo specifically in response to lowered pH. The pH-sensitive fliposomes containing one of these flipids, POPC and PEG-ceramide, and loaded with ANTS/DPX performed a content release within a few seconds at pH <5 demonstrating a potential of the piperidine derivatives as pH-switches for the design of liposomes for drug/gene delivery.

Mechanical or conformational molecular switches are molecular systems that reversibly change a relative orientation of their parts under external stimulus. They play a central role in a design of molecular machinery, controllable compounds and intelligent materials for possible use in many applications, including drug release, new sensor techniques or information storage and transmission.[1–14] The cyclohexane-based molecular systems have been designed as a new type of such switches.[13–24] In particular, we used the *trans*-2-aminocyclohexanol moiety **1** (Scheme 1) to construct the conformationally controlled crown-ethers and podands,[13–16] and the pH-sensitive 'flipids' for 'fliposomes'.[13,18–21] A pH-triggered conformational flip of these molecules (with R = alkyl chain) incorporated into the lipid bilayer disrupts the liposome membrane and causes rapid release of the liposome cargo specifically in response to lowered pH. The acid-induced release is a promising approach to the design of liposomes as drug/gene delivery systems, because increased acidity is characteristic of numerous physiological and pathological conditions including endosome processing, inflammation, ischemia and solid tumour growth.[7–12,25–34]

We recently suggested the *trans*-3,4-bis(acyloxy)-piperidine structure **2** (Scheme 1),[35,36] the *trans*-4-amino-3-piperidinol[37] and the 3,7-diazabicyclo[3.3.1]nonan-9-one[38] moieties as new platforms for the pH-triggered conformational switches. For instance, addition of acid to compounds **1** and **2** leads to protonation of the amino group and generates a strong intramolecular hydrogen bond of O···H–N$^+$ type and an electrostatic/dipole–dipole attraction stabilizing a conformer with the *gauche*-form of O–C–C–N fragment (**1AE**·H$^+$ or **2A**·H$^+$ on Scheme 1). This impulse results



Scheme 1. pH-induced conformational switch in aminocyclohexanols (**1**)[13–21] and piperidines (**2**).[35,36]

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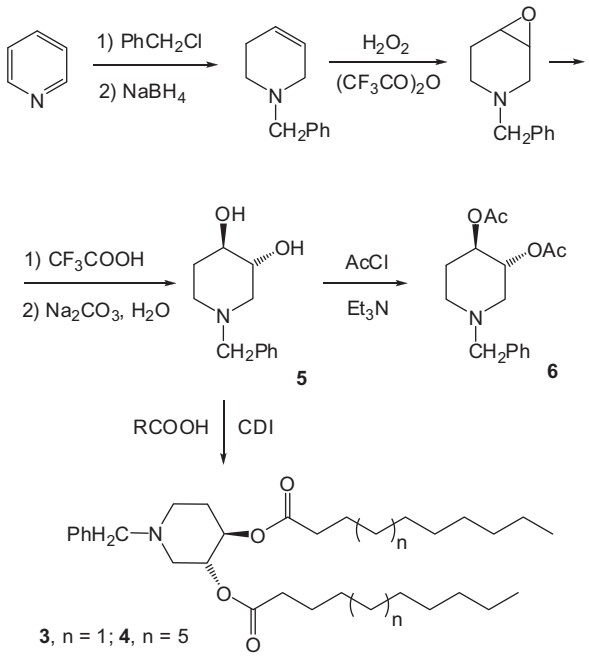
in a conformational flip of the cycle that moves the groups COOR or OR at the other end of the molecule away from each other into axial positions. The relocation of substituents changes their intra- and intermolecular interactions, for example, their ability to form complexes with cations or to pack into lipid bilayers (depending on the nature of R). Such an acid-induced conformational flip has been demonstrated by ^{1}H NMR for *trans*-3,4-diacetoxy-1-benzylpiperidine (**2**; R = Ac, R′ = $PhCH_2$).[35,36] Here we report the development of this model towards the piperidine-based latent amphiphiles of type **2** with pH-triggered conformations ('flipids', Scheme 2) and provide the first example of pH-sensitive liposomes containing these switches.

The *trans*-3,4-bis(acyloxy)-1-benzylpiperidines **3** and **4** with long lipophilic tails were synthesized by acylation of diol **5** with corresponding acids activated with *N,N′*-carbonyldiimidazole (CDI) according to Scheme 3 (see Supplementary data). The diacetate **6**[35,36] and the precursors, including diol **5**,[39–41] were prepared as described previously.

We examined the chair–chair flip of the ring in flipids **3** and **4** in solution by ^{1}H NMR (600 MHz). The vicinal coupling constants $^3J_{HH}$ between several protons attached to the piperidine moiety are strongly conformation-dependent, which allows an assignment of a predominant conformation and an estimation of the position of conformational equilibrium.[42]

The theoretically possible conformations of piperidines with an axial alkyl group at nitrogen were shown to make a negligibly small contribution to the equilibrium,[43–46] so we did not include them into consideration. The conformer populations (n_E, n_A) and the free energy difference between the conformers (ΔG_{A-E}) were estimated as described before[13–23,35–37] from ^{1}H NMR measurements for the diluted solutions (0.01–0.02 M). The observation of a single set of well-resolved multiplets attests to the high rates of both conformational and acid–base equilibria on the NMR time scale. The coupling constants of cyclic protons were used for the conformational assignment: large vicinal couplings (9–12 Hz) were observed for the *trans*-diaxial protons, and small values (2–5 Hz) were measured for the axial–equatorial and equatorial–equatorial vicinal couplings. The conformer populations were estimated using Eliel's equation[47] applied to the averaged signal width $W = \Sigma J_{HH}$ (a sum of spin–spin couplings) of the protons H^3 and/or H^4 geminal to the RO-groups: $W_{observed} = W_A \cdot n_A + W_E \cdot n_E$.[42] The parameter W was measured as a distance between terminal peaks of a multiplet (Table 1). The signals H^3 and H^4 were usually well resolved and had chemical shifts in a region apart from the signals of other coupled protons. The limiting parameters W_E were set to be equal to the values observed for H^3 and H^4 in the CD_3OD solution of **4** (W^3 = 23.3 Hz, W^4 = 24.6 Hz), assuming a complete bias of the equilibrium towards the equatorial conformer **4E** (large values of vicinal couplings). The parameter W_A for the axial conformers

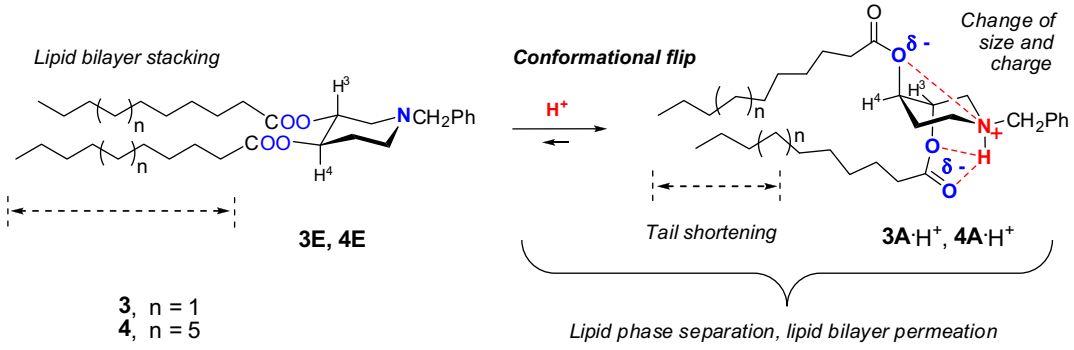


Scheme 3. Synthesis of *trans*-3,4-bis(acyloxy)-1-benzylpiperidines.

was approximately estimated as 9.5 Hz[36] from reported data for the related cyclohexanols and piperidinols.

In solutions of nonpolar $CDCl_3$, as well as in polar CD_3OD, both studied flipids **3** and **4**, and diacetate **6** strongly prefer to be in the diequatorial conformation **E** (Table 1). This is in accord with the results of calculations (see below), the experimental estimations made for diacetate **6** and diol **5** previously,[35,36,39,40] and with the well-known preference of *trans*-1,2-cyclohexanediols and other vicinal diols[48,49] and their esters[50,51] for a *gauche*-arrangement.

To study the pH-induced change of conformational equilibrium, the diesters **3**, **4** and **6** were dissolved in CD_3OD producing the starting apparent pD ≈ 7.15. The changes of their ^{1}H NMR spectra (Fig. 1) were monitored during titration of the solution with *d*-trifluoroacetic acid (*d*-TFA) (see Supplementary data for details). During the incremental addition of *d*-TFA, the signal widths W (the sum of spin–spin couplings) for protons H^3 and H^4 decreased from 23–24 Hz to 16–17 Hz for **3** and **4**, and to 13 Hz for **6** (Table 1, Fig. 1). The signals continued to narrow down with further acidification, but they became difficult to measure because of poor resolution and loss of intensity. The small values of *gauche*-coupling constants indicated equatorial positions of protons H^3 and H^4, and respectively axial positions of the acyloxy groups. Thus, the



Scheme 2. Protonation-induced conformational flip, shortening of the lipid tails, and change of the charge, effective size and shape of the polar head cause a quick perturbation of the lipid bilayer, lipid phase separation and fast content leakage from the fliposomes.

Table 1
^{1}H NMR data and conformational parameters[a]

Compound, solvent, acid	H^3		H^4		n_A (%)	ΔG_{A-E} (kJ/mol)
	δ	W (Hz)	δ	W (Hz)		
3						
$CDCl_3$	4.98	23.0	4.78	24.3	~2	~9.5
CD_3OD	4.91	23.2	4.78	24.5	~0	⩾10
CD_3OD + TFA[b]	5.09	16.3	5.01	[c]	51	-0.1
4						
$CDCl_3$	4.98	23.0	4.79	24.3	~2	~9.5
CD_3OD[d]	4.92	**23.3**	4.78	**24.6**	~0	⩾10
CD_3OD[d] + TFA[b]	5.08	17.4	5.00	[c]	43	0.7
6[35,36]						
$CDCl_3$	4.97	23.0	4.78	24.3	~2	~9.5
CD_3OD	4.89	22.8	4.77	24.2	~3	~8.5
CD_3OD + TFA[b]	5.08	13.4	5.00	[c]	72	−2.3

[a] 600 MHz (JEOL ECA-600), 295 K, 0.01–0.02 M (0.04 M for **6**).
[b] *d*-Trifluoroacetic acid was added to CD_3OD solution up to pD = 3.23 (**4**), 4.00 (**5**) and 3.07 (**6**); further acidification leads to the narrower but unresolved signals.
[c] Partially or completely overlapped with other signals and/or unresolved.
[d] Contains 5% $CDCl_3$ for solubility.

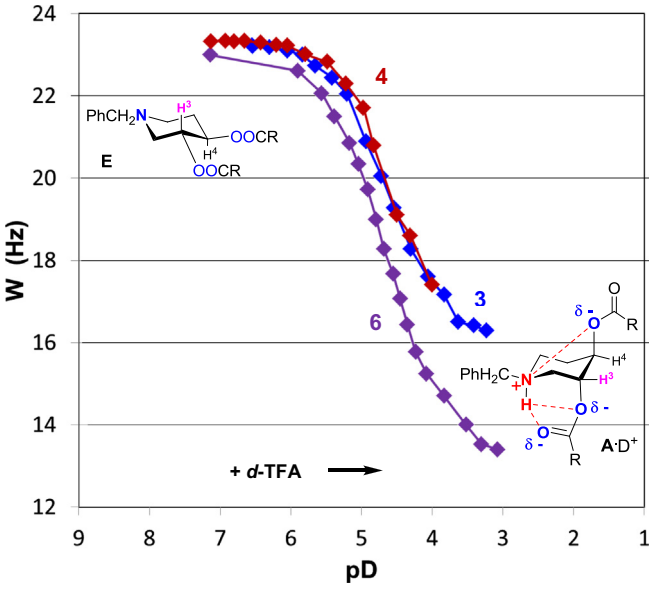


Figure 1. Dependence of the signal width ($W = \Sigma J_{HH}$) on the apparent pD for the protons H^3 in the course of incremental addition of *d*-TFA to the CD_3OD solutions of compounds **3**, **4** and **6**.

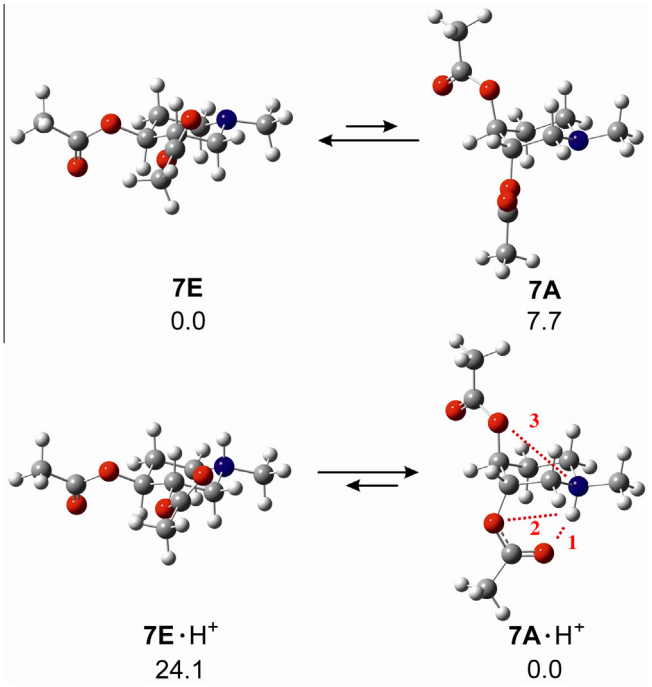


Figure 2. Calculated structures and relative enthalpies ΔH_{gas} (kJ/mol) for the diaxial **A** versus diequatorial **E** conformers of non-protonated and protonated *trans*-3,4-diacetoxy-*N*-methylpiperidine **7** at the B3LYP/AUG-cc-pVTZ//B3LYP/6-311++G(d,p)[52] level of theory. Interatomic distances: (1) 1.70 Å, (2) 2.96 Å, (3) 3.55 Å.

addition of excess *d*-TFA caused a strong protonation-induced shift of the conformational equilibrium towards the diaxial form **A**·D^+ (Schemes 1 and 2) that comprised more than 70% of the total for **6** at pD = 3.07 (Table 1). Somewhat lower population of the diaxial form in the case of flipids **3** and **4** as compared to diacetate **6** may be explained by an attraction between long lipophilic tails in strongly polar methanol-d_4. Using the change of ΔG_{A-E} values in methanol solution upon addition of acid, we estimated the power of this conformational pH-trigger as ⩾10 kJ/mol, which is close to the estimations for *trans*-2-aminocyclohexanols[13–22] and exceeds by far the conformational changes upon protonation that were observed for various RO-piperidines before. As estimated from the titration curves (Fig. 1), the basicity of the amines **3**, **4** and **6** in methanolic solutions is characterized by $pK_a \approx 4.7$ for their deuterated forms.

To better understand the nature of forces determining this conformational equilibrium and its acid-induced shift, we performed DFT calculations for a simplified model structure—*trans*-3,4-diacetoxy-*N*-methylpiperidine (**7**) and its protonated form (Fig. 2) (see Supplementary data for details). As expected, the calculations suggested a strong relative stability of the diequatorial conformers **E** for the structure **7**, which correlates well with the experimental observations for compounds **3**, **4** and **6**. The expected relative stabilization of the diaxial conformer **7A**·H^+ for the protonated form due to intramolecular interactions was also confirmed. However, the large magnitude of the calculated conformational preference for **7A**·H^+ ($\Delta H_{A-E} = -24.1$ kJ/mol) exceeded by far the experimental finding for **6**·H^+, which had only about 72% ($\Delta G_{A-E} = -2.3$ kJ/mol) of the diaxial conformer **6A**·H^+ in the equilibrium with the protonated diequatorial form. In addition to a rather small structural difference between **6** and **7** ($PhCH_2$ vs CH_3 at nitrogen), this

Table 2
Physicochemical parameters and pH-triggered leakage of fliposomes (flipid/POPC/PEG-ceramide) containing ANTS/DPX

Flipid	Molar ratio flipid/POPC/PEG-ceramide	D[a] (nm)	PDI[b]	ζ[c] (mV)	Leakage[d] (%)			
					pH 6.1	pH 5.6	pH 5.0	pH 4.0
3	25/70/5	152	0.07	−3.5	1	4	5	6
3	50/45/5	166	0.09	−2.6	2	3	5	20
4	25/70/5	225	0.25	−1.5	1	4	5	6
4	50/45/5	246	0.29	−0.9	1	9	11	32

[a] Hydrodynamic diameter, representation of two to three separate liposome preparations with less than 10 nm variation.
[b] P.I. = polydispersity index.
[c] ζ-Potential, representation of two to three separate liposome preparations with less than 3 mV variation.
[d] Percentage of ANTS released from the liposome after lowering pH from 7.4 (0% leakage) to the indicated value, average of two to three separate liposome preparations with less than 5% variation.

discrepancy may be caused partially by solvent effects. Noteworthy, a similar discrepancy between the results of calculations and of experiment was found for the relative conformer stability in *trans*-3-hydroxy-4-aminopiperidines.[37]

The flipids **3** and **4** were designed to change their predominant conformation in acidic environment thus causing disturbance in the structure of a lipid bilayer, which in turn allows a leakage of liposomal content. The ^{1}H NMR results presented above demonstrated that flipids **3** and **4** do indeed change conformation when acidity increases, and this flip occurs between apparent pD 5.5 and 3.5 in methanol-d_4 solution (Table 1, Fig. 1). To further validate the concept, we studied the pH-dependence of the leakage from liposomes comprising a flipid in their bilayer (fliposomes) (see Supplementary data for details).

PEGylated fliposomes loaded with a fluorescent dye (ANTS) and a quencher (DPX)[53] were prepared by the freeze–thawing method based on the procedure of Monnard et al.,[54] as described previously.[19–21] Using the previously optimized lipid composition,[19] we prepared, at pH 7.4, the fliposome formulations containing a flipid (**3** or **4**), a neutral phospholipid (POPC) and a PEG–lipid conjugate (PEG-ceramide) in various mole percentages and characterized their colloidal properties (Table 2). The variation of a flipid content (25 or 50 mol %) did not produce a significant difference in the liposome size, polydispersity index and ζ-potential. However, all these parameters changed substantially with elongation of the hydrocarbon tails in flipid.

We measured the leakage of ANTS/DPX after injecting a small aliquot of the fliposome preparation into buffer solutions with pH varying from 7.4 to 4.0. Upon release of these compounds from fliposomes, and stabilization of their fluorescence (λ_{ex} = 350 nm, λ_{em} = 550 nm), the value of the latter was measured and normalized by subtraction of the background fluorescence at pH 7.4 (0% leakage). The percent leakage (Table 2) was calculated by comparison with the value obtained after permeation of fliposomes through an addition of detergent (100% leakage).

As the initial data show, virtually no fliposome leakage occurs in neutral or slightly acidic medium (pH 7.4–6.1). However, the leakage becomes noticeable at pH 5.6 and substantial at pH 4.0. We did not continue acidification below this point, because at pH <4 an acid-induced drop in the ANTS fluorescence occurs, which distorts the measurements significantly.[21,53] The obtained data are in accord with the parameters of the NMR-titration curves for the flipids **3** and **4** (Fig. 1), which may be considered as an indication of the intrinsic dependence of the pH-induced fliposome leakage on the pH-triggered conformational flip of these components. As the mole percentage of flipids was increased from 25 to 50 mol %, both the rate and extent of the release of contents were increased. It was demonstrated previously that the leakage from the liposomes without flipids (POPC/PEG-ceramide = 95/5) did not exceed 3% upon acidification.[19] The results achieved so far with the flipids **3** and **4** are still below the efficiency of the fliposomes containing *trans*-2-aminocyclohexanol-based flipids.[13,18–21] However, the efficacy of flipids can be significantly improved by structural variation of the lipid tails[20] and of the polar heads.[21]

For the first time we used *trans*-3,4-bis(acyloxy)-1-benzylpiperidines as the conformationally switchable latent amphiphiles (flipids) for incorporation into lipid composition of pH-triggerable liposomes (fliposomes) that performed an instant release of their cargo in response to a weakly acidic medium. These results demonstrate a potential of the piperidine derivatives as pH-switches for the design of liposomes as drug/gene delivery systems. Further structural improvement of the piperidine-based flipids is currently in progress.

Acknowledgements

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Supplementary data

Supplementary data (the synthetic procedures, the products characterization, the procedure of NMR titration, and the details of liposome preparation and studies) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.07.156.

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