Supramolecular catalysis of H/D exchange in pyruvate by macrocyclic polyamines involving a reactive iminium intermediate

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The activity of a series of macrocyclic polyamines as catalysts for proton exchange has been investigated. Structural and physico-chemical studies demonstrate the ability of these receptors to recognize pyruvate, form a covalent iminium intermediate and catalyse H/D exchange at the CH_3 position. The reaction follows Michaelis–Menten kinetics with a rate enhancement higher than 4.8×10^5 (k_{cat}/k_{uncat}). The process also represents a potential entry into the catalysis of aldol condensation reactions.

Introduction

Numerous biological transformations[1] and organic reactions[2] involve iminium and/or enamine compounds as reactive intermediates. This is particularly the case for a series of reactions co-catalysed by vitamin B6, most of which have been reproduced in model systems.[1,3,4]

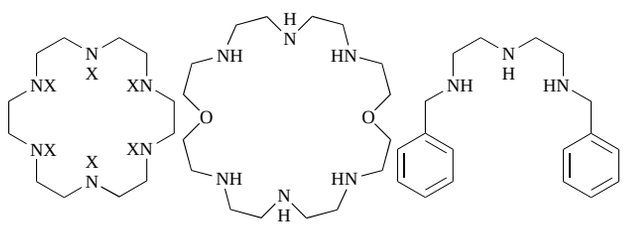
Macrocyclic polyamines possess the ability to simultaneously (1) carry a high positive charge through protonation at neutral pH,[5] so that they can serve as binding sites for anionic substrates, (2) present unprotonated[5] nucleophilic sites at neutral pH and (3) serve as a proton reservoir in a reaction on a bound substrate. These features make macrocyclic polyamines attractive systems for performing supramolecular catalysis.[6] They are involved in the catalysis of ATP hydrolysis, of phosphoryl transfer reactions[6] as well as of malonate enolization and H/D exchange.[7]

Protonated macrocyclic polyamines form stable complexes with anionic substances.[5–7] In view of its central role in biological metabolism,[1,3] pyruvate was an attractive substrate which may be bound by protonated macrocyclic polyamines because of its negative charge at neutral pH[8] and could be activated through the formation of an iminium/enamine intermediate with one of the unprotonated nitrogens. The dual nature of the acidic microenvironment provided by the ammonium sites and the nucleophilic/basic sites provided by the unprotonated nitrogens, should facilitate such a process through general acid–general base catalysis. Bifunctional catalysis of α-hydrogen exchange in carbonyl compounds by amines has been extensively studied.[11,12] Ultimately, the iminium/enamine intermediates could function as powerful electrophiles or nucleophiles for a second co-bound nucleophile/electrophile in a polytopic receptor.

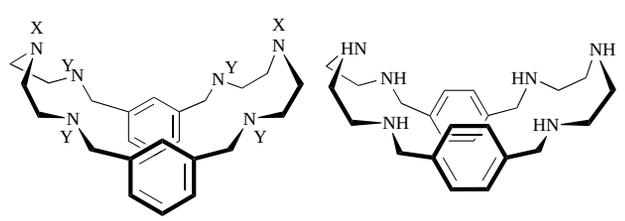
Results and discussion

Reaction of pyruvate with macrocyclic polyamines, and characterization of the intermediate

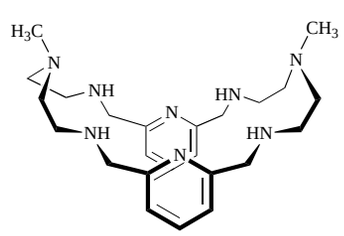
Two types of macrocyclic polyamine receptors were selected (Fig. 1). The first is rather flexible and hydrophilic (**1**–**3**) and the second is more rigid and has hydrophobic character due to aromatic subunits (**5**–**10**). The acyclic compound **4** was used for comparison purposes. The synthesis of **3**[13], **5**[14] and **9**[15] has already been reported, **1** and **2** are commercially available and



1 X = H (400)
2 X = Me (200)
3 (4000)
4 (500)



5 X = H Y = H (8500)
6 X = H Y = CH_3 (2000)
7 X = CH_3 Y = H (7000)
8 X = CH_3 Y = CH_3 (1200)
9 (950)



10 (15 000)

Fig. 1 Polyamines investigated and observed rate enhancements (in parentheses) of H/D exchange of pyruvate measured in their presence; [receptor] = 10 mM, [pyruvate] = 50 mM, pD 7, 25 °C, 60% $(CD_3)_2SO$–D_2O

4, **6**–**8** and **10** were prepared according to the synthetic scheme in Scheme 1. The key step in the preparation of the macrocyclic receptors is the [2 + 2] Schiff base condensation[16] yielding the corresponding tetraimines, which were reduced to yield the corresponding macrocyclic polyamines.

Incubation of pyruvate with any of these polyamines in aqueous dimethyl sulfoxide [60% $(CD_3)_2SO$–D_2O, pD 7, 25 °C] resulted in H/D exchange of the CH_3 group of pyruvate with the solvent. The reaction could be followed by ^1H NMR spectroscopy and the rate of reaction was dependent on the receptor

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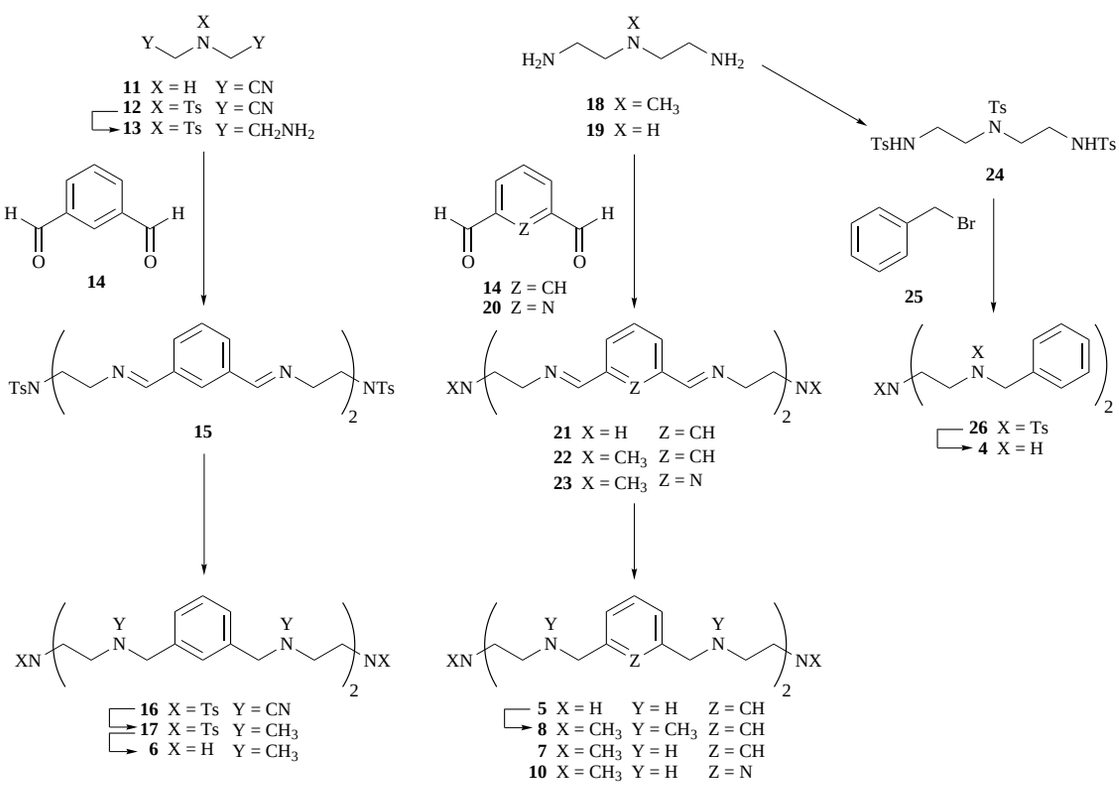
Table 1 Observed rate constants (k_{obs}) and relative rate enhancements for the H/D exchange of the α-CH_3 of pyruvate catalysed by various polyamine receptors; [receptor] = 10 mM, [pyruvate] = 50 mM, 25 °C, pD 7, 60% $(CD_3)_2SO$–D_2O

Assay	Polyamine	k_{obs}[a]/s^{-1}	$t_{1/2}$/min	Relative rates[b]	Charge[c]
1	k_{uncat}[d]	0.02	5.8×10^5	1	—
2	Diethylamine	0.03	3.9×10^5	1.5	1
3	**1**	8	1 444	400	3
4	**2**	4.0	2 888	200	3
5	**3**	80	144	4 000	4
6	**4**	10	1 155	500	2
7	**5**	170	68	8 500	3–4
8	**6**	40	289	2 000	3–4
9	**7**	140	83	7 000	3–4
10	**8**	24	481	1 200	3–4
11	**9**	19	608	950	3–4
12	**10**	300	39	15 000	3–4

[a] These data are within ± 10%. [b] Rate enhancements relative to the reaction in absence of polyamines (assay 1). [c] Overall positive charge of the polyamine upon protonation under the present experimental conditions as estimated from data obtained in H_2O for some of the receptors studied here, and from others.[21] [d] k_{uncat} is the slope of the relationship $V = k_{uncat}$ [pyruvate] for [pyruvate] = 10, 20, 30, 40, 60, 80, 100 and 150 mM.



Scheme 1 Synthetic schemes for the preparation of polyamine receptors **4**, **6**–**8** and **10**

used. Fig. 2 shows the ^{1}H NMR time course of the CH_3 signal at 2.1 ppm. The reaction is first order and the observed rate enhancements are summarized in Table 1 and Fig. 1. Under these conditions, the chemical shifts of the ^{1}H NMR signals of all the receptors were not affected significantly during the course of the reaction.

At a higher percentage of $(CD_3)_2SO$–D_2O (80%) or at a higher concentration of polyamine–pyruvate (20 mM–100 mM), the ^{1}H NMR signals corresponding to the macrocyclic polyamine **3** developed shoulders and a new signal could be observed around 2.94 ppm (insert in Fig. 2). These alterations remained constant during and after complete H/D exchange of pyruvate (5–10-fold excess) but they disappeared when the medium was diluted with D_2O, indicating that a reversible reaction was taking place between the receptor and the pyruvate. These modifications were not observed with the other polyamines, suggesting that the corresponding intermediates were less stable.[17]

The formation of a covalent intermediate was demonstrated using electrospray mass spectrometry (ESMS) (Fig. 3). Since **3** was the only polyamine that showed some alterations in the ^{1}H NMR spectra (Fig. 2), we also studied polyamine **5** in order to show that although it was undetectable by ^{1}H NMR spectroscopy, the same intermediate can be traced by ESMS. Fig. 3 presents relevant parts of the $ESMS^+$ spectra for polyamine **3** (10^{-4} M) in the presence of pyruvate (5×10^{-4} M), in DMSO–H_2O 80% (left) or DMSO–D_2O 80% (right), after incubation at 40 °C for 4 h at an apparent pH or pD of 7. In DMSO–H_2O the signal at $m = 347.5$ was assigned to the monoprotonated macrocyclic polyamine **3** ([m/z] + 1) and the signal at $m = 417.5$ to the monoprotonated iminium intermediate ([m_i/z] + 1). As a result of the H/D exchange at the nitrogen sites and at the pyruvate CH_3, when the same experiment was performed in DMSO–D_2O an increase in the molecular mass of the free polyamine and the intermediate by 7 and 9 units respectively was recorded. Similar results were obtained with **5** (data not shown). As shown in Fig. 4, the intermediate could also be the enamine or the aminal species, since they all have the same mass and could lead to the same mass shift in DMSO–D_2O. One way to discriminate between these species was to perform negative

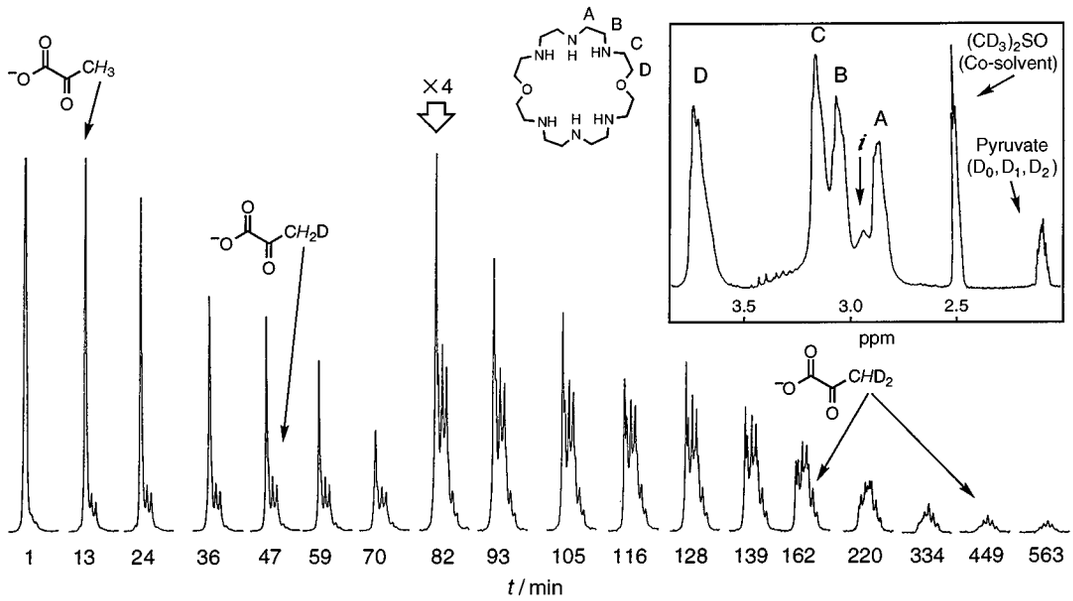


Fig. 2 Typical 200 MHz ^{1}H NMR spectra in the course of the H/D exchange of pyruvate in the presence of the macrocyclic polyamine **5**; [**5**] = 10 mM, [pyruvate] = 50 mM, pD 7, 25 °C, 60% $(CD_3)_2SO–D_2O$. Under these conditions, none of the ^{1}H NMR signals of the polyamine was affected, whereas at twice this concentration in 80% $(CD_3)_2SO–D_2O$ (insert) the ^{1}H NMR spectrum of polyamine **3** showed new peaks (***i***) that may be attributed to an intermediate iminium species; the letters A, B, C and D denote the methylene protons of the macrocycle **3**.

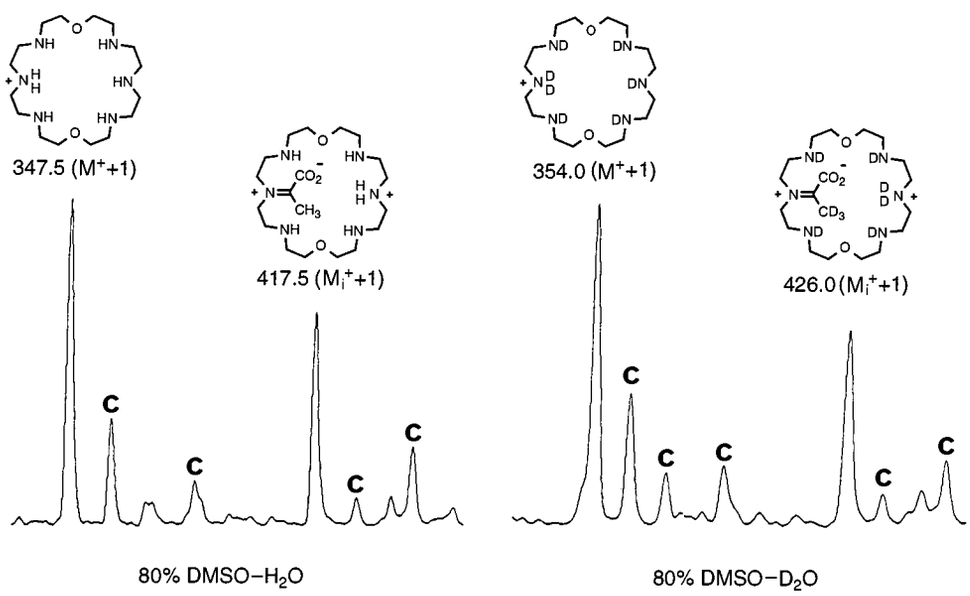


Fig. 3 Electrospray mass spectra for the characterization of the iminium intermediate; [**1**] = 10^{-4} M, [pyruvate] = 5×10^{-4} M, 25 °C, pH 7, 80% $(CD_3)_2SO–D_2O$ (left) or $(CD_3)_2SO–D_2O$ (right). The reaction media were incubated at 40 °C for 4 h before recording the spectra. The peaks indicated by the letter '**c**' are also present in the control experiments (in the absence of polyamine or pyruvate, or in the solvent alone). Similar results were obtained with the macrocycle **5**.

ESMS on the same samples. The iminium is undetectable because of its overall charge neutrality, whereas the enamine and the aminal should give rise to a signal corresponding to the monoanionic species. No signal other than that corresponding to the free pyruvate was observed (data not shown), in agreement with the iminium being the major intermediate. The enamine and aminal probably exist only as transient species. Similar results were obtained with receptor **5** (data not shown).

In order to further support this mechanism, we performed some experiments on the decarboxylation of acetoacetate in the presence of **5**. The reaction leading to acetone and carbon dioxide was accelerated by an order of magnitude.[18] Although the rate enhancement is fairly modest, this result supports the presence of the iminium/enamine intermediates.[19] Further studies to improve the catalytic performances and to elucidate the mechanistic pathway of macrocyclic polyamines catalysed decarboxylation processes are being pursued.

Structure–function relationships

Table 1 summarizes the observed rate constants k_{obs} for H/D exchange obtained with the various polyamines. The analysis of these results gives an insight into the structural and physicochemical requirements for efficient catalysis.

(1) The observed rate enhancements over the uncatalysed reaction (assay 1) vary from 1.5-fold for diethylamine (assay 2) to a factor of about 15 000 for **10** (assay 12).[20] They may result from both pyruvate binding and facilitation of the H/D exchange reaction by the polyamines.

(2) Comparison of the rate enhancements obtained with **1** (assay 3) and **2** (assay 4) to those obtained with the other receptors shows that neither the presence of unprotonated and protonated nitrogens[21] on the same receptor nor the presence or absence of secondary nitrogens is a sufficient requirement for catalysis.[20]

(3) The acyclic monotopic polyamine **4** (assay 6) which

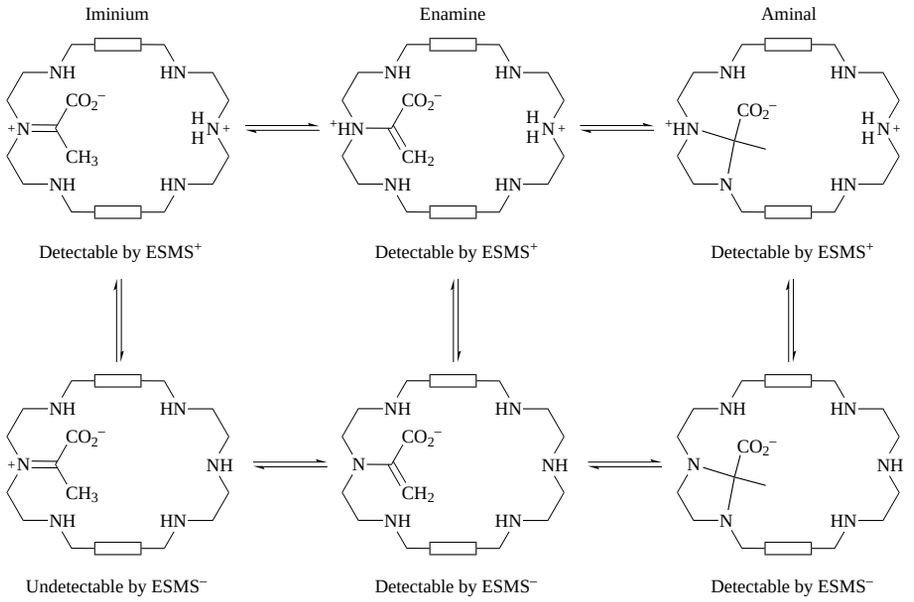


Fig. 4 Possible structures for the reactive intermediates formed between the macrocyclic polyamine and pyruvate. The top three structures are detectable by positive electrospray mass spectrometry (positive ESMS), whereas the bottom central (enamine) and right (aminal) species are detectable by negative ESMS.

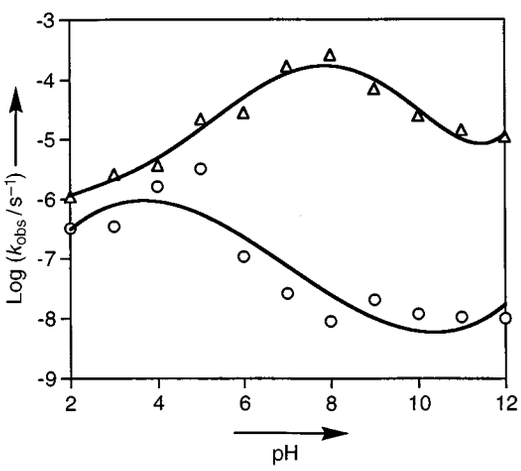


Fig. 5 pH Dependence of the observed rate constant for H/D exchange of the CH_3 group of pyruvate in the presence (△) and absence (○) of macrocyclic polyamine **10**. [**10**] = 10 mM, [pyruvate] = 50 mM, 25 °C, 60% $(CD_3)_2SO$–D_2O. The curves represented correspond to the equations: (△) in presence of polyamine, $y = -7.411 + 1.638x - 0.733x^2 + 0.173x^3 - 0.017x^4 + 0.001x^5$; (○) in absence of polyamine, $y = -8.748 = 1.680x - 0.310x^2 + 0.015x^3$.

mimics one site of the ditopic polyamine **5** (assay 7) is substantially less effective (500- *vs.* 8500-fold acceleration). This result supports a synergism between the two diethylenetriamine subunits for the H/D exchange of pyruvate, affecting either the binding or the catalytic step or both. It agrees with the much lower rate enhancement obtained with **9** (950-fold, assay 11), where the diethylene triamine subunits are more distant, than with its isomer **5** (8500-fold, assay 7).

(4) Methylation of the central nitrogens of **5** (assay 7) resulted in receptor **7** (assay 9) which has similar catalytic activity (8500 *vs.* 7000). When the four lateral nitrogens or all the nitrogens of **5** were methylated to give **6** (assay 8) and **8** (assay 10) respectively, the catalytic efficiency decreased appreciably (from 8500 for **5** to 2000 for **6** and 1200 for **8**). These results indicate that, (i) nucleophilic catalysis through iminium formation contributes only a part of the catalytic power of these receptors, the other part being provided through general acid–general base catalysis; (ii) since tertiary amines do not form an iminium group, the relative efficiency of **5**–**8** may be related to the number of secondary nitrogen sites. In addition, besides the geometrical and structural factors, the effect of the different nitrogen sites on catalysis should also be related to their pK_a values and the pH of the medium.[22,23]

(5) The macrocyclic polyamine **10**, the pyridine analogue of **7**, is the most efficient catalyst in this study and is twice as effective as **7**. This might be at least in part related to the pyridine units which could act as general base and participate in the proton abstraction from the iminium intermediate.

pH profile, Michaelis–Menten kinetics and mechanistic scheme for the catalysis of H/D exchange at CH_3 in pyruvate by the macrocyclic polyamine 10

Below pH 6 pyruvate is in equilibrium with its hydrated form.[24] The ratio of hydrate–keto forms does not vary during the course of the reaction whatever the pH. In the absence of **10**, the H/D exchange is acid catalysed with a maximum rate around pH ≈ pK_a (pyruvate) ≈ 4 (Fig. 5). This is in agreement with acid–base catalysis taking place under the conditions used.[10]

In the presence of **10** the observed rate is higher over the whole pH range investigated with a maximum around pH 8 where, interestingly, the uncatalysed reaction is at its minimum (Fig. 5). Below pH 4 pyruvate is protonated and hence it is probably no longer bound to the polyamine. Above pH 9, the macrocyclic polyamine becomes unprotonated and hence it interacts less efficiently or not at all with pyruvate. At the optimal pH 8 one expects pyruvate to be monoanionic and the polyamine to carry both protonated and neutral nitrogen sites. This coexistence should be responsible for the higher rate enhancement, in agreement with the formation of a supramolecular complex between **10** and pyruvate, prior to the chemical transformation.

Fig. 6 shows Michaelis–Menten and Lineweaver–Burk plots (insert) from which K_m^{app}, V_{max} and k_{cat} were calculated. The high K_m^{app} obtained (0.5 M, pD 7) indicates that the complex formed is very weak due to the single negative charge of pyruvate. In spite of this, the macrocyclic polyamine **10** accelerates the reaction by a factor of more than 4.8×10^5 (k_{cat}/k_{uncat}, pD 7). This rate enhancement constitutes a lower limit since isotope effects from the first exchange on the second, and the effects of the first and second on the third exchange were not taken into account in the calculation of the rate constants. Moreover, [$^2H_3$]pyruvate, the product of the reaction, acts as a competitive

inhibitor, so that the actual rate enhancement is probably even higher.

A possible mechanism for the catalytic cycle of H/D exchange of pyruvate is illustrated for the case of **10** in Fig. 7. Based on previously determined pK_a values for several macrocyclic polyamines,[21] **10** should exist essentially as the tri- and tetra-protonated species at neutral pH. After binding of one molecule of pyruvate, a nucleophilic attack from one of the lateral unprotonated nitrogens could take place leading to the formation of the covalent iminium intermediate, which should be more reactive towards nucleophiles and bases than the parent ketone.[22] Moreover, secondary amines are known to generate the most reactive iminiums.[23] For these reasons, and due also to general base catalysed proton abstraction by another unprotonated nitrogen (aliphatic or pyridinic), this intermediate is in rapid equilibrium with the enamine form. Fast capture of a deuterium from the solvent or the ammonium sites by the enamine would regenerate the iminium which may go through one or two catalytic cycles to yield respectively the di- or tri-deuterated iminium, or may hydrolyse to generate the partially deuterated pyruvate. Finally, [$^2H_3$]pyruvate is produced after three catalytic cycles.

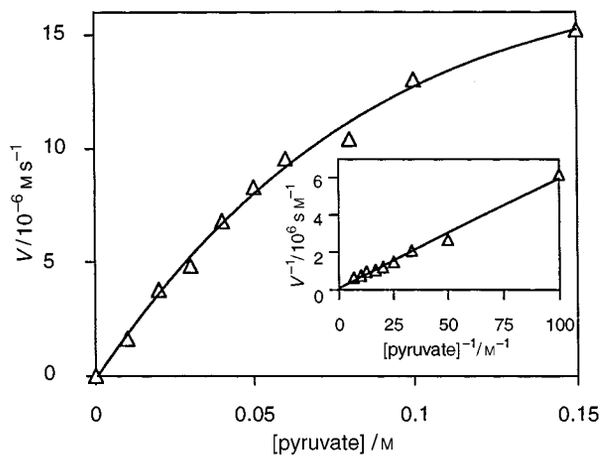


Fig. 6 Michaelis–Menten and Lineweaver–Burk (insert) plots for H/D exchange of the α-CH_3 of pyruvate catalysed by polyamine **10**; [**10**] = 10 mM, pD 7, 25 °C, 60% $(CD_3)_2SO$–D_2O; K_m = 0.55 M, $k_{cat} = 9.54 \times 10^{-3}$ s^{-1}, $V_{max} = 9.54 \times 10^{-5}$ M s^{-1}

Conclusions

Macrocyclic polyamines have been found to strongly activate pyruvate towards H/D exchange through the formation of an iminium intermediate which can in principle also participate in decarboxylation reactions and electrophilic substitutions. Conversely, the corresponding enamine form might act as an electron rich intermediate for aldol condensation and other nucleophilic reactions. Thus, a number of other important chemical and biochemical reactions might be catalysed by macrocyclic polyamines. Further exploration of the parent macrocycles and of suitably designed derivatives is warranted.

Experimental

General

NMR spectra were recorded on a Bruker AC 200 (200.1 MHz for ^{1}H and 50.3 MHz for ^{13}C spectroscopy), with the solvent as internal reference. For the ^{1}H and ^{13}C NMR spectra in D_2O, 2-methylpropan-2-ol was used as internal reference (CH_3, 1.36 ppm; $HOC(CH_3)_3$, 68.7 and 31.6 ppm). *J* Values are given in Hz. Melting points were measured on a digital Thomas–Hoover apparatus (Electrotherma). Chromatographic supports were sil-

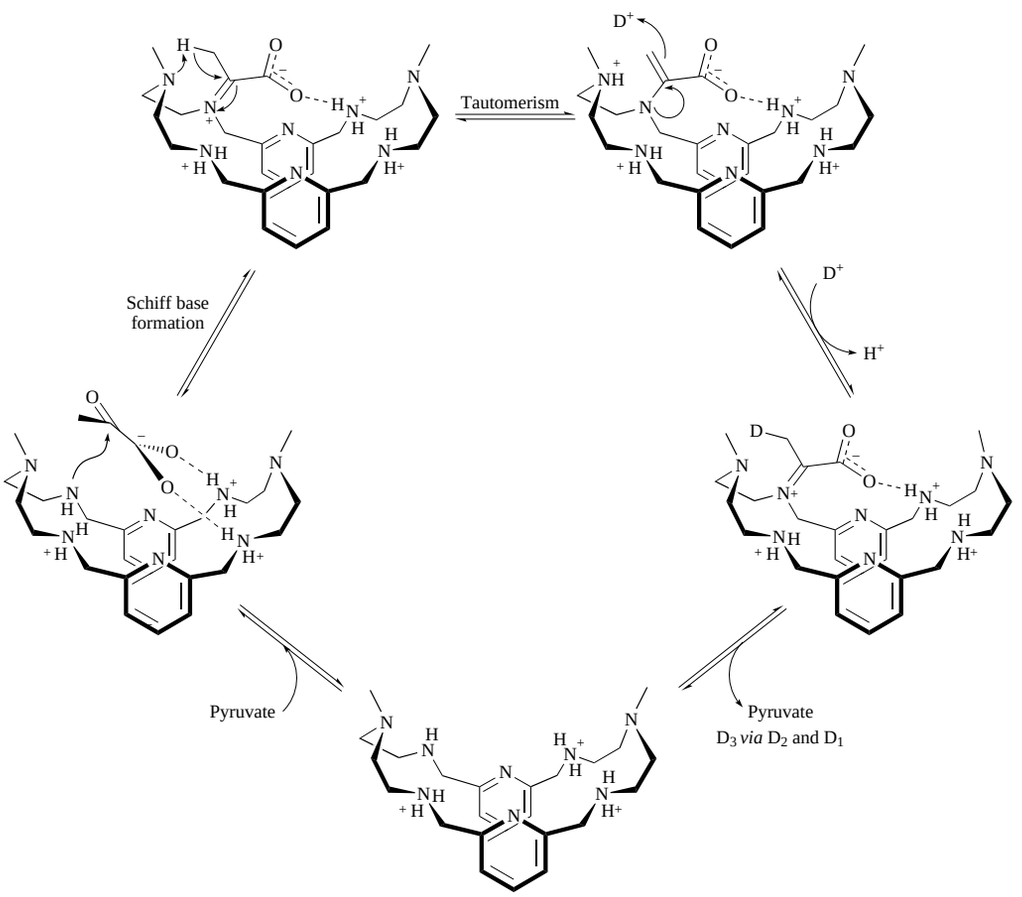


Fig. 7 Possible catalytic cycle for the H/D exchange of the CH_3 group of pyruvate catalysed by macrocyclic polyamine **10** *via* formation of iminium intermediates

ica Merck 60 (0.063–0.200 mm), silica flash Merck 60 (0.040–0.063 mm), or alumina Merck Act. II–III (0.063–0.200 mm). Elemental analyses were performed by the Service de Microanalyse de l'Université Louis Pasteur de Strasbourg or by the Service Central d'Analyse du Centre National de la Recherche Scientifique de Vernaison. The fast atom bombardment (FAB) mass spectra were recorded by the Service de Spectroscopie de Masse de l'Université Louis Pasteur de Strasbourg. ESMS measurements were performed at the Laboratoire de Spectroscopie de Masse Bioorganique, Université Louis Pasteur, Strasbourg.

Synthesis of the macrocyclic polyamines

All the reagents and solvents as well as macrocyclic polyamines **1** and **2** are commercially available. **1** and **2** were converted from the trisulfate salt to the hydrochloride salt form upon elution on Dowex 1X4 (200–400 mesh) in the hydroxide (OH^-) form, followed by acidification of the free polyamine thus obtained with 6 M HCl and crystallization from methanol to yield the corresponding hydrochloride salt. ^{1}H and ^{13}C NMR spectroscopy and microanalysis were in agreement with their structures. **3**,[13] **5**[14] and **9**[15] were prepared according to literature procedures.

1,7-Dibenzyl-1,4,7-tris(*p*-tolylsulfonyl)-1,4,7-triazaheptane, 26. 1,4,7-Tris(*p*-tolylsulfonyl)-1,4,7-triazaheptane **24**[13] (10 g, 17.7 mM), benzyl bromide **25** (12 g, 70.8 mM), and K_2CO_3 (12.2 g, 88.5 mM) were stirred in DMF (50 ml) at 100 °C for 3 days. After cooling to room temperature, the solid was filtered and washed with CH_2Cl_2 (100 ml). The organic layers were evaporated to a final volume of ~40 ml and the product was precipitated with diethyl ether (200 ml), filtered and washed with diethyl ether (200 ml), then dissolved in CH_2Cl_2 (200 ml) and washed with H_2O (2 × 150 ml). The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness to yield compound **26** ($C_{39}H_{43}N_3O_6S_3$, 745.97) as a white microcrystalline powder after recrystallization from CH_2Cl_2–diethyl ether and drying under high vacuum (11.9 g, 90%), mp 176 °C. δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 7.75 [d, ^3J(H,H) 7.9, 4H, aromatic], 7.35–7.26 (m, 14H, aromatic), 7.12 (s, 4H, aromatic), 4.25 (s, 4H, PhCH_2NTs), 3.17–3.12 (m, 4H, PhCH_2NTsCH_2CH_2), 2.45 (s, 6H, CH_3Ts), 2.79–2.71 (m, 4H, PhCH_2NTsCH_2), 2.39 (s, 3H, CH_3Ts); δ_C(50 MHz, $CDCl_3$, 25 °C, TMS) 143.52, 143.41, 136.28, 136.06, 134.66, 129.87, 129.52, 128.81, 128.74, 127.97, 127.27, 127.02 (aromatic), 53.54, 49.12, 47.92 (*C*H_2NTs), 21.49 (*C*H_3Ts); *m/z* (FAB) 746.2 ($M^+ + 1$), 590.2 [($M^+ - Ts$) + 1] [Calc. for $C_{39}H_{43}N_3O_6S_3$ (745.97): C, 62.8; H, 5.81. Found: C, 62.7; H, 5.81%].

1,7-Dibenzyl-1,4,7-triazaheptane, 4. Compound **26** (5 g, 6.7 mM) was dissolved in THF (550 ml), the solution was cooled to 0 °C and $LiAlH_4$ (6.9 g, 181 mM) was added in small portions. After the addition was completed, the reaction medium was heated at 50 °C for 24 h. After cooling to 0 °C, Celite (10 g), Na_2SO_4 (10 g) and ice were added. The latter was added slowly and carefully until complete destruction of excess $LiAlH_4$. The mixture was stirred for 1 h at room temp., filtered over Celite and the solid washed with CH_2Cl_2 (200 ml). The filtrates were evaporated to dryness and the residual solid was taken up in CH_2Cl_2 (200 ml) and extracted with a 1 M aqueous solution of NaOH (100 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residual oil was taken up in a minimum volume of ethanol (20 ml) and the desired compound was precipitated in the hydrochloride form upon addition of concentrated HCl. The precipitate was filtered, washed with ethanol (50 ml) and dried under high vacuum yielding **4** ($C_{18}H_{25}N_3 \cdot 3HCl$, 392.8) as a white hygroscopic solid (2.2 g, 85%), mp (dec.) 309 °C. δ_H(200 MHz, D_2O, 25 °C, TMS) 7.63 (s, 10H, aromatic), 4.46 (s, 4H, PhCH_2NH), 3.66 (s, 8H, NHCH_2CH_2NH); δ_C(50 MHz, D_2O, 25 °C, TMS) 131.94, 131.83, 131.35 (aromatic), 53.66, 45.49, 44.34 (*C*H_2NH); *m/z* (FAB) 284.2 [($M^+ - 3HCl$) + 1] [Calc. for $C_{18}H_{25}N_3 \cdot 3HCl$ (392.8) C, 55.04; H, 7.19; N, 10.70. Found: C, 54.96; H, 7.23; N, 10.38%].

6,20-Dimethyl-3,6,9,17,20,23-hexaazatricyclo[23.3.1.1^{11,15}]-triaconta-1(29),2,9,11(30),12,14,16,23,25,27-decaene, 22. Isophthalaldehyde (**14**, 5.02 g, 37.2 mM) and 4-methyl-1,4,7-triazaheptane (**18**, 4.36 g, 37.2 mM) were refluxed in methanol (400 ml, distilled over Mg) overnight under an argon atmosphere. The solution was evaporated to dryness and the residual oil was taken up in acetonitrile yielding a colloidal solution that was left to decant for several hours. The supernatant was slowly removed and the colloidal portion was subjected to the same procedure several times until a yellowish solid residue was isolated ($C_{26}H_{34}N_6$, 430.6, 0.8 g, 10%). This compound was dried under high vacuum and used in the next step without further purification. No attempts were made to improve the yield.[25] δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 8.3 (s, 4H, NC*H*), 7.98 (s, 2H, aromatic), 7.75 (m, 4H, aromatic), 7.4 (t, 2H, aromatic), 3.75 (t, 8H, CH_2NCH), 2.8 (t, 8H, CH_3NCH_2), 2.4 (s, 6H, CH_3N).

6,20-Dimethyl-3,6,9,17,20,23-hexaazatricyclo[23.3.1.1^{11,15}]-triaconta-1(29),11(30),12,14,25,27-hexaene, 7. Compound **22** (0.8 g, 1.84 mM) and $NaBH_4$ (0.53 g, 14 mM) were suspended in ethanol (20 ml), stirred at room temp. for 3 h and refluxed for 30 min. The solvent was evaporated to dryness and the residual solid was taken up in H_2O (10 ml) and extracted with CH_2Cl_2 (3 × 20 ml).The organic layers were combined and dried over $MgSO_4$, filtered and evaporated to dryness yielding the free polyamine **7** as a white solid. It was then converted to its hydrobromide salt by slow addition of concentrated aqueous HBr (48%, 5 ml). The solvent was evaporated to dryness yielding a white solid which was taken up in H_2O (5 ml) and precipitated by addition of methanol followed by propan-2-ol. The white solid thus obtained was filtered and recrystallized from H_2O–ethanol yielding the hydrobromide salt of **7** after drying under high vacuum ($C_{26}H_{42}N_6 \cdot 6HBr$, 924.11) as a white microcrystalline powder (1.26 g, 74%). δ_H(200 MHz, D_2O, 25 °C, TMS) 7.78 (s, 2H, aromatic), 7.75 (s, 6H, aromatic), 4.54 (s, 8H, NHCH_2Ph), 3.55–3.37 (m, 16H, CH_3NCH_2CH_2NH), 2.95 (s, 6H, CH_3N); δ_C(50 MHz, D_2O, 25 °C, TMS) 133.4, 133.2, 132.4, 131.7 (aromatic), 54.1, 53.3, 43.8 (*C*H_2N), 42.6 (*C*H_3N); *m/z* (FAB) 601.2 [($M^+ - 4HBr$) + 1] [Calc. for $C_{26}H_{42}N_6 \cdot 6HBr$ + 1/2 EtOH (947.15): C, 34.24; H, 5.43; N, 8.87. Found: C, 33.97; H, 5.74; N, 8.67%].

3,6,9,17,20,23-Hexamethyl-3,6,9,17,20,23-hexaazatricyclo-[23.3.1.1^{11,15}]triaconta-1(29),11(30),12,14,25,27-hexaene, 8. Macrocyclic polyamine **5** in the hexahydrobromide form (1.08 g, 1.21 mM)[14] was converted to the corresponding free polyamine by elution on Dowex 1X4 (200–400 mesh) in the OH^- form. After solvent evaporation, the free polyamine was refluxed in formic acid (99%, 1.7 ml, 43.6 mM) and formaldehyde (40%, 1.3 ml, 18.2 mM) for 9 h. The reaction medium was cooled to room temp. and concentrated HCl (12 M, 0.5 ml) was added before refluxing for an additional 3.5 h. The reaction was cooled in an ice bath, treated with an aqueous KOH solution (5 M, 6 ml) and extracted with CH_2Cl_2 (3 × 20 ml). The organic layers were combined, extracted with basic brine, dried over $MgSO_4$, filtered and evaporated to dryness. The free macrocyclic polyamine **8** ($C_{30}H_{50}N_6$, 494.77) obtained as a colourless oil was converted to the corresponding hexahydrobromide by dissolution in concentrated aqueous HBr (48%, 5 ml), evaporation to dryness, recrystallization from H_2O–ethanol and drying under high vacuum ($C_{30}H_{50}N_6 \cdot 6HBr$, 980.22, 0.84 g, 71%). Free polyamine δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 7.3–7.0 (m, 8H, aromatic), 3.4 (s, 8H, NCH_2Ph), 2.5–2.35 (m, 16H, CH_3NCH_2CH_2NCH$_3$), 2.19 (s, 6H, CH_3N), 2.18 (s, 12H, CH_3N); hexahydrobromide form δ_H(200 MHz, D_2O, 25 °C, TMS) 8.1 (s, 2H, aromatic), 7.8 (s, 6H, aromatic), 4.82 (s, 8H, NCH_2Ph), 3.92 (s, 16H, CH_3NCH_2CH_2NCH$_3$), 3.10 (s, 12H, CH_3N), 3.09 (s, 6H, CH_3N); free polyamine δ_C(50 MHz, $CDCl_3$, 25 °C, TMS) 138.7, 129.7, 127.8 (aromatic), 62.6, 55.1, 54.3

(CH_2N), 43.1, 42.8 (CH_3N); hexahydrobromide form δ_C(50 MHz, D_2O, 25 °C, TMS) 135.7, 135.6, 132.5, 131.2 (aromatic), 62.2, 52.9, 51.1 (CH_2N), 43.2, 42.3 (CH_3N); *m/z* (FAB) free polyamine: 495.3 ($M^+ + 1$) [Calc. for $C_{30}H_{50}N_6 \cdot 6HBr + 3H_2O + 1EtOH$ (1080.34): C, 35.58; H, 6.35; N, 7.78. Found: C, 35.73; H, 6.23; N, 7.89%].

6,20-Dimethyl-3,6,9,17,20,23,29,30-octaazatricyclo-[23.3.1.1^{11,15}]triaconta-1(29),2,9,11(30),12,14,16,23,25,27-decaene, 23. Pyridine-2,6-dicarboxaldehyde **20** (1.25 g, 9.25 mM) in dry acetonitrile (150 ml, distilled over CaH_2) was added dropwise over 1 h under a nitrogen atmosphere to a solution of 4-methyl-1,4,7-triazaheptane (**18**, 1.08 g, 9.25 mM) in dry acetonitrile (250 ml, distilled over CaH_2) and stirred for an additional 24 h. After evaporation of the solvent to dryness, the residual oil was taken up in diethyl ether (400 ml) and the insoluble material was eliminated. The solvent was evaporated to dryness and the same procedure repeated with 300 ml of diethyl ether. The desired compound **23** ($C_{24}H_{32}N_8$, 432.58, 1.21 g, 60%), obtained as a yellow solid, was dried under high vacuum and used in the next step without further purification. δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 8.05 (s, 4H, NC*H*Ph), 7.85 [d, ^3J(H,H) 8, 4H, aromatic], 7.56 [t, ^3J(H,H) 9, 2H, aromatic], 3.73 [t, ^3J(H,H) 5.5, 8H, CH_2NCHPh], 2.81 [t, ^3J(H,H) 5.5, 8H, CH_3NCH_2], 2.3 (s, 6H, CH_3N).

6,20-Dimethyl-3,6,9,17,20,23,29,30-octaazatricyclo-[23.3.1.1^{11,15}]triaconta-1(29),11(30),12,14,25,27-hexaene, 10. The tetraimine **23** (1.21 g, 2.8 mM) and $NaBH_4$ (2.1 g, 14 mM) were stirred in EtOH (50 ml) at room temp. for 20 h. The solvent was evaporated and the residue was taken up in CH_2Cl_2–H_2O (50 ml–50 ml). The organic layer was evaporated to dryness and the residual solid was taken up in ethanol (10 ml) and the solution was acidified with concentrated aqueous HBr (48%, 1 ml). The precipitate thus generated was filtered, dried under high vacuum and recrystallized from H_2O–methanol yielding **10** ($C_{24}H_{40}N_8 \cdot 6HBr$, 926.10) as a white microcrystalline powder (1.5 g, 50%), mp (dec.) 254 °C. δ_H(200 MHz, D_2O, 25 °C, TMS) 8.08 [s, ^3J(H,H) 9, 2H, aromatic], 7.61 [d, ^3J(H,H) 7.8, 4H, aromatic], 4.68 (s, 8H, NCH_2Ph), 3.78 (s, 16H, $CH_3NCH_2CH_2NH$), 2.97 (s, 6H, CH_3N); δ_C(50 MHz, D_2O, 25 °C, TMS) 152.04, 141.54, 125.16 (aromatic), 54.86, 53.05, 44.29 (CH_2N), 42.32 (CH_3N); *m/z* (FAB): 441.3 [(M^+ − 6HBr) + 1], 521.3 [(M^+ − 5HBr) + 1], 603.2 [(M^+ − 4HBr) + 1], 683.1 [(M^+ − 3HBr) + 1] [Calc. for $C_{24}H_{40}N_8 \cdot 6HBr + 4H_2O$ (998.16): C, 28.89; H, 5.45; N, 11.23. Found: C, 28.86; H, 5.48; N, 11.26%].

4-(*p*-Tolylsulfonyl)-1,4,7-triaza-hepta-1,6-diyne, 12

To a solution of *p*-toluenesulfonyl chloride (33.2 g, 174 mM) in pyridine (100 ml) at 0 °C, iminodiacetonitrile **11** (15 g, 158 mM) in pyridine (100 ml) was added dropwise over a period of 1 h. Stirring was maintained for 3 h at 0 °C and 2 h at room temp. Ice (300 ml) was added to the solution and the reaction medium was vigorously stirred for 1 h. The precipitate formed was filtered, washed with H_2O (500 ml), ethanol (200 ml) and diethyl ether (200 ml), recrystallized from CH_2Cl_2–hexane and dried under high vacuum. **12** ($C_{11}H_{11}N_3O_2S$, 249.29) was obtained as a white crystalline solid (30.7 g, 78%), mp 99 °C. δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 7.74 [d, ^3J(H,H) 8.4, 2H, aromatic], 7.42 [d, ^3J(H,H) 8.2, 2H, aromatic], 4.3 (s, 4H, CH_2NTs), 2.47 (s, 3H, CH_3Ts); δ_C(50 MHz, $CDCl_3$, 25 °C, TMS) 146.16, 132.82, 130.58, 127.71 (aromatic), 112.65 (*C*N), 35.94 (CN*C*H_2NTs), 21.72 (CH_3Ts); *m/z* (EI): 249.1 (M^+) [Calc. for $C_{11}H_{11}N_3O_2S$ (249.29): C, 52.99; H, 4.45; N, 16.86. Found: C, 53.11; H, 4.49; N, 16.97%].

4-(*p*Tolylsulfonyl)-1,4,7-triazaheptane, 13. Compound **12** (24.9 g, 100 mM) in dry THF (100 ml, distilled over Na) and 1 M B_2H_6 in dry THF (400 ml) were refluxed under a nitrogen atmosphere for 20 h. The reaction mixture was cooled in an ice bath and the excess B_2H_6 was slowly and carefully destroyed by addition of 50% aqueous THF (100 ml). After evaporation of the solvent to dryness the residual solid was taken up in an aqueous solution of HCl (6 M, 200 ml) and refluxed for 2 h. The solvent was evaporated to dryness and the residual solid was taken up in H_2O (100 ml), basified to pH ~13 upon dropwise addition of a 5 M aqueous solution of KOH and extracted with CH_2Cl_2 (2 × 200 ml). The organic layers were combined, evaporated to dryness and the resulting oil was taken up in ethanol (100 ml), acidified to pH ~1 with aqueous concentrated HCl (12 M, 20 ml) and evaporated to dryness. The white solid thus obtained was recrystallized from hot ethanol and dried under high vacuum yielding the desired compound **13** ($C_{11}H_{19}N_3O_2S \cdot 2HCl$, 330.27) as white hygroscopic crystals (11.8 g, 46%), mp 279 °C. δ_H(200 MHz, D_2O, 25 °C, TMS) 7.90 [d, ^3J(H,H) 8, 2H, aromatic], 7.6 [d, ^3J(H,H) 8, 2H, aromatic], 3.63 [t, ^3J(H,H) 5.9, 4H, CH_2NH_2], 3.4 [t, ^3J(H,H) 5.8, 4H, CH_2NTs], 2.55 (s, 3H, CH_3Ts); δ_C(50 MHz, D_2O, 25 °C, TMS) 148.08, 134.33, 132.43, 128.33 (aromatic), 50.2, 40.84 ($NH_2CH_2CH_2NTs$), 22.79 (CH_3Ts); *m/z* (FAB) 258.1 [(M^+ − 2HCl) + 1] [Calc. for $C_{11}H_{19}N_3O_2S \cdot 2HCl$ (330.27): C, 40.00; H, 6.41; N, 12.72. Found: C, 39.78; H, 6.61; N, 12.41%].

6,20-Bis(*p*-toluenesulfonyl)-3,6,9,17,20,23-hexaazatricyclo-[23.3.1.1^{11,15}]triaconta-1(29),2,9,11(30),12,14,16,23,25,27-decaene, 15. Compound **13** was converted quantitatively to the corresponding free polyamine (yellowish oil) upon elution on a Dowex 1X4 (200–400 mesh) column in the hydroxide form and evaporation. After drying under high vacuum, it was taken up in dry acetonitrile (1040 ml, distilled over CaH_2). Isophthalaldehyde **14** (5.2 g, 39 mM) in dry acetonitrile (650 ml, distilled over CaH_2) was added dropwise under a nitrogen atmosphere to this solution over 6 h at room temp. Stirring was maintained for an additional 24 h. The precipitate formed was filtered, washed with acetonitrile and dried under high vacuum. **15** ($C_{38}H_{42}N_6O_4S_2$, 710.92) was obtained as a microcrystalline powder from CH_2Cl_2 (24.12 g, 87%), mp 216 °C. δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 8.17 (s, 4H, NC*H*Ph), 7.93 (s, 2H, aromatic Ph), 7.76 [d, ^3J(H,H) 8.3, 4H, aromatic Ts], 7.67 [d, ^3J(H,H) 7.6, 4H, aromatic Ph], 7.41 [7, ^3J(H,H) 7.8, 2H, aromatic Ph], 7.30 [d, ^3J(H,H) 10, 4H, aromatic Ts], 3.84 [t, ^3J(H,H) 5.9, 8H, CH_2NCHPh], 3.54 [t, ^3J(H,H) 6, 8H, TsNCH_2], 2.43 (s, 6H, CH_3Ts); δ_C(50 MHz, $CDCl_3$, 25 °C, TMS) 161.93 (N*C*HPh), 143.37, 136.42, 130.69, 130.43, 129.77, 128.96, 127.34, 126.94 (aromatic), 51.43, 61.43 (TsN*C*H_2CH_2NCHPh), 21.53 (CH_3Ts); *m/z* (FAB) 711.2 ($M^+ + 1$) [Calc. for $C_{38}H_{42}N_6O_4S_2 + 1/2H_2O$ (719.93): C, 63.40; H, 6.02; N, 11.67. Found: C, 63.46; H, 5.79; N, 11.65%].

3,9,17,23-Tetracyano-6,20-bis(*p*-toluenesulfonyl)-3,6,9,17,20,23-hexaazatricyclo[23.3.1.1^{11,15}]triaconta-1(29),11(30),12,14,25,27-hexaene 16. The tetraimine **15** (5.5 g, 7.74 mM) and $NaBH_4$ (2.9 g, 77 mM) were suspended in DMSO–EtOH (75 ml–30 ml), refluxed until complete dissolution (~2 h) and then for an additional 3 h. The solvent was evaporated under reduced pressure and the residue taken up in H_2O (75 ml) and extracted with CH_2Cl_2 (5 × 125 ml). The organic layers were combined, washed with brine (200 ml), dried over $MgSO_4$, filtered and evaporated to dryness. The residual white solid was dried under high vacuum yielding **16** ($C_{38}H_{50}N_6O_4S_2$, 718.98) which was used in the next step without further purification, *m/z* (FAB) 720.0 ($M^+ + 1$).

3,9,17,23-Tetramethyl-6,20-bis(*p*-toluenesulfonyl)-3,6,9,17,20,23-hexaazatricyclo[23.3.1.1^{11,15}]triaconta-1(29),11(30),12,14,25,27-hexaene, 17. The macrocyclic polyamine **16** was suspended in a mixture of formic acid (90%, 7.6 ml, 186 mM) and formaldehyde (5.3 ml, 77.4 mM) and refluxed for 9 h. The suspension dissolved slowly during the course of the reaction. After cooling, concentrated HCl (12 M, 1.5 ml) was added and the medium was refluxed for an additional 4 h. The reaction mixture was cooled, basified with 5 M KOH and extracted with CH_2Cl_2 (4 × 60 ml). The organic layers were combined, washed with brine (2 × 75 ml), dried over $MgSO_4$, filtered and evaporated to dryness. The residual yellowish solid was chromato-

graphed (SiO_2 flash, methanol–CH_2Cl_2 1–5%) yielding **17** ($C_{42}H_{60}N_6O_4S_2$, 777.1) as a white solid (2.82 g, 47% from **15**). δ_H(200 MHz, $CDCl_3$, 25 °C, TMS) 7.7 (d, 4H, aromatic), 7.2 (d, 4H, aromatic), 7.25–7.0 (m, 8H, aromatic), 3.25 (t, 8H, NCH_2Ph), 3.2 (t, 8H, $TsNCH_2CH_2N$), 2.45 (t, 8H, $TsNCH_2CH_2N$), 2.4 (s, 12H, NCH_3), 2.13 (s, 6H, CH_3Ts); δ_C(50 MHz, $CDCl_3$, 25 °C, TMS) 143.0, 138.9, 136.9, 129.5, 129.3, 128.1, 127.7, 127.2 (aromatic), 62.4, 55.7, 46.4 (NCH_2), 42.4 (NCH_3), 21.5 (CH_3Ts).

3,9,17,23-Tetramethyl-3,6,9,17,20,23-hexaazatricyclo-[23.3.1.1^{11,15}]triaconta-1(29),11(30),12,14,25,27-hexaene, 6. The macrocyclic polyamine **17** (0.21 g, 0.27 mM), HBr–AcOH (33%, 6 ml) and phenol (0.4 g) were refluxed for 24 h during which a brown solid was formed. After cooling, diethyl ether (100 ml) was added to the reaction medium and the brown solid was filtered and washed with diethyl ether (100 ml). The residual solid was taken up in H_2O (40 ml) and washed with CH_2Cl_2 (3 × 40 ml). The aqueous phase was eluted on a Dowex 1X4 (200–400 mesh) column in the OH^- form yielding the free polyamine **6** after evaporation of the eluent (H_2O). It was then converted to the corresponding hexahydrobromide upon dissolution in concentrated aqueous HBr (48%, 5ml), evaporation to dryness, recrystallization from methanol and drying under high vacuum. The desired compound **6** ($C_{28}H_{46}N_6 \cdot 6HBr$, 952.17) was obtained as a white microcrystalline powder (0.22 g, 85%). δ_H(200 MHz, D_2O, 25 °C, TMS) 7.9 (s, 2H, aromatic), 7.8 (s, 6H, aromatic), 4.6 (s, 8H, NCH_2Ph), 3.66 (s, 16H, $NHCH_2CH_2NCH_3$), 3.1 (s, 12H, NCH_3); δ_C(50 MHz, D_2O, 25 °C, TMS) 135.5, 135.4, 132.6, 131.6 (aromatic), 62.1, 52.6, 44.5 (CH_2N), 42.5 (CH_3N); *m/z* (FAB): 466 [(M^+ − 6HBr) + 1] [Calc. for $C_{28}H_{46}N_6 \cdot 6HBr + 1\ CH_3OH$ (984.21): C, 35.39; H, 5.74; N, 8.54. Found: C, 35.44; H, 5.69; N, 8.52%].

Kinetic measurements

1–5 ml stock solutions (100 mM in D_2O) of the macrocyclic polyamines obtained in their protonated forms were prepared in volumetric flasks and the pH was adjusted to 6 by adding very small volumes of NaOH (0.1–5 M) and HCl (0.1–5 M) using a Metrohm 636 pH-Meter equipped with a microelectrode. A few assays on a small sample (0.5 ml) were performed in order to determine approximately the amount of base or acid that should be added in order to reach a given pH. Care was taken to account for the solvent molecules that could not be removed from the polyamine solids even after drying under high vacuum for 48 h. A 5 ml stock solution (500 mM in D_2O) of sodium pyruvate was prepared in a volumetric flask and used as such. All the solutions could be stored at +4 °C for several months without apparent deterioration.

For each kinetic assay, using a set of micropipettes, the appropriate volume of the polyamine receptor that yields the desired concentration was added to a solution containing $(CD_3)_2SO$ (60% v/v of the final volume) and the appropriate volume of D_2O so that when the pyruvate solution was added, the final volume was 600 μl. For instance, for the preparation of a 60% (v/v) $(CD_3)_2SO$–D_2O solution of polyamine (10 mM) and pyruvate (50 mM) at pH 7, 60 μl of the polyamine stock solution were added to a mixture of $(CD_3)_2SO$ (360 μl) and of D_2O (120 μl) in an Eppendorf tube. 60 μl of the stock solution of pyruvate was added and the pH was rapidly adjusted to 7, under magnetic stirring, by adding a few microlitres of NaOH (0.1–5 M) or HCl (0.1–5 M). In any case, the total volume of base and/or acid added to adjust the pH did not exceed 10 μl. The solution was then immediately transferred to a 5 mm NMR tube and the course of the H/D exchange reaction was monitored at 25 ± 3 °C by integration of the ^{1}H NMR CH_3 signal of pyruvate over several half-lives. The spectra were recorded 1–5 min after sample mixing. The ^{1}H NMR signal of DMSO was used as an internal reference (2.5 ppm). The semi-logarithmic representation of the normalized surface of the ^{1}H NMR CH_3 signal as a function of time yields a straight line, the slope of which corresponds to the observed rate constant of the overall H/D exchange process. The isotope effect of the first exchange on the second, and the first and second on the third exchange were not taken into account for the calculation of the rate constants. The correlation coefficients (r^2) for all the curves obtained were >0.98.

The ^{1}H NMR acquisition parameters used on the Bruker AC 200 spectrometer were as follows: SF, 200.1328; SW, 3000; PW, 3.5 μs; O1, 3800; RG = 10–100 (depending on the sample concentration); AQ, 2.736 seconds; LB, 0.1; NS, 16–32 scans (depending on the sample concentration); TD, 16K; SI, 16K; NE, 20–50. Depending on the speed of the reaction, the time interval between two consecutive spectra varied from 1 to 30 min. For the slowest reactions (uncatalysed) the NMR tubes were sealed with Teflon tape and kept in a thermostatted bath at 25 ± 0.1 °C. The spectra were recorded once every two weeks over several months under the same conditions.

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