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An artificial aspartic proteinase system

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Abstract—A series of aza crown ether derivatives with or without carboxyl groups in their side arms were synthesized and the former showed deacylation activities toward amino acid *p*-nitrophenyl ester hydrohalides. Substrate-selective phenomena were also observed. The relationship between the structures and deacylation activities of corresponding compounds suggested a nucleophilic catalytic mechanism. The results partially simulate some aspartic proteinases in the case of catalytic mechanism and are also useful for us to understand the detailed catalytic process of aspartic proteinases.
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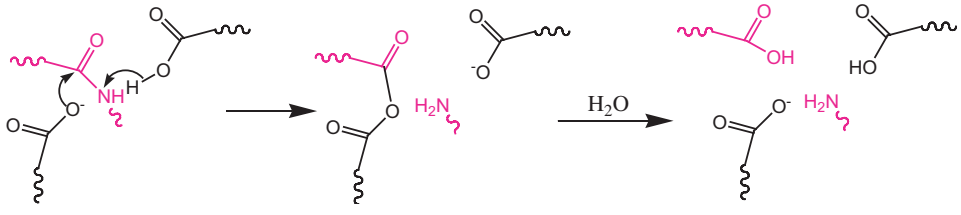
1. Introduction

Aspartic proteinases are an important family of proteolytic enzymes associated with several pathological disorders such as hypertension, gastric ulcers, muscular dystrophy, and neoplastic diseases. X-ray crystallographic data for the various aspartic proteinases reveal that their active site regions are very similar in structure: two Asp residues lie close to each other at the center of a deep and extended active site cleft.[1] It is generally agreed that these two Asp residues play an essential role in the catalysis of peptide hydrolysis.[2] But the detailed knowledge of the structure and the elucidation of a catalytic mechanism has been the subject of persisting controversy.[3] A nucleophilic mechanism and a general-acid/general-base mechanism were presented as two possible mechanisms to illustrate the catalytic process of aspartic proteinases (Schemes 1 and 2). A distinctive mark of the nucleophilic mechanism is the intermediate formed covalently. But a great deal of effort to find this kind of covalent intermediates failed for a variety of peptide substrates.[4] On the other hand, the formation of a non-covalent enzyme-bound amide hydrate intermediate in some peptide substrates had offered unmistakable evidence for general-acid/general-base mechanisms.[5] Does this mean the nucleophilic mechanism is unreasonable in an actual enzyme reaction? Lee and co-workers thought the anhydrides formed via nucleophilic mechanisms might not accumulate to an experimentally detectable amount.[3] Furthermore, some evidence of existence of anhydride intermediates were also presented in studies of enzyme models[4c] and hydrolysis of model peptide substrates.[6] Lee and co-workers also pointed out that either a nucleophilic mechanism or a general-acid/general-base mechanism was operative in an actual enzyme reaction on the basis of the results of quantum chemical



Scheme 1. Nucleophilic mechanism of aspartic proteinases.

Keywords: Crown ether derivatives; Carboxyl; Aspartic proteinases; Artificial enzymes; Nucleophilic catalytic mechanism.
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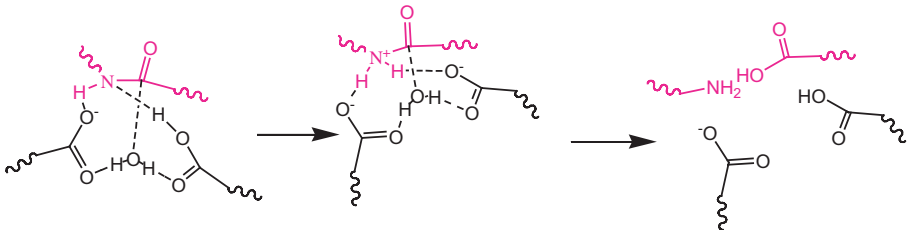


Scheme 2. General-acid/general-base mechanism of aspartic proteinases.

calculation.[3] But explicit evidence from artificial aspartic proteinases supporting nucleophilic mechanism has not been presented as yet.[7]

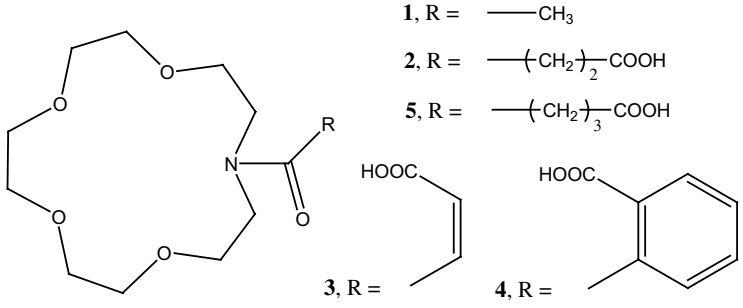
The primary aim of artificial enzymes is to reproduce catalytic features of natural enzymes and another major subjective is to obtain information useful for studies on specific target enzymes.[8] We expected to offer support for nucleophilic mechanism from the aspect of artificial aspartic proteinases.

In the present work, we found that crown ether compounds with or without carboxyls in their molecules showed a different deacylation activity as artificial enzymes toward glycine *p*-nitrophenyl ester hydrobromide. The relationship between structures and deacylation activities of series compounds **1**–**11** (Schemes 3 and 4) suggested there were anhydride intermediates formed and the carboxyls in the molecules of hosts participate as nucleophiles in the catalytic process. The nucleophilic mechanism of two alternative mechanisms of aspartic proteinases was supported in artificial enzymes.

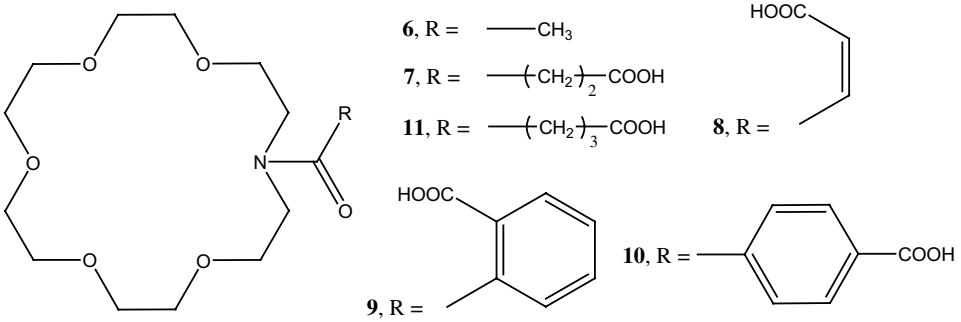
2. Results and discussion

The A_{max} of the substrates is at about 268 nm and that of the product, *p*-nitrophenol, is at 315 nm in 5% CH_3OH–CH_2Cl_2. The release of the product, *p*-nitrophenol, was monitored at 320 nm by a UV–vis spectrometer when compounds **1**–**11** acted on the substrates in 5% CH_3OH–CH_2Cl_2 and the results are described in Table 1. All the rate constants were calculated by the method of half time.

From the results in Table 1 we can still draw the following apparent conclusions easily: (1) Acetic acid and the crown ethers without carboxyls in their side arms (**1** and **6**) alone can hardly enhance the rate constants obviously, while the compounds with carboxyls in their molecules show a significant acceleration effect on the rate of reaction; (2) The compounds with 18-membered rings show more significant catalytic efficiency than the corresponding ones with 15-membered rings, respectively; (3) In the same ring size series, the compounds whose side arms include four carbon atoms are better catalysts than



Scheme 3. Compounds **1**–**5**.



Scheme 4. Compounds **6**–**11**.

Table 1. Rate constants for *p*-nitrophenol release from substrates[a]

Substrate	Run	Host[b] no.	k_1[c] 10^{-4} s^{-1}	k_{rel}
Glycine *p*-nitrophenyl ester hydrobromide	1	None	0.03	1
	2	AcOH	0.42	14
	3	**1**	0.03	1
	4	**2**	7.12	237
	5	**3**	5.91	197
	6	**4**	32.54	1085
	7	**5**	1.45	48
	8	**6**	0.03	1
	9	**7**	10.12	338
	10	**8**	24.50	833
	11	**9**	119.00	3967
	12	**10**	1.26	42
	13	**11**	2.31	77
Glycine *p*-nitrophenyl ester hydrochloride	14	None	0.02	0.67
	15	**9**	108.24	3608
	16	**9** + Inhibitor[d]	8.36	279
Valine *p*-nitrophenyl ester hydrochloride	17	None	0.01	0.33
	18	**4**	5.94	198
Valine *p*-nitrophenyl ester hydrobromide	19	None	0.01	0.33
	20	**4**	6.06	202
p-Nitrophenyl acetate	21	None	No detectable reaction	—
	22	**4**	No detectable reaction	—

[a] The system was self-buffered by acidic host compounds in it and no additional buffer was needed; A_{320} (1.04 for 1.0×10^{-4} M) of *p*-nitrophenol in the reactive solution almost equals that (1.02 for the same concentration) in 5% CH_3OH–CH_2Cl_2 alone.
[b] [Crown] = 1.0×10^{-2} M, [substrate] = 1.0×10^{-4} M, $T = 25$ °C.
[c] The rate constants were calculated by the method of initial rate and all were corrected from background.
[d] Inhibitor = glycine hydrochloride, [inhibitor] = [**9**] = 1.0×10^{-2} M.

are other compounds, such as **2**, **3**, **4** and **7**, **8**, **9**; (4) The compounds with more rigid side arms show a higher catalytic efficiency when they have side arms with an approximately equal length; (5) The deacylation of amino acid *p*-nitrophenyl ester hydrobromides is greatly accelerated, while that of *p*-nitrophenyl acetate is not; (6) The deacylation of glycine *p*-nitrophenyl ester hydrobromide is accelerated more significantly than that of valine *p*-nitrophenyl ester hydrobromide; (7) The anions of substrates show little effect on rates of deacylation.

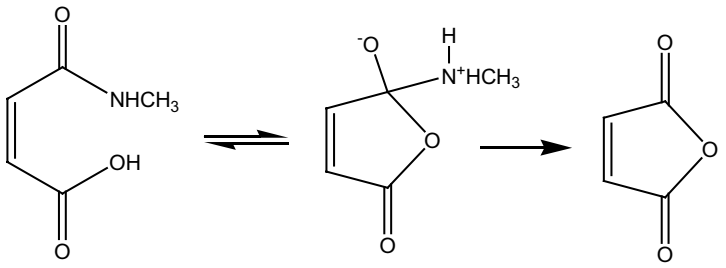
What factors make the above difference in rate constants? Yashima reported[9] that a polymer modified with N-carbonyl aza crown ethers could form complexes with amino acid perchlorates through the hydrogen bonds between primary amine cations and the oxygen atoms of crown rings. Obviously, there were also complexes formed between our N-carbonyl aza crown ethers and the substrates amino acid *p*-nitrophenyl ester hydrohalides. The fact that the deacylation of *p*-nitrophenyl acetate cannot be accelerated also supports this point. The reason is that there is no primary amine cation in its molecule. When the 'enzyme' and its 'substrate' meet the complex formed can be regarded as a pseudo-molecule. Some proper functional groups if they are there in the pseudo-molecules can now participate in the cleavage of ester bonds of substrates easily. The difference of catalytic efficiency between **1**, **6**, and other compounds with carboxyls in their molecules suggests the carboxyl just serves as the above proper functional group.

It is not surprising that a carboxyl shows catalytic activity because there were precedents both in chemical research and in nature. Bruice et al. reported[10] intramolecular nucleophilic catalysis of ester hydrolysis could occur very rapidly by the carboxyl group of monoesters of dicarboxylic acids. He also pointed out that a mixed hydride intermediate was formed when the carboxylate anion attacked the carbonyl of ester, which was similar to the work of Kirby (Scheme 5).[11] In the introduction of this paper, we noticed that the active sites of aspartic proteinases were composed of two carboxyls from aspastyl (Asp) residues. In the nucleophilic catalysis mechanism of aspartic proteinases, there was also a mixed intermediate was formed. We can easily see some similarity in the above two fields. Bruice also thought over four decades ago that the intramolecular nucleophilic catalysis of monoesters of dicarboxylic acids had received particular attention just because of its similarity to enzymic catalysis.[10]

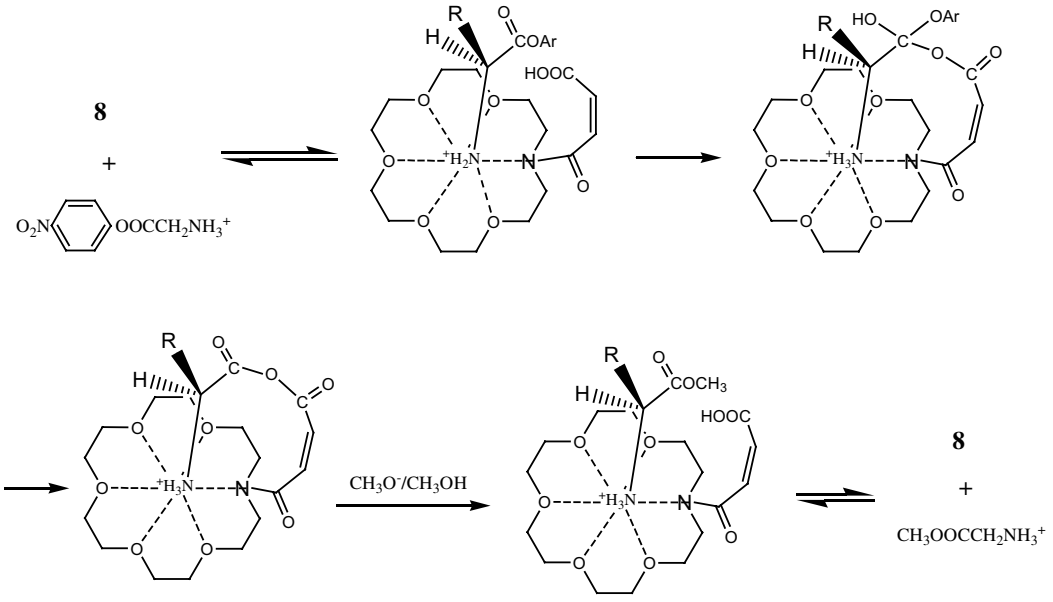
We supposed a mechanism (Scheme 6) for the catalytic solvolysis of the substrates under host compounds according to the literature[3,10,11] and our experimental results. It is expected to explain all experimental phenomena.

2.1. Effect of the length of side arms of artificial enzymes on catalysis

From the center oxygen atom between two carbonyls of the intermediate there are two paths by which we can get to the oxygen or nitrogen atoms of the crown ether



Scheme 5. Intramolecular nucleophilic catalysis of maleamic acid.



Scheme 6. Nucleophilic mechanism under the participation of carboxyl of **8**.

rings. The substrate path includes five all kinds of chemical bonds and the side arm (of the crown ethers) path also includes five chemical bonds when the side arms include four carbon atoms. It will benefit the stability of the intermediate when the length of the two paths match each other well. Compounds **2**, **3**, **4** and **7**, **8**, **9**, including four carbon atoms in their side arms, can catalyze the reaction more remarkably than other compounds. The paths of the side arms (of crown ethers) will include six chemical bonds when compounds **5** and **11** act on the substrates. One bond being longer than the optimal length of the paths makes the latter two compounds **5** and **11** not so effective catalysts like **2**, **3**, **4** and **7**, **8**, **9**. The existence of an optimal length of the side arms offers the most unmistakable evidence for the existence of an anhydride intermediate.

2.2. Effect of rigidness of side arms of artificial enzymes on catalysis

For obvious reasons compounds **4** and **9** are two most effective catalysts. The more rigid the side arms are, the more easily they are directed to the carbonyls of substrates. Compound **4** with the most rigid side arm leads to substrate solvolysis with the highest efficiency increase. But the sequence of catalytic efficiency of compounds **2** and **3** are not like their 18-membered ring counterparts. The reason may be related to the way of complexing. Further work needs to be done to explain this phenomenon. Compound **10**, the isomer of **9**, shows little catalytic activity toward the deacylation of glycine *p*-nitrophenyl ester hydrobromide. The reason is that carboxyl of **10** is substituted at the 4-position and deviates from the center of the crown ether ring. It is difficult for the carboxyl of **10** to participate in the catalytic process like that of compound **9**.

2.3. Effect of substrates on catalysis

Glycine *p*-nitrophenyl ester hydrobromide has a smaller R group (Scheme 6) than valine *p*-nitrophenyl ester hydrobromide. A bigger R group means a greater spacial resistance with the side arms of the crown ethers. So valine *p*-nitrophenyl ester hydrobromide is not so easily catalyzed as glycine *p*-nitrophenyl ester hydrobromide. The anions of substrate salts show little effect on the rate of deacylation because the concentration of crown ethers is 100-fold that of substrates and almost all the substrates form complexes. Anyway the anions of substrates hardly affect the concentration of the complexes. The influences of anions and other factors on complexes of primary amine cations and crown ethers have been

studied by Cram systematically.[12] The equilibrium constants of the complexes decrease in the order perchlorate > thiocyanate ~ picrate.

2.4. Effect of ring size of crown ethers on the catalysis

The effect of ring size on rate constants may be more complicated than other factors. We notice that compounds with 18-membered rings have a higher catalytic efficiency than the corresponding ones with 15-membered rings, respectively. We think the way of interaction of substrates and crown ethers will be responsible for this. The crown ether rings and the substrate cations can form complexes having both *cis*- and *trans*-relationships between the side arms and the substrates. Maybe the concentration of *cis*-complexes of 18-membered series compounds is higher than that of 15-membered series because only the *cis*-complexes are useful for catalysis. Sutherland and co-workers studied[13] the complexes between N-methyl aza crown ethers and primary amine cations by ^{1}H NMR, which may help us to understand the analogous process. He pointed out that N-methyl aza 15-crown-5 could form complexes in which the primary alkylammonium cation had a *cis*-relation to the N-methyl group, while N-methyl aza 18-crown-6 could form an equilibrium mixture of complexes of both *cis* and *trans* in ca. 1:1 ratio. The circumstance of N-carbonyl aza crown ethers may be contrary to that of N-methyl aza crown ethers when forming complexes with primary amine cations. Further work needs to be done to support this point.

2.5. Inhibition experiment

In our research, the concentration of the crown ethers is much higher than that of substrates. So it is questionable whether substrate binding is actually involved in the catalysis since all experiments were performed at high concentration of catalysts. To eliminate this worry and to illustrate the importance of substrate binding, an inhibition experiment was performed with the best catalyst **9**. Glycine hydrochloride was used as inhibitor since it had the same primary amine cation as glycine *p*-nitrophenyl ester hydrochloride while it would not release detectable *p*-nitrophenol at 320 nm. The results in Table 1 (runs 14–16) showed that equimolecular glycine hydrochloride inhibited the catalysis significantly. Most rings of **9** were occupied by primary amine cations of glycine hydrochloride and almost no binding site was offered to that of the substrate. Of course there was still little release of *p*-nitrophenol since the binding between **9** and substrate or inhibitor was in dynamic equilibrium. The inhibition experiment and the fact that *p*-nitrophenyl acetate could not be catalyzed suggested actual binding of the catalysts and substrates.

2.6. pH dependence of the kinetics

The dominant acids or bases in our kinetic system are exclusively the 0.01 M host compounds most of which include a carboxyl group in their molecules. The system can be buffered by itself and the product *p*-nitrophenol can be detected as a fixed unionized form. The difference of pH value of each kinetic run lies in the difference of pK_a between aromatic acid hosts and saturated acid hosts, such as the difference of pK_a between host **9** and host **7** (about 0.6 pK_a unit in water solution). We can approximately calculate the pH values of the systems including 0.01 M of host **9** and host **7** provided they are in water:

$$pH_9 \approx -1/2\ \lg\ (Ka_9 \times c) = -0.5 \times \lg(10^{-4.2} \times 0.01) = 3.36$$

$$pH_7 \approx -1/2\ \lg\ (Ka_7 \times c) = -0.5 \times \lg(10^{-4.8} \times 0.01) = 3.40$$

The pH values of the reaction medium are almost fixed if we can ignore the difference. However, we still think the measurement of the pH dependence of the kinetic data is very necessary for a selected host since we proposed a nucleophilic mechanism. The carboxylate anions of compounds **2**–**11** should have a much greater nucleophilicity compared with their respective unionized carboxyl species according to the proposed nuleophilic mechanism. Of **2**–**11** representative aromatic acid **9** and saturated acid **7** were tested in pH-controlled medium in which $Et_4N^+OAc^-$/AcOH was used as buffer. The rate constants were calculated by the half time method and the influence of buffer was deducted. We observed a weak pH dependence of kinetic data (Fig. 1). The rate constants increased very tardily with the increasing of pH of the medium, which ranged from pH 3.2 to 6.4. The reason, we think, can be attributed to two aspects: (1) the concentration of the crown ether acid is much higher than that of the substrate, so only very few ionized carboxyl groups are enough for cleaving ester bonds of the substrate. (2) The hydrogen atoms of the buffer can compete with the substrate to form hydrogen bonds with the crown ether, which decreases the combination of the substrate and the crown ether. Koga and co-workers[14] used mercapto crown ether

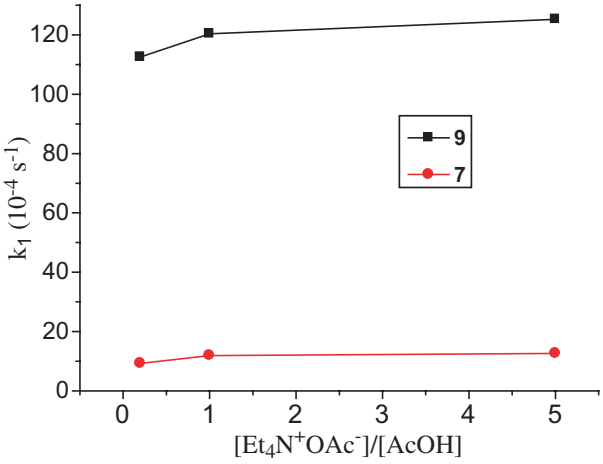


Figure 1. pH dependence of kinetic data of an aromatic acid **9** and a saturated acid **7**.

compounds as catalysts with the same substrate and their kinetic studies were performed only under one fixed pH medium (0.01 M AcOH, 0.02 M pyridine, pH 5.4 in water). We think there was also a weak pH dependence of kinetic data for the same reason although the active form of the nucleophile was the ionized mercapto group. Anyway the weak pH dependence of kinetic data still offers certain evidence for the nucleophilic mechanism (Scheme 6).

The catalytic process can be summarized as follows: The crown ether compounds first form complexes with the substrates with primary amine cations. *P*-Nitrophenyl acetate has no primary amine cation in its molecule and so cannot be catalyzed by title compounds. Only those with a rigid and proper length of carboxyl arms can catalyze the reaction effectively. The position of the carboxyl and the R groups of substrates (Scheme 6) also have a great effect on the catalytic process. The intermediates are attacked by methanol in solvent to get products and original hosts.

3. Conclusion

In summary, the series of crown ether compounds with carboxyl groups selectively recognized substrates and some of them showed catalytic activity against the deacylation of amino acid *p*-nitrophenyl ester hydrohalides.

Each active site of aspartic proteinases includes an ionized carboxyl and a protonated carboxyl because of their different microenvironment. Both in the nucleophilic mechanism and in the general-acid/general-base mechanism the task of the protonated carboxyl is to offer a proton to assist the leaving of the hydrolytic product. The action of the ionized carboxyl makes the difference of the two catalytic mechanisms. In the nucleophilic mechanism the ionized carboxyl attacked the carbonyl of peptides and the protonated one offered a proton to the nitrogen atom to help it to leave (Scheme 1).

There is at the most one carboxyl in each molecule of our series compounds and they can be regarded as partial artificial enzymes of aspartic proteinases. A possible nucleophile is methanol in the medium. But the methanol is free in the reactive system, not like the water molecule fixed by two carboxyls of the active site of aspartic proteinases (Scheme 2). The exclusive carboxyl as nucleophile alone attacks the carbonyl of substrates to get products without the help of another carboxyl. This seems to explain the relatively low catalytic efficiency of the artificial enzymes. It is impossible for carboxyl, the exclusive catalytic element in each molecule, to catalyze the reaction as a general acid or general base. The relationship of structures and deacylation activities of host compounds suggest the existence of an anhydride intermediate. The results support the nucleophilic mechanism of aspartic proteinases experimentally.

If there is a second carboxyl in an artificial aspartic proteinase we think a higher catalytic efficiency can be obtained. The experimental results and the mechanism of aspartic proteinases also tell us that some artificial aspartic proteinases with two carboxyls as catalytic elements need to be developed. Good artificial aspartic proteinases or proper inhibitors of aspartic proteinases may, in the future, be used as drugs for diseases related to Apases.

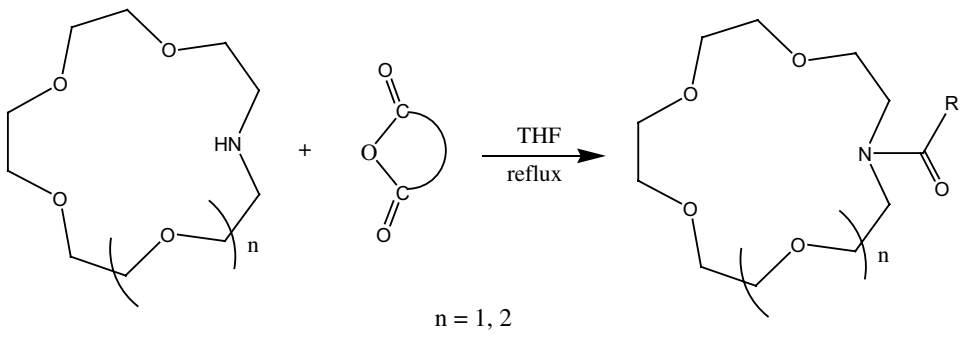
4. Experimental

4.1. Materials and methods

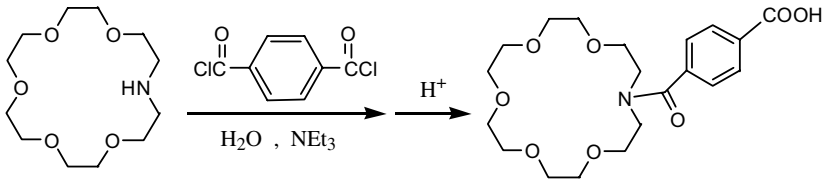
Proton nuclear magnetic resonance (^{1}H NMR) was recorded using Bruker 400 in $CDCl_3$. Chemical shifts are reported as δ values in parts per million from tetramethylsilane as internal standard. Mass spectroscopy was performed with TRIO 2000. HSMS were performed with Bruker Daltonics. Kinetic measurements were operated on a UV–vis spectrometer of Hitachi U-3010. The release of *p*-nitrophenol was monitored at 320 nm. All solvents were pretreated just before use.

4.2. Synthesis

Aza-18-crown-6 and aza-15-crown-5 were purchased from Acros. Compounds **1**–**9** and **11** were prepared from aza-18-crown-6 or aza-15-crown-5 and corresponding cyclic and linear (only acetic anhydride for **1**) anhydrides with yields of about 90% (Scheme 7). Compound **10** was prepared from aza-18-crown-6 and terephthaloyl chloride in water in the presence of triethyl amine (Scheme 8).



Scheme 7. Synthesis of compounds **1**–**9**, **11**.



5 40%

Scheme 8. Synthetic route of compounds **10**.

4.2.1. *N*-(*o*-Carboxy) benzoyl-1,4,7,10-tetraoxa-13-azacyclopenta-decane (4). A solution of aza 15-crown-5 (0.4818 g, 2.2 mM) and phthalic anhydride (0.3256 g, 2.0 mM) in 50 mL of dry tetrahydrofuran was refluxed for 3 h. After TLC experiments showed there was a new main product formed in the reactive solution, the solvent was evaporated under reduced pressure and the residue was dissolved in 100 mL of chloroform. The organic solution was extracted with about 10 mL of 1.0 M hydrochloride and dried with anhydrous magnesium sulfate overnight. After removal of solvent, pure product **6** was obtained as a colorless thick oil (90%). ^{1}H NMR ($CDCl_3$): δ (ppm) 10.5–10.3 (broad, –COOH), 3.28–3.90 (m, 20H), 7.31 (q, 1H), 7.45 (m, 1H), 7.57 (m, 1H), 8.08 (q, 1H); MS *m/z* (relative intensity) 368 ($M+H^+$, 6.43), 236 (2.12), 232 (4.50), 220 (34.58), 188 (13.67), 162 (15.42), 74 (100). High-resolution MS: calcd for $(M-H)^-$ $C_{18}H_{24}O_7N$, *m/e* 366.1558; found, 366.1558.

4.2.2. *N*-Acetyl-1,4,7,10-pentaoxa-13-azacyclopentadecane (1). This compound was prepared, using a method similar to that used for **4**, from aza 15-crown-5 (0.4818 g, 2.2 mM) and acetic anhydride (2.0 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 3.52–3.84 (m, 20H), 2.05 (s, 3H); MS *m/z* (relative intensity) 262 ($M+H^+$, 100), 218 (6.87), 200 (2.10), 188 (2.59), 174 (5.73); High-resolution MS: calcd for $(M+H)^+$ $C_{12}H_{24}O_5N$, *m/e* 262.1649; found, 262.1646.

4.2.3. *N*-Carboxypropanoyl-1,4,7,10-pentaoxa-13-azacyclooctadecane (2). This compound was prepared, using a method similar to that used for **4**, from aza 15-crown-5 (0.4818 g, 2.2 mM) and succinic anhydride (0.2000 g, 2.0 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 8–11 (broad, COOH), 3.58–3.88 (m, 20H), 2.75 (t, 2H), 2.70 (t, 2H); MS *m/z* (relative intensity) 320 ($M+H^+$, 100), 302 (M−OH, 14.81), 276 (M−43, 4.51), 232 (3.52), 220 (13.21), 188 (7.23); High-resolution MS: calcd for $(M-H)^-$ $C_{14}H_{24}O_7N$, *m/e* 318.1558; found, 318.1552.

4.2.4. *N*-Carboxyacryl-1,4,7,10-pentaoxa-13-azacyclopentadecane (3). This compound was prepared, using a method similar to that used for **6**, from aza 15-crown-5 (0.4818 g, 2.2 mM) and maleic anhydride (0.1960 g, 2.0 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 13–17 (broad, COOH), 6.38 (d, 1H), 6.84 (d, 1H), 3.60–3.92 (m, 20H); MS *m/z* (relative intensity) 318 ($M+H^+$, 4.01), 274 (2.23), 220 (7.97), 188 (10.06), 176 (5.75), 56 (100); High-resolution MS: calcd for $(M-H)^-$ $C_{14}H_{22}O_7N$, *m/e* 316.1402; found, 316.1398.

4.2.5. *N*-Carboxybutanoyl-1,4,7,10-tetraoxa-13-azacyclopentadecane (5). This compound was prepared, using a method similar to that used for **6**, from aza 15-crown-5 (0.4818 g, 2.2 mM) and pentanedioic anhydride (0.2280 g, 2.0 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 7.5–9 (broad, COOH), 3.53–3.85 (m, 20H), 2.52 (t, 2H), 2.46 (t, 2H), 2.00 (m, 2H); MS *m/z* (relative intensity) 334 ($M+H^+$, 100), 316 (M−OH, 8.25), 290 (3.75), 246 (2.74), 220 (8.25); High-resolution MS: calcd for $(M-H)^-$ $C_{15}H_{25}O_7N$, *m/e* 332.1715; found, 332.1710.

4.2.6. *N*-Acetyl-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (6). This compound was prepared, using a method similar to that used for **4**, from aza 18-crown-6 (0.5786 g, 2.2 mM) and acetic anhydride. ^{1}H NMR ($CDCl_3$): δ (ppm) 3.55–3.72 (m, 24H), 2.12 (s, 3H); MS *m/z* (relative intensity) 306 (M+H, 45.95), 262 (M−43, 21.79), 244 (2.83), 232 (5.28), 218(6.97), 202 (5.45), 86 (100).

4.2.7. *N*-Carboxypropanoyl-1,4,7,10,13-pentaoxa-16-azacycloocta-decane (7). This compound was prepared, using a method similar to that used for **4**, from aza 18-crown-6 (0.5786 g, 2.2 mM) and succinic anhydride. ^{1}H NMR ($CDCl_3$): δ (ppm) 8–11(broad, COOH), 2.68 (t, 2H, CH_2CON), 2.77 (t, 2H, CH_2COOH), 3.55–3.85 (m, 24H); MS *m/z* (relative intensity) 365 (M+2H, 64.43), 347 (6.15), 320 (15.32), 302 (3.95), 288 (4.40), 276 (6.62), 264 (71.15), 144 (100); High-resolution MS: calcd for $(M-H)^-$ $C_{16}H_{28}O_8N$, *m/e* 362.1820; found, 362.1813.

4.2.8. *N*-Carboxyacryl-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (8). This compound was prepared, using a method similar to that used for **4**, from aza 18-crown-6 (0.5786 g, 2.2 mM) and maleic anhydride (2.2 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 13–17 (broad, COOH), 3.55–3.90 (m, 24H), 6.35 (d, 2H), 6.88 (d, 2H); MS *m/z* (relative intensity) 361 (M, 1.12), 344 (M−OH, 64.43), 55 (100).

4.2.9. *N*-(*o*-Carboxy) benzoyl-1,4,7,10,13-pentaoxa-16-azacycloocta-decane (9). This compound was prepared, using a method similar to that used for **4**, from aza 18-crown-6 (0.5786 g, 1.90 mM) and phthalic anhydride (2.2 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 10.5–10.3 (broad, COOH), 3.55–3.90 (m, 24H), 7.30 (d, 1H), 7.45 (t, 1H), 7.56 (t, 1H), 8.07 (d, 1H); MS *m/z* (relative intensity) 412 (M+H, 2.22), 264 (100).

4.2.10. *N*-Carboxybutanoyl-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (11). This compound was prepared,

using a method similar to that used for **4**, from aza 18-crown-6 (0.5786 g, 2.2 mM) and pentanedioic anhydride (2.0 mM). ^{1}H NMR ($CDCl_3$): δ (ppm) 9–10 (broad, COOH), 3.40–3.90 (m, 24H), 2.42 (t, 2H), 2.37 (t, 2H), 1.90 (m, 2H); MS *m/z* (relative intensity) 378 ($M+H^+$, 66.17), 360 (M−OH, 5.55), 334 (M−43, 26.88), 320 (4.70), 302 (2.22), 290 (4.14), 272 (5.17), 262 (9.73), 246 (8.22), 218 (8.36); High-resolution MS: calcd for $(M-H)^-$ $C_{17}H_{30}O_8N$, *m/e* 376.1978; found, 376.1975.

4.2.11. *N*-(*p*-Carboxy)benzoyl-1,4,7,10,13-pentaoxa-16-azacycloocta-decane (10). Compound **10** was prepared from aza-18-crown-6 and terephthaloyl chlorides (Scheme 8). Terephthaloyl chloride (3.82 g, 0.019 M) and 3 mL of triethyl amine were added into a solution of aza 18-crown-6 (0.50 g, 1.90 mM) of 500 mL of water. The solution was agitated for 6 h at room temperature and then filtered. Concentrated hydrochloride was added to the clear water solution, then a great amount of precipitate formed. On removal of solid by filtration, a clear solution was obtained. The water phase was extracted with chloroform twice and we then combined the organic phase and dried with anhydrous magnesium sulfate overnight. On removal of the solvent, compound **5** (40%) was obtained as a colorless thick oil. ^{1}H NMR ($CDCl_3$): δ (ppm) 9.6 (broad, COOH), 3.45–3.95 (m, 24H), 7.51 (d, 2H), 8.09 (d, 2H); MS *m/z* (relative intensity) 412 (M+H, 100), 394 (8.96), 368 (9.70), 324 (7.18), 307 (3.26), 279 (3.08), 262 (5.34). High-resolution MS: calcd for $(M-H)^-$ $C_{20}H_{28}O_8N$, *m/e* 410.1820; found, 410.1812.

4.2.12. Amino acid *p*-nitrophenyl ester hydrohalides. The substrates were prepared according to the published literature.[12] The data of the compounds were coincident to those of the literature.

4.3. Kinetics

Spectral-grade solvents were used. Each run of deacylation was initiated by adding 0.020 mL of 1.0×10^{-2} M amino acid *p*-nitrophenyl ester hydrohalides in EtOH to 2.0 mL of a reaction medium in a cell thermostated at 25.0 °C. The reaction mixtures were quickly stirred and the rates of *p*-nitrophenol liberation were followed at 320 nm with a Hitachi UV spectrometer model 3010. The pseudo-first-order rate constants were calculated by using Eq. 1,

$$k_{obs} = \ln 2/t_{1/2} \quad (1)$$

Over three kinetic measurements were made and averaged to determine k_{obs} values. Rate constants, k values listed in Table 1, were calculated by using Eq. 2,

$$k = k_{obs} - k_0 \quad (2)$$

in which k_0 is the first-order rate constant of each substrate in 5% CH_3OH–CH_2Cl_2 alone.

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References and notes

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