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Design, synthesis and catalytic activity of a serine protease synthetic model

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SUMMARY

The design, synthesis and catalytic properties of a cyclic branched peptide carrier that possesses the catalytic triad residues of the serine proteases is reported. The synthesis of the peptide model was totally completed on solid support using three different orthogonal amino protecting groups. Hydrolytic activity measurements against Suc-Ala-Ala-Ala-pNA substrate showed that it is hydrolysed by the peptide model to a small extent. Despite this small hydrolytic activity, it is the first time, to our knowledge, that hydrolysis of such a substrate is reported by an enzyme model compound. Contrary, this enzyme model peptide showed considerable activity against the Boc-Ala-pNP substrate (k_{cat} = 0.414 min^{-1} and K_m = 0.228 mm). These results suggest that the designed carrier brings in appropriate contact the catalytic triad residues (Ser, His, Asp) resulting in the obtained hydrolytic activity.

INTRODUCTION

Serine proteases are a large class of proteolytic enzymes found in many organisms [1] and they are believed to play an important role in many biochemical processes, like protein catabolism, digesting, blood pressure regulation etc. Beyond their biological functions in vivo, the last decades they have gained great attention for in vitro applications like organic reaction catalysts [2,3] and biotechnological usage in biomass degradation [4]. Serine proteases catalyse the cleavage of a peptide bond in two steps. In the first step, acylation of the reactive serine takes place to form the acyl-enzyme intermediate and in the second one deacylation of serine and release of products occurs. Both steps involve a nucleophilic attack on a carbonyl group and the formation of a negative charge. Rate acceleration of the proteolytic process derives from the charge stabilisation, accomplished by two backbone NH groups in the chymotrypsin family, and one NH group and one carboxamide group in the subtilisin family of serine proteases [5]. All necessary residues for catalysis constitute the active site of the enzyme,

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Abbreviations: Boc, *t*-butyloxycarbonyl; Alloc, allyloxycarbonyl; Bzl, benzyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Dnp, dinitrophenyl; Fmoc, fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; MD, Molecular Dynamics; NMP, *N*-methylpyrrolidone; pMBHA, 4-methylbenzhydrylamine resin; pNA, 4-nitroanilide; pNP 4-nitrophenyl; RP-HPLC, reversed-phase high-performance liquid chromatography; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate; TFA, trifluoroacetic acid.

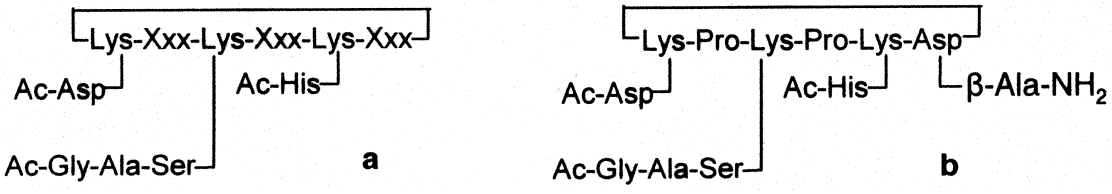

Fig. 1. The initial starting peptide model (a) where Xaa = Pro, Ala, Gly and the peptide model selected for synthesis after molecular modeling studies (b).

while residues responsible for the substrate binding and specificity constitute the binding site of the enzyme. This is the so-called two site approximation for enzyme function, which explains many features of the enzymatic reactions; however it cannot be fully generalised, for example allosteric control of enzymes cannot be interpreted by the above simplification. Although several serine protease mimicking compounds have been reported [6–8], the question of modelisation and synthesis of effective organic catalysts which take advantage of the structure and mechanism of reaction of enzymes remains under dispute [9–11] and still biochemists are not familiar with the idea of using enzyme models [12] instead of their originates.

We present here a new molecule (Fig. 1a) designed to mimic the serine proteases active site, based on the attachment of all the active site residues to a cyclic peptide carrier. It possesses the catalytic triad residues: Asp, His and Ser and a Gly residue to mimic the oxyanion hole [5,13]. The aspartic residue found in natural enzymes between Gly and Ser was replaced by Ala, since it does not participate in the hydrolytic process. At this stage in our study we omitted the modelization of the binding site and its inclusion in the proposed model, despite its obvious significance in catalysis. Our attention is focused on the construction of a leader compound with serine protease-like activity, without further specificity. The starting peptide model, shown in Fig. 1a, comprises a cyclic hexapeptide-carrier on which the active site residues can be attached to the Lys-$N^{\varepsilon}H$ groups. The main restriction taken in consideration was that the expected conformation of the model should bring in close proximity the Ser, His and Asp residues, so that the distances between them would be relatively similar to those found in crystal structures of serine proteases.

MATERIALS AND METHODS

Amino acid derivatives were purchased from Bachem. Solvents were obtained from Merck and Fluka. DMF, pyridine and DIEA were purified by standard protocols [14]. Fmoc-Asp-OAllyl was synthesized from Fmoc-Asp(tBu)-OH (Bachem) as described in the literature [15]. Alloc-Lys(Fmoc)-OH was synthesized by reaction of H-Lys(Fmoc)-OH (Bachem) with allyl chloroformate [16] in 1,4-dioxane/10% Na_2CO_3 for 30 min in an ice-water bath. After extraction workup, the residue was taken up in ethyl acetate. The solution was dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue crystallized upon treatment with ethyl acetate/hexane. The product was homogeneous in TLC and gave the expected ^{1}H-NMR spectrum. Suc-Ala-Ala-Ala-pNA and Boc-Ala-pNP (Sigma) were used as substrates.

Computational procedure

Molecular modeling studies were performed using the InsightII/Discover (Biosym, San Diego, CA) package on an SGI R4000 workstation. The initial structures were built in the Biopolymer and Build modules of InsightII, with all amide groups, both backbone and Lys side chains, in the *trans* configuration. The aspartate residue had an ionized side chain, making the total charge of the molecule –1. All calculations were performed with the cvff force field. A linear distance dependent dielectric constant $1{\cdot}r$ was applied to all simulations. In order to find the residues that best support the desired geometry we tested with molecular simulations techniques the Ala, Gly, and Pro residues in the place of Xaa (Fig. 1a). Proline was chosen for its known tendency to form turns, thus facilitating cyclisation, Gly was chosen for its flexibility, and Ala as a medium sized

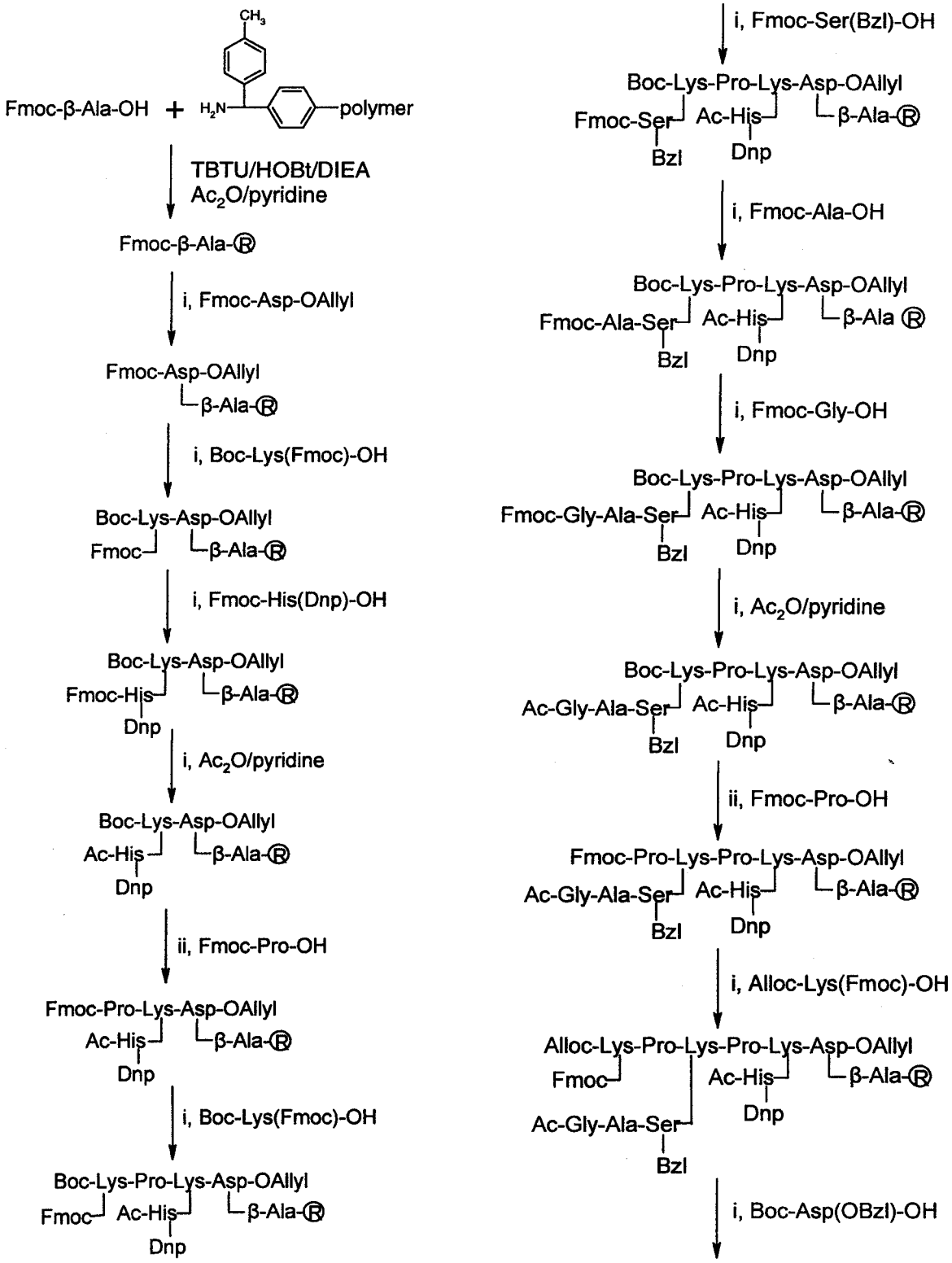

Fig. 2. The synthetic scheme followed to prepare the peptide model. Fmoc, *t*-Boc and Alloc/Allyl deprotections are denoted by i, ii and iii, respectively.

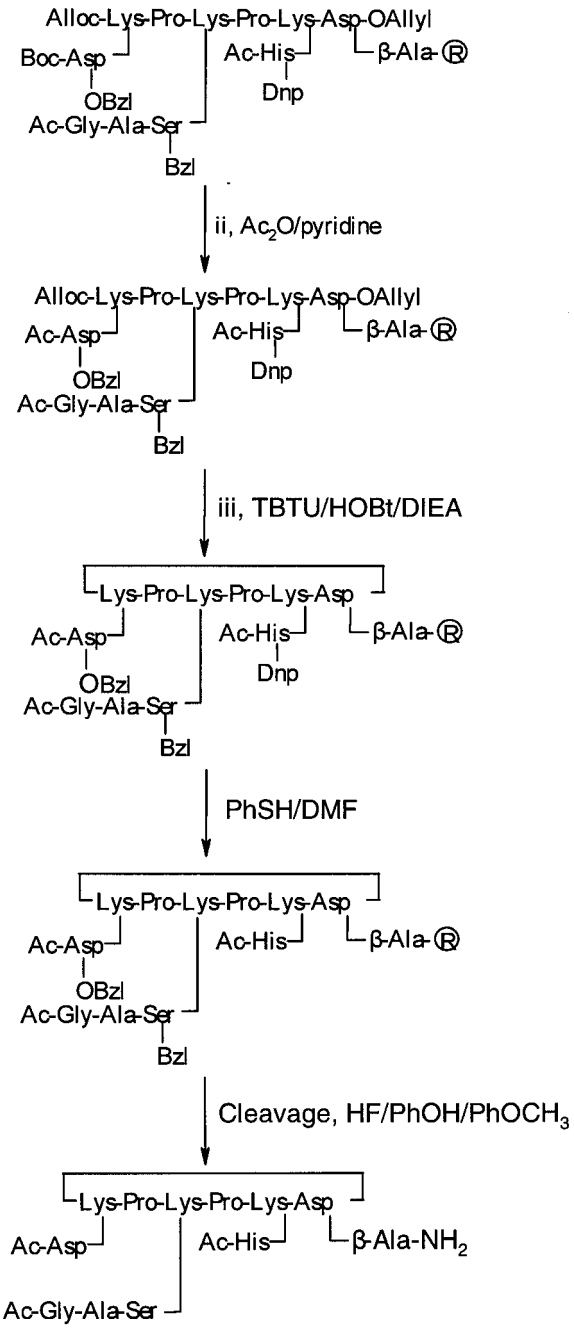

Fig. 2. (continued).

non functional residue. Short time molecular dynamics (100 ps) were performed and the molecule that best resembled the active geometry was selected, that was the one containing Pro in place of Xaa. In order to facilitate the synthesis of the molecule we replaced one proline by aspartic acid having a β-alanine conjugated on its side chain (Fig. 1b). The resulting molecule was then energy minimized with conjugate gradients until the maximum derivative was less than 0.1 kcal mol^{-1} $Å^{-1}$ and subjected to a 1000 ps molecular dynamics (time step 1 fs) run at 900 K with 20 ps equilibration period. Structures were stored every 1 ps resulting in a trajectory of 1000 frames. Each structure was energy minimized with conjugate gradients until the maximum derivative was less than 0.1 kcal mol^{-1} $Å^{-1}$. For the final analysis only the last 500 frames were used. This minimized trajectory contained several stable conformers, and some of them showed significant interaction of the three active residues.

Peptide synthesis

The synthesis of the peptide started by anchoring Fmoc-β-Ala-OH on 5 g of pMBHA resin (0.13 mmol g^{-1}, substitution estimated by measuring the Fmoc-piperidine adduct at 301 nm, ε = 7800, after Fmoc removal with piperidine in DMF in small weighed aliquots of the peptide resin), followed by acetylation of the unreacted amine groups. A low substitution level of the resin was desired in order to facilitate the last cyclization step, while β-Ala was included to avoid sterical hindrance of the α-carboxylate group of the Asp with the benzene rings at the cyclization step. Fig. 2 describes the step by step synthetic procedure of the final peptide model. Amino acid derivatives were preactivated before the addition in the reaction vessel, 3 equiv of properly protected amino acid and 3 equiv of HOBt were dissolved in 15 ml of DMF/NMP (1:1, v/v) solution, 3 equiv of TBTU [17] was added to the mixture under magnetic stirring and activation was completed with the addition of 6 equiv of DIEA. The mixture was stirred for 10 min and added to the resin. Couplings were performed for 30–120 min, and repeated if necessary. Fmoc deprotection was carried out with 40% piperidine in DMF, while *t*-Boc deprotection was performed with 40% TFA in DCM followed by neutralisation with 10% DIEA in DCM. Alloc and allyl groups were removed simultaneously

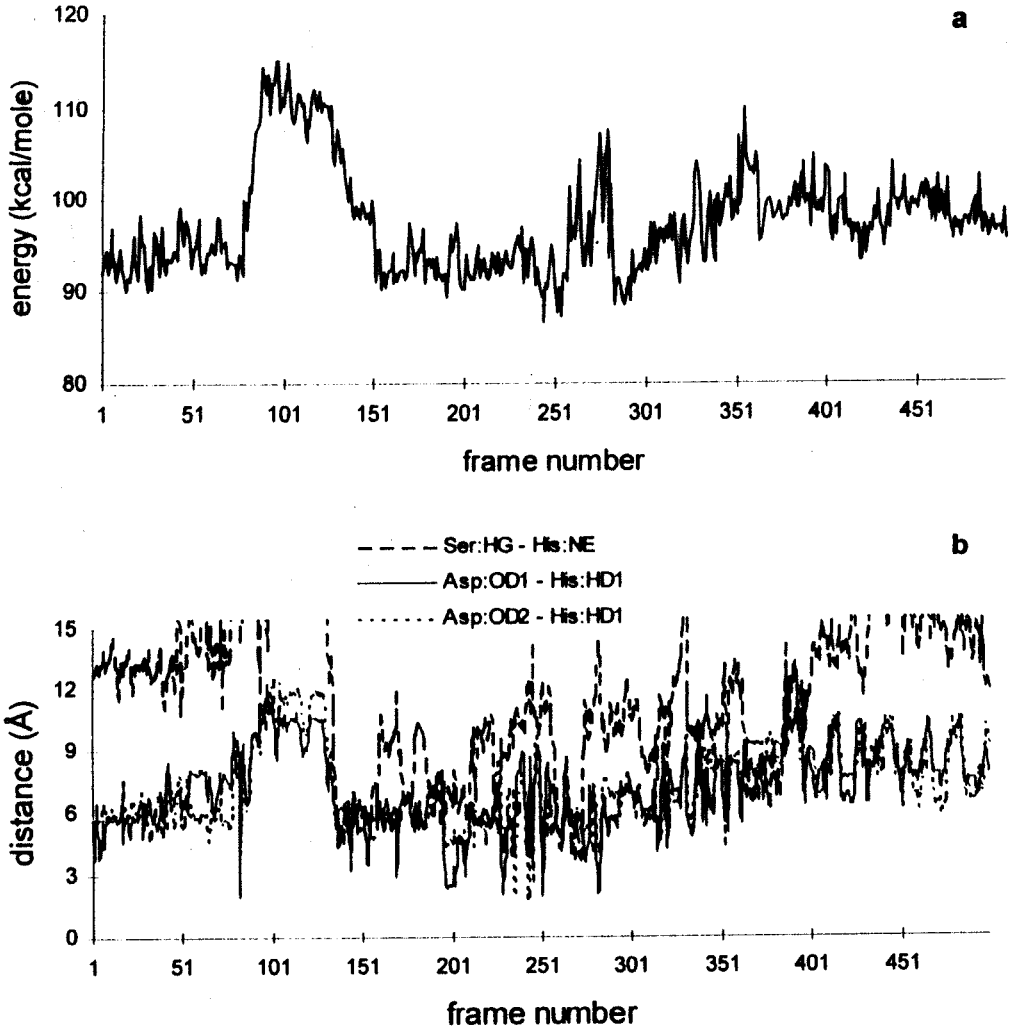

Fig. 3. Energy evalution of the last 500 frames derived from energy minimization of the high temperature MD trajectory (a) and distance variations between the active site residues in the same structures (b).

with $PdCl_2(PPh_3)_2$/Bu_3SnH/AcOH (0.04/3/3 per allyl group in DMF, 2×30 min). Cyclization was performed in the presence of TBTU/HOBt/DIEA (equimolar quantities to carboxylate) overnight. All steps were ensured by the ninhydrin test. After cyclization the peptide-resin was treated with thiophenol in DMF to remove the Dnp protecting group of the histidine side chain. Deprotection and cleavage of the peptide from the resin was achieved by anhydrous HF treatment in the presence of phenol and anisole as scavengers. The crude peptide was purified by preparative RP-HPLC on a C_{18} column applying a linear gradient of 20–60% B in 40 min, A = 0.1% TFA in water, B = 0.1% TFA in methanol. The overall yield of the HPLC purified product was ca. 14%. Amino acid analysis of the purified product gave: Lys 2.58 (3), Pro 1.67 (2), Asx 2.00 (2), Ala 1.00 (1), Gly 1.25 (1), His 1.08 (1), Ser 0.42 (1). In addition, mass spectrometric analysis showed the correct molecular mass (M^+ calculated: 1357.5; found:1355 by MALDI-TOF and M^+ found: 1357 by FAB/MS).

Kinetic studies

Hydrolytic activity of the peptide model was followed on a Shimadzu UV2100 spectrometer using 1 ml assays. A constant concentration of the peptide model of 200 μM was used to hydrolyse various concentrations of the Suc-Ala-Ala-Ala-pNA (410 nm) and Boc-Ala-pNP (400 nm) substrates in 0.1 m Tris and 0.5 m NaCl buffer adjusted to pH 7.5 at 25 °C. The product release was followed by the progress curve method. Buffer hydrolysis of the substrates was directly subtracted by placing in the reference cuvette the same substrate concentration. Data were analysed with the adapted Shimadzu software to calculate the initial slopes within the first 5 min. Experiments were performed in doublicates. Eadie–Hofstee plots were used to derive K_m and V_{max}.

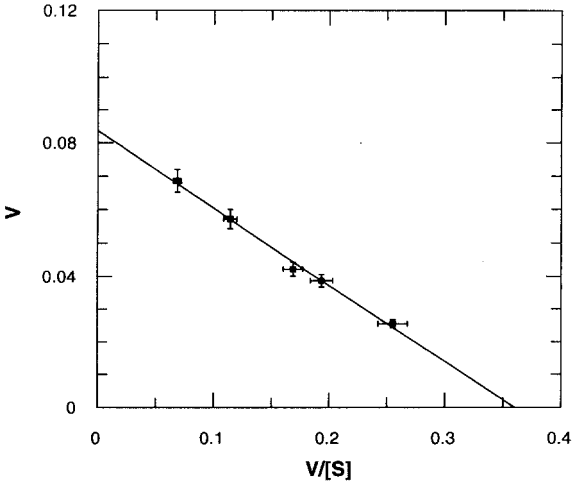

Fig. 4. Eadie–Hofstee plot (V in mM min^{-1} against V/[S] in min^{-1}) of the Boc-Ala-pNP hydrolysis from the peptide model.

RESULTS AND DISCUSSION

Figures 3a and b show the energy evaluation of the minimized structures and the distances between the active site residues, respectively. In several low energy conformations the His and Asp side chains are in close contact (the corresponding distance of heavy atoms in the crystal structure [18] – PDB entry code 5cha – is 2.67 Å), while the hydroxyl group of the Ser residue shows less preference for interacting with imidazole (the corresponding distance of heavy atoms in the crystal structure is 3.01 Å). It is interesting to note that the very high energy frames correspond to conformers without interactions between Ser, His and Asp side chains. These results prompted us to synthesize the molecule.

The hydrolytic activity of the enzyme model compound was followed using the Suc-Ala-Ala-Ala-pNA and Boc-Ala-pNP substrates. The model peptide showed little activity against Suc-Ala-Ala-Ala-pNA ($\Delta A = \sim 0.07$ in 24 h). Despite the small product release using this compound, it is the first time that an anilide bond [CONH-(4-NO_2)-Ph], comparable to a peptide bond, is hydrolysed by an enzyme model. The achieved hydrolytic activity of the constructed compound indicates that the designed molecule mimicks sufficiently the serine protease active site. Taking into account that the binding site of the enzyme was not included in our model compound, as well as the fact that the Ser side-chain showed a limited preference for interacting with the His side-chain, we could explain the small hydrolysis of the Suc-Ala-Ala-Ala-pNA. Obviously, this model compound can be a starting molecule for further improvement.

Using the Boc-Ala-pNP substrate, the kinetic constants of the enzymatic model were estimated k_m = 0.492±0.011 mM^{-1} and V_{max} = 82.9 ± 2.0 μmol min^{-1} (Fig. 4) and k_{cat} = 0.414 min^{-1}. This substantial hydrolytic activity further supports that the designed molecule is a good starting compound towards an effective enzymatic model. ^{1}H-NMR and ^{13}C-NMR studies are now in progress for the structural refinement of our enzymatic model for designing more potent compounds.

CONCLUSIONS

Molecular modeling was used successfully for the design of a new synthetic carrier that incorporates the catalytic triad residues of serine proteases. Also, it is the first time that a branched homothetic cyclic peptide was totally synthesized on a solid support using three different orthogonal amino protecting groups. It is concluded that the concept of our branched peptide carrier can be successfully used for

the development of enzymatic active site mimicking compounds.

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