Catalytic Hydrolysis of Phosphate Monoester by Supramolecular Complexes Formed by the Self-Assembly of a Hydrophobic Bis(Zn\(^{2+}\)-cyclen) Complex, Copper, and Barbital Units That Are Functionalized with Amino Acids in a Two-Phase Solvent System

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Abstract: We previously reported on the preparation of supramolecular complexes by the 2:2:2 assembly of a dinuclear Zn\(^{2+}\)-cyclen (cyclen = 1,4,7,10-tetraazacyclododecane) complex having a 2,2\(^{\prime}\)-bipyridyl linker equipped with 0–2 long alkyl chains (Zn\(_2\)L\(_1\)–Zn\(_2\)L\(_3\)), 5,5-diethylbarbituric acid (Bar) derivatives, and a copper(II) ion (Cu\(^{2+}\)) in aqueous solution and two-phase solvent systems and their phosphatase activities for the hydrolysis of mono(4-nitrophenyl) phosphate (MNP). These supermolecules contain Cu\(_2\)(µ-OH)\(_2\) core that mimics the active site of alkaline phosphatase (AP), and one of the ethyl groups of the barbital moiety is located in close proximity to the Cu\(_2\)(µ-OH)\(_2\) core. The generally accepted knowledge that the amino acids around the metal center in the active site of AP play important roles in its hydrolytic activity inspired us to modify the side chain of Bar with various functional groups in an attempt to mimic the active site of AP in the artificial system, especially in two-phase solvent system. In this paper, we report on the design and synthesis of new supramolecular complexes that are prepared by the combined use of bis(Zn\(^{2+}\)-cyclen) complexes (Zn\(_2\)L\(_1\), Zn\(_2\)L\(_2\), and Zn\(_2\)L\(_3\)), Cu\(^{2+}\), and Bar derivatives containing amino acid residues. We present successful formation of these artificial AP mimics with respect to the kinetics of the MNP hydrolysis obeying Michaelis–Menten scheme in aqueous solution and a two-phase solvent system and to the mode of the product inhibition by inorganic phosphate.

Keywords: supramolecular chemistry; zinc; copper; hydrolysis; dephosphorylation; self-assembly

1. Introduction

The regulation of protein phosphorylation and dephosphorylation is an important process in terms of cellular signal transduction, apoptosis, and the cell cycle among other issues. The phosphorylation of proteins is mediated by protein kinases, such as tyrosine kinases which are related to signal transduction pathways in living cells [1]. Indeed, several molecular targeted drugs that target tyrosine kinases in cancer cells and inhibit their action have been developed for the treatment of cancer [2,3]. On the other hand, dephosphorylation is promoted by the action of protein phosphatases such as alkaline phosphatase (AP) which contains two Zn\(^{2+}\) ions in its active center [4–10]. Although some artificial
phosphatases that mimic the active center of metallophosphatases have been reported [11–22], very few of them function as catalysts for the hydrolysis of a phosphate monoester such as mono(4-nitrophenyl) phosphate (MNP). Hence, dephosphorylation by artificial catalysts that mimic protein phosphatases remains a great challenge.

It has been described that chemically synthesized enzyme models have several disadvantages including, (i) a lack of long-range interactions between catalysts and substrate and/or between functional groups in their active sites, (ii) the synthesis of such compounds is time-consuming, and (iii) they are active only in organic solvents in many cases [23]. Indeed, the synthesis of artificial enzyme models that are constructed by covalent bonds require long and complex synthetic routes especially for their functionalization, as a result, only a limited number of structures have been reported. These drawbacks, however, could be overcome by a supramolecular strategy utilizing the self-assembly of the artificial and functionalized molecular building blocks that are readily available [24–52].

In this context, we previously reported on the formation of the supramolecular complex 8a, by the 2:2:2 assembly of a bis(Zn$^{2+}$-cyclen) complex (cyclen = 1,4,7,10-tetraazacyclododecane) containing 2,2′-bipyridyl (bpy) linker 1 (Zn$_2$L$_1$), a dianion of barbital 4a (Bar), and a copper(II) ion in an aqueous solution which is stabilized by non-covalent bonds (Scheme 1a) [19,24]. The X-ray crystal structure of the supermolecule 8a revealed that it contains a Cu$_2$(µ-OH)$_2$ core, which resembles the active centers of metallophosphatases such as AP, and that one of the ethyl groups of the Bar unit is located in close proximity to the Cu$_2$(µ-OH)$_2$ core [24]. More importantly, 8a accelerates the hydrolysis of MNP, although the yield is low, possibly due to product inhibition by inorganic phosphate (HPO$_{4}^{2−}$), a byproduct of the MNP hydrolysis.

We also reported on the formation of the hydrophobic supramolecular complexes 9a–c and the amphiphilic supramolecular complexes 10a–c (Scheme 1a) by the 2:2:2 assembly of a hydrophobic bis(Zn$^{2+}$-cyclen) complex containing two long alkyl chains 2 (Zn$_2$L$^2$) and a complex that contained one long alkyl chain 3 (Zn$_2$L$^3$), respectively, with Bar$^{2−}$ derivatives and Cu$^{2+}$ in a two-phase solvent system (CHCl$_3$/H$_2$O) [53,54]. As shown in Scheme 2, we expected that the hydrophobic or amphiphilic 2:2:2 complexes 9 or 10 would be formed mainly in the organic layer, and that product inhibition by HPO$_{4}^{2−}$ could be avoided, since the hydrophilic HPO$_{4}^{2−}$ would be released into the aqueous layer, thus permitting the Cu$_2$(µ-OH)$_2$ core to be regenerated. The results showed that 9 and 10 accelerated the hydrolysis of MNP and one of most important findings was that the hydrolysis of MNP by 9 and 10 in the two-phase solvent system obeyed Michaelis–Menten kinetics, suggesting that these reaction systems consisting of a supramolecular complex in two-phase solvent system closely mimic the active sites of AP. However, catalytic activity was observed only in the case of 10 (the catalytic turnover numbers (CTN) were 3–4 [54]), but not in 9 [53]. In addition, the product inhibition of 9 and 10 by inorganic phosphate (HPO$_{4}^{2−}$) was not competitive, although it is well known that HPO$_{4}^{2−}$ inhibits natural AP in a competitive manner. These results raised next question of how to design and synthesize good and appropriate models for natural enzymes such as AP based on a supramolecular strategy.
Scheme 1. (a) Formation of the 2:2:2 supramolecular complexes 8–10 by the self-assembly of Zn2L complexes (1–3), 5,5-diethylbarbital (Bar) derivatives (4a–c) and Cu2+ in an aqueous solution at neutral pH or in a two-phase solvent system (CHCl3/H2O) as was used in our previous study. (b) Formation of the 2:2:2 supramolecular complexes 15–17 by a self-assembly of Zn3L complexes (1–3), 5,5-diethylbarbital (Bar) derivatives (11a–p) and Cu2+ in an aqueous solution at neutral pH or a two-phase solvent system (CHCl3/H2O) in this work.
It is generally accepted that amino acids in the active site of AP contribute to the hydrolysis of phosphomonoesters; for example, the arginine residue captures and fixes the conformation of the phosphomonoester, and the serine residue attacks the phosphorous to promote the hydrolysis [4–10]. These well-grounded assumptions prompted us to introduce amino acids (alanine, serine, arginine, phenylalanine, and tyrosine) into the side chains of the Bar units for the easy and versatile construction of a combinatorial library of supramolecular phosphatases (Scheme 3). For example, functionalization with arginine (Arg) unit would be expected to stabilize the transition state that is produced in the hydrolysis of MNP (Scheme 3a) by electrostatic interactions. Second, the serine (Ser) residue would participate in a nucleophilic attack on the phosphorous of the MNP (Scheme 3b). Third, a phenylalanine (Phe) unit was introduced to support π–π interactions with MNP for a stronger complexation (Scheme 3c).

In this paper, we report on the combinatorial construction of 2:2:2 supramolecular complexes 15–17 from dinuclear Zn\(^{2+}\)-cyclen complexes 1 (Zn\(_2\)L\(_1\)), 2 (Zn\(_2\)L\(_2\)), or 3 (Zn\(_2\)L\(_3\)) with the Bar derivatives that are functionalized with amino acids 11d–p as well as their synthetic intermediates 11a–c and Cu\(^{2+}\) (Scheme 1b), their catalytic activity for the hydrolysis of MNP, and the results of a Michaelis–Menten kinetic study. These findings indicate that hydrophobic active sites of the supramolecular phosphatases well mimic the mode of the substrate (MNP) recognition and the product inhibition (by inorganic phosphate), and both of hydrophobic and hydrophilic property of active sites are important for the catalytic turnover of the system.
was performed using Merck Silica gel 60 F254 plate or Fuji Silysia Chemical CHROMATOREX NH-TLC PRATE, and Fuji Silysia Chemical FL-100D or Fuji Silysia Chemical CHROMATOREX NH chromatography Silica Gel, respectively.

**2. Materials and Methods**

### 2.1. General Information

All reagents and solvents were of the highest commercial quality and were used without further purification, unless otherwise noted. Anhydrous N,N-dimethylformamide (DMF) was obtained by distillation from calcium hydride. MNP was purchased from Nacalai Tesque (Kyoto, Japan). All aqueous solutions were prepared using deionized and distilled water. The Good’s buffer reagents (Dojindo, pH 7.6) were obtained from commercial sources: HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl))ethanesulfonic acid, pH 7.6. For the measurement of UV/Vis spectra, CHCl₃ was purchased from Nacalai Tesque (Kyoto, Japan). UV/Vis spectra were recorded on a JASCO V-550 spectrophotometer with quartz cuvettes (path length: 10 mm). IR spectra were recorded on a Perkin-Elmer attenuated total reflectance (ATR)-IR spectrometer 100 at room temperature. Melting points were measured on a Yanaco MP-J3 Micro Melting Point apparatus and are uncorrected. ¹H- (300 and 400 MHz) and ¹³C- (75 and 100 MHz) NMR spectra at 25 ± 0.1 °C were recorded on a JEOL Always 300 spectrometer and a JEOL Lamda 400 spectrometer. Tetramethylsilane (TMS) was used as the internal reference for ¹H- and ¹³C-NMR measurements in CDCl₃ and CD₂OD. 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP) was used as the external reference for ¹H- and ¹³C-NMR measurements in D₂O. Mass spectra was recorded on a JEOL JMS-700 and Varian 910-MS spectrometer. Elemental analyses were performed on a Perkin-Elmer CHN 2400 analyzer. Optical rotations were measured with a JASCO-P-1030 digital polarimeter in 50 mm cells using the D line of sodium (589 nm). Thin-layer chromatography (TLC) and silica gel column chromatography was performed using Merck Silica gel 60 F254 plate or Fuji Silysia Chemical CHROMATOREX NH-TLC PRATE, and Fuji Silysia Chemical FL-100D or Fuji Silysia Chemical CHROMATOREX NH chromatography Silica Gel, respectively.

Scheme 3. Predicted effect of new barbital derivatives functionalized with amino acids on the hydrolysis of MNP. (a) It is considered that Arg stabilizes the transition state of the MNP hydrolysis by the electrostatic interactions between them, (b) Ser is supposed to undergo the nucleophilic attack to the phosphorous of MNP, (c) Phe is supposed to contribute to the π–π interactions with MNP.
2.2. *Synthesis of Compounds*

5,5-Bis[3-(toluenesulfonyl)oxypropyl]barbituric acid (18)

A solution of 4c (698 mg, 2.86 mmol) and DMAP (35 mg, 0.29 mmol) in pyridine (5 mL) was stirred at 0 °C for 5 min, to which p-toluenesulfonyl chloride (1.53 g, 8.00 mmol) was slowly added, and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was quenched by adding an aqueous solution saturated with NaHCO₃, and the resulting suspension was extracted with CHCl₃ (50 mL × 3). The combined organic layer was washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (CHCl₃/MeOH = 1/0 to 50/1) to give 18 as a colorless amorphous solid (819 mg, 54% yield). ¹H NMR (300 MHz, CDCl₃, TMS): δ = 7.75 (d, J = 8.3 Hz, 4H), 7.35 (d, J = 8.3 Hz, 4H), 3.98–3.92 (m, 4H), 2.45 (s, 6H), 2.12–1.94 (m, 4H), 1.68–1.56 (m, 4H) ppm; ¹³C NMR (75 MHz, CDCl₃, TMS): δ = 171.7, 149.0, 145.1, 132.5, 129.9, 127.9, 69.4, 43.8, 34.0, 24.3, 21.6 ppm; IR (ATR): v = 3357, 3203, 3090, 2977, 2935, 1755, 1686, 1516, 1448, 1392, 1365, 1275, 1248, 1163, 924, 811, 660, 575, 552, 493. HRMS (ESI): m/z calcld for [M+N]⁺, C₂₂H₂₉N₂O₅NaSO₂, 575.1124; found, 575.1129.

5,5-Bis[3-azidopropyl]barbituric acid (11a)

A solution of 18 (850 mg, 1.54 mmol) and sodium azide (280 mg, 4.31 mmol) in DMF (4 mL) was stirred at 80 °C for 5 h. After allowing the solution to cool to room temperature, the reaction mixture was poured into H₂O and the resulting mixture was extracted with Et₂O (40 mL × 3). The combined organic layer was washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (CHCl₃/MeOH = 1/0 to 10/1) to give 11a as a white solid (434 mg, 96% yield). ¹H NMR (300 MHz, CDCl₃, TMS): δ = 8.71 (s, 2H), 3.29 (t, J = 6.6 Hz, 4H), 2.11–2.05 (m, 4H), 1.59–1.49 (m, 4H) ppm; ¹³C NMR (75 MHz, CDCl₃, TMS): δ = 171.5, 55.5, 50.7, 35.6, 24.5 ppm; mp 118–119 °C; IR (ATR): v = 3232, 3106, 2962, 2856, 1756, 1696, 1596, 1419, 1346, 1171, 1096, 953, 914, 811, 660, 575, 552, 493. HRMS (ESI): m/z calcld for [M+Na]⁺, C₂₄H₂₈N₂O₅Na₂S, 575.1124; found, 575.1129.

5,5-Bis[3-(azidopropyl)]barbituric acid (11b)

Solutions of 11a (420 mg, 1.43 mmol) in AcOEt (2 mL) and Boc₃O (760 mg, 3.48 mmol) in AcOEt (2 mL) were added to a suspension of 10% Pd/C (50 mg) in AcOEt (2 mL) at room temperature under a N₂ atmosphere. The reaction mixture was hydrogenated with H₂ (at 1 atm) at room temperature for 24 h. After the reaction reached completion, the reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography (AcOEt/Hexanes = 2/3) to give 11b as a colorless amorphous solid (440 mg, 71% yield). ¹H NMR (300 MHz, CDCl₃, TMS): δ = 9.32 (brs, 2H), 4.78–4.68 (m, 2H), 3.12–3.00 (m, 4H), 2.01–1.96 (m, 4H), 1.47–1.40 (m, 22H) ppm; ¹³C NMR (75 MHz, CDCl₃, TMS): δ = 172.7, 156.0, 150.0, 79.4, 55.5, 39.9, 35.7, 28.3, 25.4 ppm; mp 268 °C; IR (ATR): v = 3357, 3203, 2977, 2935, 1755, 1686, 1516, 1448, 1392, 1365, 1275, 1248, 1163, 1040, 854, 756, 666, 494. HRMS (ESI): m/z calcld for [M+H]⁺, C₁₀H₁₃N₉O₃, 293.1122; found, 293.1116. Anal. Calcd. for C₁₀H₁₃N₉O₃: C, 40.82; H, 4.80; N, 38.08; found: C, 40.91; H, 4.74; N, 37.79.

5,5-Bis[3-(l-butoxycarbonylamino)propyl]barbituric acid (11c)

Compound 11b (440 mg, 0.99 mmol) was dissolved in MeOH (2 mL), to which conc. HCl (2 mL) was added at 0 °C. The mixture was stirred at room temperature for 1 h and concentrated under reduced pressure to give 11c as a white solid (276 mg, 86% yield as the 2HCl salt). ¹H NMR (300 MHz, CD₃OD, TMS): δ = 2.90 (t, J = 7.2 Hz, 4H), 2.05–2.00 (m, 4H), 1.66–1.56 (m, 4H) ppm; ¹³C NMR (100 MHz, D₂O, TSP): δ = 173.8, 150.5, 55.0, 38.9, 34.2, 22.3 ppm; mp 268 °C; IR (ATR): v = 2967, 2826, 1749, 1721, 1694, 1591, 1485, 1443, 1397, 1346, 1283, 1220, 981, 819, 492. HRMS (ESI): m/z calcld for [M+H]⁺,
C_{10}H_{16}N_{4}O_{3}, 243.1456; found, 243.1452. Anal. Calcd. for C_{10}H_{20}N_{4}O_{3}Cl_{2}: C, 38.11; H, 6.40; N, 17.78; found: C, 38.31; H, 6.42; N, 17.85.

5,5-Bis-[3-[N-(t-butoxycarbonyl)-l-allyl]aminopropyl]barbituric acid (11d)

To a suspension of 11c (10 mg, 0.03 mmol) in anhydrous DMF (0.5 mL) and diisopropylethylamine (22 mg, 0.17 mmol), Boc-l-Alanine-OH (13 mg, 0.07 mmol) and PyBOP (36 mg, 0.07 mmol) were added and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure, dissolved in CHCl_{3} (10 mL), washed with a 50% NH_{4}Cl solution (10 mL × 3), then with brine, dried over Na_{2}SO_{4} and concentrated under reduced pressure. The resulting oil was purified by silica gel column chromatography (AcOEt/z/61.18; H, 7.18; N, 11.26. [\alpha]_D^{20} = +16.7 (c = 1.0, CHCl_{3}).

5,5-Bis-[3-[N-(t-butoxycarbonyl)-O-(t-butyl)-l-seryl]aminopropyl]barbituric acid (11e)

Compound 11e was synthesized as a white solid (29 mg, 84% yield) from 11c (10 mg, 0.03 mmol), Boc-l-Serine(OtBu)-OH (18 mg, 0.07 mmol), diisopropylethylamine (22 mg, 0.17 mmol), and PyBOP (36 mg, 0.07 mmol) in anhydrous DMF (0.5 mL) using a procedure similar to that used for 11d. ^1H NMR (300 MHz, CDCl_{3}, TMS): δ = 9.27 (brs, 2H), 6.71 (t, J = 6.0 Hz, 2H), 5.47 (d, J = 6.9 Hz, 2H), 4.20–4.10 (m, 2H), 3.80–3.76 (m, 2H), 3.42–3.36 (m, 2H), 3.30–3.10 (m, 4H), 1.98–1.92 (m, 4H), 1.48–1.39 (m, 22H), 1.18 (s, 18H) ppm; ^13C NMR (75 MHz, CDCl_{3}, TMS): δ = 172.2, 171.0, 155.7, 148.8, 80.1, 74.0, 61.8, 55.6, 54.4, 39.0, 35.8, 28.3, 27.4, 25.0 ppm; mp 136–139 °C; IR (ATR): ν = 3315, 3092, 2977, 2935, 1754, 1692, 1653, 1522, 1448, 1365, 1246, 1162, 1055, 1021, 856, 759, 495. HRMS (ESI): m/z calcd for [M+H]^+: C_{34}H_{61}N_{4}O_{11}·H_{2}O: C, 54.68; H, 8.37; N, 11.25; found: C, 54.82; H, 8.43; N, 11.20. [\alpha]_D^{25} = +16.7 (c = 1.0, CHCl_{3}).

5,5-Bis-[3-[N-(t-butoxycarbonyl)-l-phenylalanyl]aminopropyl]barbituric acid (11f)

Compound 11f was synthesized as a white solid (20 mg, 86% yield) from 11c (10 mg, 0.03 mmol), Boc-l-Phenylalanine-OH (19 mg, 0.07 mmol), diisopropylethylamine (22 mg, 0.17 mmol), and PyBOP (36 mg, 0.07 mmol) in anhydrous DMF (0.5 mL) using a procedure similar to that used for 11d. ^1H NMR (300 MHz, CDCl_{3}, TMS): δ = 9.43 (brs, 2H), 7.24–7.16 (m, 10H), 6.36–6.26 (m, 2H), 5.32 (d, J = 8.7 Hz, 2H), 4.39–4.30 (m, 2H), 3.20–2.98 (m, 8H), 1.90–1.78 (m, 4H), 1.38 (s, 18H) ppm; ^13C NMR (100 MHz, CDCl_{3}, TMS): δ = 172.5, 171.8, 155.8, 149.5, 136.8, 129.3, 128.5, 126.8, 80.2, 55.9, 55.4, 39.8, 35.5, 28.3, 24.6 ppm; mp 117–118 °C; IR (ATR): ν = 3319, 3088, 2978, 2933, 2871, 1754, 1697, 1655, 1498, 1390, 1363, 1243, 1163, 1085, 1019, 861, 755, 668, 494. HRMS (ESI): m/z calcd for [M+H]^+: C_{38}H_{33}N_{5}O_{12}: 737.3869; found, 737.3869. Anal. Calcd. for C_{38}H_{33}N_{5}O_{12}·0.5H_{2}O: C, 61.19; H, 7.16; N, 11.27; found: C, 61.18; H, 7.18; N, 11.26. [\alpha]_D^{25} = +2.73 (c = 1.0, CHCl_{3}).

5,5-Bis-[3-[N-(t-butoxycarbonyl)-l-tyrosyl]aminopropyl]barbituric acid (11g)

Compound 11g was synthesized as a white solid (18 mg, 72% yield) from 11c (10 mg, 0.03 mmol), Boc-l-Tyrosine-OH (20 mg, 0.07 mmol), diisopropylethylamine (22 mg, 0.17 mmol), and PyBOP (36 mg, 0.07 mmol) in anhydrous DMF (0.5 mL) using a procedure similar to that used for 11d. ^1H NMR (300 MHz, CD_{2}OD, TMS): δ = 7.02 (d, J = 8.7 Hz, 4H), 6.70 (d, J = 8.7 Hz, 4H), 4.18–4.04 (m, 2H), 3.13–3.03 (m, 4H), 2.92 (dd, J = 3.6 Hz, 2H), 2.76–2.64 (m, 2H), 1.88–1.74 (m, 4H), 1.41–1.28 (m, 4H), 1.37 (s, 18H) ppm; ^13C NMR (100 MHz, CD_{2}OD, TMS): δ = 174.5, 157.5, 157.2, 151.2, 131.3, 129.2, 116.2, 80.6, 57.8, 56.5, 40.1, 40.0, 38.8, 37.0, 28.7, 25.8 ppm; mp 150–152 °C; IR (ATR): ν = 3320, 2975, 2930, 1693, 1651,
5,5-Bis[3-\(N^\alpha, N^\nu\), \(N^\delta\)-tril-(butoxycarbonyl)-\(\nu\)-arginyl]aminopropyl]barbituric acid (11h)

Compound 11h was synthesized as a white solid (30 mg, 82% yield) from 11c (10 mg, 0.03 mmol), Boc-\(\nu\)-Arginine(Boc)\(_2\)-OH (35 mg, 0.07 mmol), diisopropylethylamine (22 mg, 0.17 mmol), and PyBOP (36 mg, 0.07 mmol) in anhydrous DMF (0.5 mL) using a procedure similar to that used for 11d. \(^1\)H NMR (300 MHz, CDCl\(_3\), TMS): \(\delta = 9.33\) (s, 2H), 7.14–7.08 (m, 2H), 6.00–5.92 (m, 2H), 4.32–4.22 (m, 2H), 4.04–3.90 (m, 2H), 3.73–3.60 (m, 2H), 3.35–3.05 (m, 4H), 1.94–1.82 (m, 4H), 1.82–1.74 (m, 4H), 1.52 (s, 18H), 1.48 (s, 18H), 1.47–1.30 (m, 12H), 1.44 (s, 18H) ppm; \(^{13}\)C NMR (100 MHz, CDCl\(_3\), TMS): \(\delta = 172.3, 171.9, 163.2, 161.0, 155.7, 155.0, 148.5, 84.2, 79.8, 79.4, 55.5, 53.7, 44.0, 39.1, 36.1, 28.9, 28.5, 28.4, 28.1, 25.1, 24.5 ppm; mp 123–125 °C; IR (ATR): \(\nu = 3380, 2977, 2934, 1755, 1709, 1607, 1508, 1452, 1390, 1366, 1271, 1249, 1145, 1099, 981, 886, 852, 811, 779, 512, 496. HRMS (ESI): \(m/z\) calcd for [M+H]^+; \(C_{52}H_{91}N_{12}O_{17}\), 1155.6612; found, 1155.6620. Anal. Calcd. for \(C_{52}H_{91}N_{12}O_{17}\): C, 53.64; H, 7.88; N, 14.44; found: C, 53.69; H, 8.11; N, 14.21. [\(\alpha\)]\(^{25}\)D = +22.3 (c = 1.0, CHCl\(_3\)).

5,5-Bis[3-\(\nu\)-alanyl]aminopropyl]barbituric acid (11i)

Compound 11i (20 mg, 0.03 mmol) was dissolved in MeOH (0.5 mL), to which conc. HCl (0.5 mL) was added and the reaction mixture was stirred at room temperature for 12 h. The resulting solution was evaporated under reduced pressure to give 11i as a white solid (16 mg, quant.). \(^1\)H NMR (300 MHz, CD\(_2\)OD, TMS): \(\delta = 3.88\) (q, \(J = 7.2\) Hz, 2H), 3.26–3.10 (m, 4H), 1.96–1.91 (m, 4H), 1.49 (d, \(J = 7.2\) Hz, 6H), 1.46–1.39 (m, 4H) ppm; \(^{13}\)C NMR (100 MHz, CD\(_2\)OD, TMS): \(\delta = 174.6, 170.8, 56.5, 50.3, 40.1, 37.1, 26.1, 18.4, 17.7 ppm; mp 194 °C; IR (ATR): \(\nu = 2933, 1749, 1722, 1666, 1563, 1494, 1447, 1355, 1268, 1206, 1115, 1043, 829, 760, 667, 496. HRMS (ESI): \(m/z\) calcd for [M+H]^+; \(C_{16}H_{29}NO_{12}\), 538.2195; found, 538.2194. Anal. Calcd. for \(C_{16}H_{29}NO_{12}\): C, 53.64; H, 7.88; N, 14.44; found: C, 40.13; H, 7.01; N, 16.09. [\(\alpha\)]\(^{25}\)D = +2.24 (c = 0.5, CH\(_3\)OH).

5,5-Bis[3-\(\nu\)-seryl]aminopropyl]barbituric acid (11j)

Compound 11j (10 mg, 0.01 mmol) was dissolved in MeOH (0.3 mL), to which conc. HCl (0.3 mL) was added and the reaction mixture was stirred at room temperature for 12 h. The resulting solution was evaporated under reduced pressure to give 11j as a white solid (7 mg, quant.). \(^1\)H NMR (400 MHz, CD\(_2\)OD, TMS): \(\delta = 3.95–3.90\) (m, 4H), 3.80 (q, \(J = 7.2\) Hz, 2H), 3.19 (t, \(J = 7.2\) Hz, 4H), 1.96–1.92 (m, 4H), 1.47–1.40 (m, 4H) ppm; \(^{13}\)C NMR (100 MHz, CD\(_2\)OD, TMS): \(\delta = 173.2, 166.8, 149.7, 60.4, 55.0, 38.8, 35.6, 33.5, 24.6 ppm; mp 197 °C; IR (ATR): \(\nu = 3197, 3059, 2932, 1748, 1667, 1562, 1495, 1448, 1418, 1356, 1338, 1308, 1272, 1210, 1145, 1045, 761, 498. HRMS (ESI): \(m/z\) calcd for [M+H]^+; \(C_{16}H_{29}NO_{12}\), 417.2090; found, 417.2092. Anal. Calcd. for \(C_{16}H_{29}NO_{12}\): C, 37.24; H, 6.96; N, 14.89; found: C, 36.96; H, 6.44; N, 14.72. [\(\alpha\)]\(^{25}\)D = +5.90 (c = 0.5, CH\(_3\)OH).

5,5-Bis[3-(\(\nu\)-phenylalanyl)]aminopropyl]barbituric acid (11k)

Compound 11k (25 mg, 0.03 mmol) was dissolved in MeOH (0.5 mL), to which conc. HCl (0.5 mL) was added and the reaction mixture was stirred at room temperature for 12 h. The resulting solution was evaporated under reduced pressure to give 11k as a white solid (22 mg, quant.). \(^1\)H NMR (300 MHz, CD\(_2\)OD, TMS): \(\delta = 7.35–7.24\) (m, 10H), 3.98 (t, \(J = 7.2\) Hz, 2H), 3.19–3.13 (m, 2H), 3.10 (d, \(J = 7.2\) Hz, 4H), 2.97–2.90 (m, 2H), 1.81 (t, \(J = 8.4\) Hz, 4H), 1.23–1.15 (m, 4H) ppm; \(^{13}\)C NMR (100 MHz, CD\(_2\)OD, TMS): \(\delta = 174.5, 169.3, 151.2, 135.7, 130.5, 130.1, 128.9, 56.3, 55.9, 40.0, 38.8, 37.1, 25.9 ppm; mp 183–185 °C; IR (ATR): \(\nu = 3189, 3029, 2933, 2818, 1749, 1721, 1668, 1563, 1495, 1454, 1355, 1335, 1303, 1268, 1207, 1144, 1080, 1041, 825, 746, 700, 497. HRMS (ESI): \(m/z\) calcd for [M+H]^+; \(C_{29}H_{37}N_{6}O_{3}\), 537.2820; found, 537.2820. Anal. Calcd. for \(C_{29}H_{37}N_{6}O_{3}\): C, 52.10; H, 6.78; N, 12.57; found: C, 52.17; H, 6.59; N, 12.64. [\(\alpha\)]\(^{25}\)D = +34.9 (c = 0.5, CH\(_3\)OH).
5,5-Bis[3-(l-tyrosyl)aminopropyl]barbituric acid (11I)

A solution of 11g (25 mg, 0.03 mmol) and conc. HCl (0.5 mL) in MeOH (0.5 mL) was stirred at room temperature for 12 h. The resulting solution was evaporated under reduced pressure to give 11I as a white solid (21 mg, quant.). 1H NMR (300 MHz, CD2OD, TMS): δ = 7.04 (d, J = 8.7 Hz, 4H), 6.78 (d, J = 8.7 Hz, 4H), 3.91 (t, J = 7.2 Hz, 2H), 3.16–2.90 (m, 8H), 1.87–1.82 (m, 4H), 1.37–1.31 (m, 4H) ppm; 13C NMR (100 MHz, CD2OD, TMS): δ = 174.5, 169.7, 158.2, 151.1, 131.6, 126.2, 116.9, 56.5, 56.1, 40.2, 38.0, 37.1, 25.8 ppm; mp 196–198 °C; IR (ATR): ν = 3019, 2934, 1749, 1722, 1668, 1613, 1564, 1514, 1443, 1355, 1306, 1210, 1041, 824, 780, 632, 497. HRMS (ESI): m/z calc for [M+H]+, C28H32N6O7, 569.2707; found, 569.2718. Anal. Calcld. for C28H36N6O7·4HCl·H2O·MeOH: C, 45.56; H, 6.06; N, 10.99; found: C, 45.42; H, 5.87; N, 11.13. [α]D25 = +35.6 (c = 1.0, CH3OH).

5,5-Bis[3-(l-arginyl)aminopropyl]barbituric acid (11m)

A solution of 11h (40 mg, 0.03 mmol) and conc. HCl (0.5 mL) in MeOH (0.5 mL) was stirred at room temperature for 12 h. The resulting solution was evaporated under reduced pressure to give 11m as a white solid (22 mg, quant.). 1H NMR (300 MHz, CD2OD, TMS): δ = 3.89 (t, J = 6.0 Hz, 2H), 3.30–3.26 (m, 8H), 3.18–3.09 (m, 2H), 1.98–1.87 (m, 8H), 1.74–1.67 (m, 4H), 1.45–1.43 (m, 4H) ppm; 13C NMR (100 MHz, CD2OD, TMS): δ = 174.6, 169.9, 158.7, 151.0, 56.5, 54.1, 41.9, 40.2, 37.2, 29.8, 26.0, 25.6 ppm; mp 182–184 °C; IR (ATR): ν = 3157, 3058, 2936, 1749, 1722, 1655, 1561, 1498, 1446, 1355, 1303, 1270, 1209, 1162, 1042, 827, 663, 496. HRMS (ESI): m/z calc for [M+H]+, C22H43N12O5, 555.3480; found, 555.3479. Anal. Calcld. for C22H42N12O5·4HCl·H2O·MeOH: C, 35.94; H, 7.08; N, 21.87; found: C, 35.87; H, 6.89; N, 21.83. [α]D25 = +10.7 (c = 1.0, CH3OH).

5,5-Bis-[N-(9-fluorenylmethoxycarbonyl)-O-(l-buty1)-l-seryl]aminopropyl]barbituric acid (11n)

Compound 11n was synthesized as a white solid (31 mg, 98% yield) from 11c (10 mg, 0.03 mmol), Fmoc-l-Serine(Obu)-OH (27 mg, 0.07 mmol), diisopropylethylamine (22 mg, 0.17 mmol), and PyBOP (36 mg, 0.07 mmol) in anhydrous DMF (0.5 mL) using a procedure similar to that used for 11d. 1H NMR (300 MHz, CDCl3, TMS): δ = 9.03 (brs, 2H), 7.75 (d, J = 7.2 Hz, 4H), 7.62–7.54 (m, 4H), 7.39 (t, J = 7.2 Hz, 4H), 7.29 (t, J = 7.2 Hz, 4H), 6.70–6.58 (m, 2H), 5.80 (d, J = 7.2 Hz, 2H), 4.40 (d, J = 6.9 Hz, 4H), 4.21 (t, J = 7.2 Hz, 4H), 3.76 (dd, J = 3.9 Hz, 2H), 3.38 (t, J = 8.1 Hz, 2H), 3.28–3.10 (m, 4H), 2.02–1.88 (m, 4H), 1.50–1.36 (m, 4H), 1.17 (s, 18H) ppm; 13C NMR (100 MHz, CDCl3, TMS): δ = 172.0, 170.5, 156.2, 148.6, 143.8, 141.3, 127.7, 127.1, 125.2, 120.0, 74.2, 67.1, 61.7, 55.6, 54.6, 47.1, 39.0, 35.7, 27.4, 24.9 ppm; mp 122–124 °C; IR (ATR): ν = 3313, 3064, 2972, 2934, 2870, 1698, 1659, 1516, 1449, 1410, 1389, 1363, 1330, 1225, 1081, 812, 784, 758, 738, 664, 620, 539, 496. HRMS (ESI): m/z calc for [M+H]+, C54H64N6O11, 973.4707; found, 973.4706. Anal. Calcld. for C54H64N6O11·H2O·MeOH: C, 65.44; H, 6.71; N, 8.48; found: C, 65.23; H, 6.77; N, 8.30. [α]D25 = +20.4 (c = 1.0, CHCl3).

5,5-Bis[3-(l-buty1)-l-seryl]aminopropyl]barbituric acid (11o)

To a solution of 11m (15 mg, 0.02 mmol) in anhydrous DMF (0.4 mL), piperidine (0.1 mL) was added and the reaction mixture was stirred at room temperature for 20 min. The reaction mixture was concentrated under reduced pressure, and resulting residue was purified by silica gel column chromatography (AcOEt/MeOH = 1/0 to 6/1) to give 11o as a colorless amorphous solid (7 mg, 92% yield). 1H NMR (400 MHz, CDCl3, TMS): δ = 7.53–7.48 (m, 2H), 3.62 (q, J = 4.0 Hz, 2H), 3.55 (q, J = 4.0 Hz, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.24–3.14 (m, 4H), 1.98–1.90 (m, 2H), 1.50–1.36 (m, 4H), 1.18 (s, 18H) ppm; 13C NMR (100 MHz, CDCl3, TMS): δ = 173.2, 173.0, 150.2, 73.6, 64.0, 55.6, 55.5, 38.8, 35.9, 27.6, 25.1 ppm; IR (ATR): ν = 3299, 3072, 2971, 2928, 2856, 1723, 1693, 1646, 1533, 1448, 1411, 1388, 1362, 1335, 1306, 1258, 1192, 1078, 1022, 874, 759, 670, 493. HRMS (ESI): m/z calc for [M+H]+, C24H45N6O7, 529.3344; found, 529.3344. [α]D25 = −14.5 (c = 1.4, CHCl3).
5,5-Bis[3-(N-(9-fluorenylmethoxycarbonyl)-l-seryl]aminopropyl]barbituric acid (11p)

To a solution of 11n (11 mg, 0.01 mmol) in dichloromethane (0.2 mL), trifluoroacetic acid (0.2 mL) was added and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was concentrated under reduced pressure, suspended in H₂O, extracted with AcOEt (10 mL × 3), washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (AcOEt/Hexanes = 4/1 to 1/0) to give 11p as a white solid (5 mg, 54% yield). ¹H NMR (400 MHz, CD₂OD, TMS): δ = 7.78 (d, J = 7.6 Hz, 4H), 7.67 (t, J = 7.6 Hz, 4H), 7.38 (t, J = 7.6 Hz, 4H), 7.30 (t, J = 7.6 Hz, 4H), 4.39 (dd, J = 2.4 Hz, 4H), 4.23 (t, J = 6.8 Hz, 2H), 4.12 (t, J = 6.8 Hz, 2H), 3.72 (d, J = 5.2 Hz, 4H), 3.17–3.08 (m, 4H), 1.96–1.84 (m, 4H), 1.43–1.34 (m, 4H) ppm; ¹³C NMR (100 MHz, CD₂OD, TMS): δ = 174.6, 172.9, 158.5, 151.0, 145.4, 145.3, 142.6, 128.8, 128.2, 126.3, 121.0, 68.1, 63.3, 58.7, 56.5, 40.0, 36.9, 25.9 ppm; mp 124–125 °C.

2.3. Hydrolysis of MNP

The hydrolysis of MNP was carried out in 10 mM HEPES buuffer (pH 7.4) with I = 0.1 (NaNO₃) (for 8, 15) and in a CHCl₃/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (2/8) (for 9, 10, 16, 17) at 37 °C. Stock solutions of 1 (3.0 mM in H₂O), 4 or 11 (6.0 mM in H₂O), and Cu(NO₃)·3H₂O (10 mM in H₂O) were used for the preparation of sample solutions of 8 and 15 (20 μM) in 10 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (3.0 mL). Stock solutions of 2 (1.0 mM in CHCl₃), 4 or 11 (3.0 mM in H₂O or CHCl₃), and Cu(ClO₄)₂·6H₂O (20 mM in H₂O) were used for the preparation of sample solutions of 9 and 16 (20 μM in total solution) in CHCl₃/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (2/8) (total volume 3.0 mL). A stock aqueous solution of MNP (20 mM) was used for the hydrolysis reaction. Prior to the hydrolysis of MNP in the two-phase solvent system, the reaction mixtures of 2 or 3, barbital derivatives (4a-c, 11a-p), and Cu²⁺ in CHCl₃/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (2/8) were incubated at 37 °C overnight using a shaking water bath (shaking speed: 150 rpm) (Tokyo Glass, Kikai, FWB-1) to form 9, 10, 16, and 17 in situ, and the aqueous solution of MNP was added. All of the hydrolysis experiments were performed in triplicate at 37 °C using a shaking water bath (shaking speed: 150 rpm). The yields of the hydrolysis product from MNP in the presence of the supramolecular complexes were calculated based on the increase in the absorption of the released 4-nitrophenoxy (NP) at 400 nm ([NP produced in the presence of the supramolecular complex]–[NP produced in the absence of the supramolecular complex]) (ε₄₀₀ value of NP is 1.35 × 10⁴ M⁻¹ cm⁻¹ at pH 7.4) [17] in aqueous layer. The partition ratio of NP (79% in aqueous solution), which had been determined in the previous report [53], was used for the calculation of the yields in the CHCl₃/H₂O system.

3. Results and Discussion

3.1. Synthesis of Barbital Derivatives

The barbital derivatives that were functionalized with amino acid residues were synthesized as shown in Scheme 4. Compound 4c was synthesized from diethyl malonate via 4b according to our previous paper [24] and was reacted with p-toluene sulfonyl chloride to afford the ditosylate 18. The reaction of 18 with sodium azide gave compound 11a. Our attempt to carry out Staudinger reactions [55,56] of 11a with the o-phosphynothioesters of amino acids resulted in failure. Therefore, the reduction of the azide groups of 11a were carried out to obtain the diamino intermediate 11c. It should be noted that two amino groups of 11c produced by the reduction of 11a were directly protected with a Boc group in situ for easy purification as a Boc-protected compound 11b and the subsequent deprotection of the Boc groups afforded 11c. The condensation reactions of 11c with Boc-protected amino acids in DMF in the presence of PyBOP and DIEA gave 11d-h. The deprotection of the Boc groups by treatment with HCl afforded the corresponding compounds 11i-m in quantitative yield.
The barbital derivative equipped with serine 11n was obtained from 11c and Fmoc-\(\text{t-Ser(OtBu)}\)-OH and then reacted with piperidine and TFA to give compound 11o and 11p, respectively.

Scheme 4. Synthesis of barbital derivatives 11a–p as building units for supramolecular complexes 15, 16, and 17.

3.2. Complexation Behavior of 1 (Zn\(\text{L}_{1}\)) with Barbital Derivatives and Cu\(^{2+}\) by UV/Vis Titrations

In order to examine the formation of the 2:2 and 2:2:2 supramolecular complexes from the bis(Zn\(^{2+}\)-cyclen) complexes 1 (Zn\(\text{L}_{1}\)) and 2 (Zn\(\text{L}_{2}\)) with the synthesized Bar units and Cu\(^{2+}\) as shown in Scheme 1, UV/Vis titrations of the bis(Zn\(^{2+}\)-cyclen) complexes with 11d and 11i, and then with Cu(NO\(_{3}\))\(_{2}\)·3H\(_{2}\)O were attempted. While the titrations of 2 in the two-phase solvent system (CHCl\(_{3}\)/50 mM HEPES buffer (pH 7.4, I = 0.1 (NaNO\(_{3}\))) with 11d and 11i and Cu(ClO\(_{4}\))\(_{2}\)·6H\(_{2}\)O were unsuccessful, the UV/Vis titrations of 1 with 11d and 11i were successfully carried out in 10 mM HEPES buffer (pH 7.4, I = 0.1 (NaNO\(_{3}\))) at 37 °C. As shown in Figure 1a, the absorption maxima (\(\lambda_{\text{max}}\)) at 287 nm, which increased upon the addition of 11d and decreased upon the addition of 11i, reaching a plateau at a 1:1 ratio, suggesting a 2:2 assembly of 1 with 11d or 11i as same as that with 4a. In...
addition, as shown in Figure 1c,d, the addition of Cu$^{2+}$ induced a red shift from 287 nm to 309 nm, which reached a plateau at [12d] or [12i]:[Cu$^{2+}$] = 1:2, suggesting the quantitative formation of the 2:2:2 supermolecules 15 with newly synthesized barbital derivatives at μM order concentrations.

![Figure 1](image)

Figure 1. (a) UV/Vis titration of 1 (80 μM) upon the addition of 11d in 10 mM HEPES buffer (pH 7.4 with I = 0.1 (NaNO$_3$)) at 25 °C; (b) UV/Vis titration of 1 (80 μM) upon the addition of 11i in 10 mM HEPES buffer (pH 7.4 with I = 0.1 (NaNO$_3$)) at 25 °C; (c) UV/Vis titration of 12d (40 μM) upon the addition of Cu$^{2+}$ in 10 mM HEPES buffer (pH 7.4 with I = 0.1 (NaNO$_3$)) at 25 °C; (d) UV/Vis titration of 12i (40 μM) upon the addition of Cu$^{2+}$ in 10 mM HEPES buffer (pH 7.4 with I = 0.1 (NaNO$_3$)) at 25 °C. The insets in (a,b) show the change in the absorbance at 287 nm, and the insets in (c,d) show the decrease in absorbance at 287 nm (closed circles) and the increase in absorbance at 309 nm (open circles).

3.3. Location of Complexes 13 and 16 in the Two-Phase Solvent System, as Determined by UV/Vis Spectra

The distribution of 13 and 16 in the organic phase and the aqueous phase of the two-phase solvent system was determined from the UV/Vis absorption spectra of both layers, as described in our previous report [54]. For the in situ formation of 13 and 16, 11d and 11i were selected as more hydrophobic barbital units, and 4a, 11i, and 11k were selected as more hydrophilic barbital units. Two-phase solutions of Zn$_2$L$_2$ (2) alone (40 μM), 2:2 complex (20 μM) of Zn$_2$L$_2$ and barbital derivatives (6a, 13d, 13f, 13i, and 13k), and 2:2:2 complex (20 μM) of Zn$_2$L$_2$, barbital derivatives, and Cu$^{2+}$ (9a, 16d, 16f, 16i, and 16k) were prepared in CHCl$_3$/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO$_3$) (1/1), incubated for 18 h at 37 °C, and centrifuged (2000 rpm × 10 min) at room temperature. Pictures of each complex and their distribution ratios are summarized in Figure 2 and Table 1. The findings indicate that 2 is located mostly in the organic layer (ca. 99%), and the addition of barbital derivatives did not significantly affect the distribution of 6a, 13d, 13f, 13i, and 13k (ca. 96%–99%), implying that these complexes are distributed mainly in the organic layer. Since the addition of Cu$^{2+}$ quenched the emission from the bpy units, it was not possible to determine the distribution of 9a, 16d, 16f, 16i, and 16k in both layers. These behaviors of 6a, 13d, 13f, 13i, and 13k are different from 7a prepared from 3 (Zn$_2$L$_2$) and 4a (Bar), which were distributed both in the aqueous and the organic layers [54].
3.4. Hydrolysis of MNP by 2:2:2 Complexes in a Two-Phase Solvent System

We conducted the hydrolysis of MNP (100 μM) in the single-phase solvent system (10 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (1/1)) at 37 °C in the presence of 8a or 15i–m (prepared from 1, 11i–m,
and Cu$^{2+}$) (100 μM) [24] and in the two-phase solvent system (CHCl$_3$/50 mM HEPES buffer (pH 7.4) with $I = 0.1$ (NaNO$_3$)) (2/8) at 37 °C in the presence of 9a or 16i–m (prepared from 2, 11i–m, and Cu$^{2+}$) (20 μM in the total solution including the aqueous phase and the CHCl$_3$ phase). The results showed that the MNP hydrolysis in the presence of 15i–m was much slower than that of 8a in the single-phase solvent system, as shown in Figure 3.

As shown in Figure 4a, the activity of 16i–m (20 μM) was similar to that of 9a in the two-phase solvent system with negligible catalytic turnover (note that [16i–m] = 20 μM), suggesting that more hydrophobic supermolecules have higher hydrolysis activity in the two-phase solvent system than in the single-phase solvent system. Interestingly, the MNP hydrolysis activity for 16d–h (prepared from 2, 11d–h, and Cu$^{2+}$) was higher than that of 16i–m under the same conditions, as shown in Figure 4b, and the hydrolysis yields for 16d and 16f were in excess of 20% after 1 day and in excess of 25%–40% after 7 days, indicating that these complexes had catalytic activities. Moreover, the activities of 16a and 16b were similar to that for 16d–h (Figure 5), suggesting that the azide and Boc-protected amino groups on the Bar unit provide a similar effect on the hydrolysis of MNP as those of the protected amino acid residues in 16d–g.
Figure 4. Hydrolysis of MNP (100 μM in the total solution) by (a) 9a (open circles with dashed curve), 16i (open squares), 16j (closed circles), 16k (open triangles), 16l (closed triangles), or 16m (closed squares) ([9, 16] = 20 μM in the total solution); (b) 9a (open circles with dashed curve), 16d (open squares), 16e (open circles), 16f (open triangles), 16g (closed triangles), or 16h (closed squares) ([9, 16] = 20 μM in the total solution) in CHCl₃/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (2/8) at 37 °C. The concentrations of the product (4-nitrophenol) were determined in the total two-phase solvent system.

Figure 5. Hydrolysis of MNP (100 μM in the total solution) by 9a (open circles), 9b (closed triangles), 9c (open triangles), 16a (closed circles), 16b (open squares), and 16c (closed squares) ([9, 16] = 20 μM in the total solution) in CHCl₃/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (2/8) at 37 °C. The concentrations of the product (4-nitrophenol) were determined in the total two-phase solvent system.

Figure 6 summarizes the results for the hydrolysis of MNP by 16e, 16j, 16n, 16o, and 16p, which contain serine units ([MNP] = 100 μM and [16e, 16j, 16n, 16o, and 16p] = 20 μM). The findings suggest that 16e and 16n consisting of the fully protected Ser have a higher activity than 16j, 16o, and 16p in which the Bar units contain fully or partially deprotected Ser.
The hydrolysis of MNP (100 μM) by the amphiphilic supramolecular complexes 17d or 17f (prepared from 3, 11d, or 11f, and Cu²⁺) (20 μM) was also examined. As shown in Figure 7, the hydrolytic activity of 17d and 17f were higher than 9a, but slightly lower than 16d and 16f.

The hydrolysis of MNP by 16d–m, 17d and 17f (20 μM) was carried out at higher concentrations of MNP (200, 300, 400, 500, and 1000 μM) and the typical results at [MNP] = 1000 μM are summarized in Figure 8 (the results for 9a are included in all of the graphs as standard data), in which 16d–g formed from the N-protected barbital units 11d–g showed higher hydrolysis activities. It should be noted that 16f demonstrated the highest MNP hydrolysis yield as shown in Figure 8b, and the catalytic turnover number (CTN) was more than 2 after 1 day. The CTN values for 9a, 10a, 16b, 16d–n, 17d, and 17f at [MNP] = 1000 μM, which are summarized in Figure 9, imply that the CTNs of 16b, 16e, 16f, 16g, 17d, and 17f are over 2, while these values are smaller than that of our previously reported 10a (~4) [54].
Figure 8. Hydrolysis of MNP (1000 μM in the total solution) by (a) 9a (open circles with dashed curve), 16i (open squares), 16j (open triangles), 16k (closed squares), or 16m (closed squares); (b) The hydrolysis of MNP by 9a (open circles with dashed curve), 16d (open squares), 16e (closed circles), 16f (open triangles), 16g (closed triangles), or 16h (closed squares); (c) The hydrolysis of MNP by 9a (open circles with dashed curve), 10a (closed circles with dashed curve), 16d (open squares), 16f (open triangles), 17d (closed squares), 17f (closed triangles) ([9, 16, 17] = 20 μM in the total solution) in CHCl₃/50 mM HEPES buffer (pH 7.4) with I = 0.1 (NaNO₃) (2/8) at 37 °C. The concentrations of the product (4-nitrophenol) were determined in the total two-phase system.
The results for the hydrolysis of MNP by the supramolecular phosphatases at \([\text{MNP}] = 100, 200, 300, 400, \text{ and } 500\) μM (in the total solution) were analyzed based on Michaelis–Menten kinetics, as described in our previous reports (the hydrolysis of MNP by 16d, 16h, and 16i were carried out at \([\text{MNP}] = 100, 200, 300, 400, \text{ and } 500\) μM (in the total solution)) \([19,24,53,54]\). Based on the Lineweaver–Burk plots shown in Figure 10, \(V_{\text{max}}\) (the maximum velocity for the formation of NP from MNP promoted by 9a, 16, and 17, \(\mu\)M min\(^{-1}\)), \(K_m\) (Michaelis constant, \(\mu\)M), and \(k\) (the first-order rate constant defined by Equation (1), min\(^{-1}\)) \([57]\) values were determined and summarized in Table 2.

\[
k = V_{\text{max}}/K_m
\]  

(1)

**Figure 9.** Comparison of catalytic turnover numbers (CTN) of 9a, 10a, 16b, 16d–n, 17d, and 17f at \([\text{MNP}] = 1000\) μM and \([9, 16, 17] = 20\) μM (in the total solution) in CHCl\(_3/50\) mM HEPES buffer (pH 7.4) with \(l = 0.1\) (NaNO\(_3\)) (2/8) at 37 °C.

**3.5. Michaelis–Menten Kinetics for Hydrolysis of MNP by 9, 16, and 17 in the Two-Phase Solvent System**

Figure 10. (a) Lineweaver–Burk plots for the hydrolysis of MNP catalyzed by 9a (cross with plain line), 16i (open squares with plain line), 16j (closed circles with dashed line), 16k (open triangles with plain line), 16l (closed triangles with plain line), and 16m (closed squares with dashed line). (b) Lineweaver–Burk plots for the hydrolysis of MNP by 9a (crosses with plain line), 16d (open squares with plain line), 16e (closed circles with dashed line), 16f (open triangles with dashed line), 16g (closed triangles with plain line), 16h (closed squares with plain line), and 16n (open circles with plain line).
Table 2. Kinetics parameters for the hydrolysis of MNP by 8a and AP in single-phase solvent system (10 mM HEPES buffer (pH 7.4) with \( I = 0.1 \) (NaNO₃)) at 37 °C, and 9a, 10a, 16b, 16d–n, 17d, and 17f in two-phase solvent system (CHCl₃/50 mM HEPES buffer (pH 7.4) with \( I = 0.1 \) (NaNO₃) (2/8)) at 37 °C.

| Entry | Cat. | \( V_{\text{max}} \) (\( \mu \text{M min}^{-1} \)) | \( K_m \) (\( \mu \text{M} \)) | \( k \) (min\(^{-1}\)) \(^{b} \) | \( K_i \) (\( \mu \text{M} \)) | \( K_m/K_i \) | CTN \(^{c} \) |
|-------|------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1     | 8a \(^{d} \) | \((8.9 \pm 0.2) \times 10^{-2}\) | \((4.1 \pm 0.3) \times 10^{2}\) | \((2.2 \pm 0.2) \times 10^{-4}\) | \((2.2 \pm 0.2) \times 10^{-4}\) | ca. 15 (mixed-type) \(^{d} \) | ca. 27 | 0.4 |
| 2     | AP \(^{e} \) | \((1.3 \pm 0.1)\) | \((7 \pm 4)\) | \((2.9 \pm 1.8) \times 10^{-1}\) | \((3 \pm 1 \) (competitive) \(^{e} \) | ca. 2.3 | >10\(^{3}\) | |
| 3     | 9a \(^{g} \) | \((1.4 \pm 0.4) \times 10^{-2}\) | \((5.4 \pm 0.5) \times 10^{2}\) | \((2.7 \pm 1.0) \times 10^{-5}\) | ca. 15 (competitively) \(^{g,h} \) | ca. 36 | 1.0 |
| 4     | 10a \(^{h} \) | \((6.8 \pm 0.3) \times 10^{-2}\) | \((3.8 \pm 0.2) \times 10^{2}\) | \((1.8 \pm 0.2) \times 10^{-4}\) | ca. 80 (mixed-type) \(^{h} \) | ca. 4.8 \(^{h} \) | -4 |
| 5     | 16b \(^{i} \) | \((3.6 \pm 0.2) \times 10^{-2}\) | \((2.5 \pm 0.3) \times 10^{2}\) | \((1.4 \pm 0.3) \times 10^{-4}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 2.1 |
| 6     | 16d \(^{i} \) | \((3.9 \pm 0.2) \times 10^{-2}\) | \((1.9 \pm 0.1) \times 10^{2}\) | \((2.1 \pm 0.2) \times 10^{-4}\) | 16 (competitive) | ca. 12 | 1.8 |
| 7     | 16e \(^{i} \) | \((2.4 \pm 0.2) \times 10^{-2}\) | \((1.1 \pm 0.1) \times 10^{2}\) | \((2.2 \pm 0.4) \times 10^{-4}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 2.0 |
| 8     | 16f \(^{i} \) | \((2.9 \pm 0.1) \times 10^{-2}\) | \((1.2 \pm 0.1) \times 10^{2}\) | \((2.4 \pm 0.3) \times 10^{-4}\) | 23 (competitive) | ca. 5.4 | 2.7 |
| 9     | 16g \(^{i} \) | \((2.3 \pm 0.2) \times 10^{-2}\) | \((1.0 \pm 0.1) \times 10^{2}\) | \((2.4 \pm 0.4) \times 10^{-4}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 2.1 |
| 10    | 16h \(^{i} \) | \((1.2 \pm 0.1) \times 10^{-2}\) | \((3.6 \pm 0.5) \times 10^{-4}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | n.d. \(^{i} \) | 1.1 |
| 11    | 16i \(^{i} \) | \((8.1 \pm 0.2) \times 10^{-3}\) | \(13 \pm 1\) | \((6.0 \pm 0.4) \times 10^{-4}\) | 0.67 (competitive) | ca. 20 | 1.3 |
| 12    | 16j \(^{i} \) | \((1.0 \pm 0.1) \times 10^{-2}\) | \((4.7 \pm 0.2) \times 10^{2}\) | \((2.2 \pm 0.2) \times 10^{-5}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 1.6 |
| 13    | 16k \(^{i} \) | \((1.4 \pm 0.1) \times 10^{-2}\) | \((7.6 \pm 0.2) \times 10^{2}\) | \((1.9 \pm 0.2) \times 10^{-5}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 1.0 |
| 14    | 16l \(^{i} \) | \((4.5 \pm 0.2) \times 10^{-3}\) | \((3.5 \pm 0.1) \times 10^{2}\) | \((1.3 \pm 0.1) \times 10^{-5}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 1.6 |
| 15    | 16m \(^{i} \) | \((7.8 \pm 0.3) \times 10^{-3}\) | \(40 \pm 1\) | \((2.0 \pm 0.1) \times 10^{-4}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 1.2 |
| 16    | 16n \(^{i} \) | \((2.2 \pm 0.2) \times 10^{-2}\) | \(72 \pm 2\) | \((3.0 \pm 0.3) \times 10^{-4}\) | n.d. \(^{i} \) | n.d. \(^{i} \) | 1.7 |
| 17    | 17d \(^{i} \) | \((2.5 \pm 0.2) \times 10^{-2}\) | \((1.2 \pm 0.1) \times 10^{2}\) | \((2.2 \pm 0.4) \times 10^{-4}\) | 17 (mixed-type) | ca. 7.4 | 2.0 |
| 18    | 17f \(^{i} \) | \((2.3 \pm 0.2) \times 10^{-2}\) | \(47 \pm 2\) | \((5.0 \pm 0.6) \times 10^{-4}\) | 5.7 (competitive) | ca. 8.3 | 2.3 |

\(^{a}\) Cat = catalyst. \(^{b}\) Calculated by eq. (1). \(^{c}\) Catalytic turnover numbers determined at [MNP] = 1000 \( \mu \text{M} \) (see Figure 9). \(^{d}\) From ref. 24 (in a single aqueous solution (10 mM HEPES buffer, pH 7.4 with \( I = 0.1 \) (NaNO₃)). \(^{e}\) From ref. 19 (in a single aqueous solution (10 mM HEPES buffer, pH 7.4 with \( I = 0.1 \) (NaNO₃)). \(^{f}\) Calculated from data in ref. 19. \(^{g}\) From ref. 53. \(^{h}\) From ref. 54. \(^{i}\) Determined in CHCl₃/50 mM HEPES buffer (pH 7.4) with \( I = 0.1 \) (NaNO₃) (2/8). \(^{j}\) Not determined.
The V\textsubscript{max} and K\textsubscript{m} values for 9a were reported to be (1.4 ± 0.4) × 10\textsuperscript{−2} µM min\textsuperscript{−1} and (5.4 ± 0.5) × 10\textsuperscript{2} µM (entry 3 in Table 2), respectively, in a two-phase solvent system in our previous report [53]. Among 16b, 16d–n, 17d, and 17f tested in this work (entry 5–18 in Table 2), 16d had the highest V\textsubscript{max} (3.9 × 10\textsuperscript{−2} µM min\textsuperscript{−1}) (entry 6) and 16f had the highest CTN (2.7) (entry 8).

The hydrolysis of MNP by 9a, 16d, 16f, 16i, 17d, and 17f in the presence of HPO\textsubscript{4}\textsuperscript{2−}, a product and an inhibitor of MNP hydrolysis, was also carried out to determine the K\textsubscript{i} values of HPO\textsubscript{4}\textsuperscript{2−} as listed in Table 2. Interestingly, it was indicated that 9a, 16d, 16f, and 16i are inhibited by inorganic phosphate in a competitive manner and the K\textsubscript{i} values for 16d (16 µM) and 16f (23 µM) (entries 6 and 8) was almost comparable to that for 9a (ca. 15 µM) (entry 3). In contrast, the K\textsubscript{i} value for 16i is 0.67 µM (entry 11), which is much smaller than the others. We previously reported that smaller K\textsubscript{m}/K\textsubscript{i} values are good parameters for assessing the catalytic activity of the MNP catalysts [54]. For example, the K\textsubscript{m}/K\textsubscript{i} values for 10a (entry 4) and 16f, both of which function as catalysts are ca. 4.8 and 5.4 (entries 4 and 8), respectively, and these values are close to that for AP (ca. 2.3) (entry 2), and smaller than the corresponding values for 16d (ca. 12) and 16i (ca. 20) (entries 6 and 11), whose CTN values are 1.2–1.8.

Another important point is that 16d, 16f, 16i, and 17f are inhibited by HPO\textsubscript{4}\textsuperscript{2−} in a competitive manner (entries 6, 8, 11, and 18), like that of AP. This type of inhibition is different from our previous supramolecular phosphatases such as 8a (entry 1) that functions in a single-phase aqueous solution and 10a that functions in a two-phase solvent system. Based on the above findings, we conclude that the quantitative distribution of 16d, 16f, 16i, and 17f in the organic phase (Table 1) adequately mimics the binding mode of AP with the substrate and inhibitors (MNP and HPO\textsubscript{4}\textsuperscript{2−}, respectively, in this case).

The K\textsubscript{m} values for 16d–h (entries 6–10) (0.3–1.9 × 10\textsuperscript{2} µM) are lower than that for 10a (entry 4) (3.8 × 10\textsuperscript{2} µM), although the K\textsubscript{i} values for 16d, 16f, and 16i (16, 23, and 0.67 µM, respectively) are also lower than that for 10a (80 µM). The proposed structure of 16f generated by BIOVIA Discovery Studio (Ver: 17.2.0) based on the crystal structure of 8a (Figure 11a, in which Cu\textsuperscript{2+}-bound H\textsubscript{2}O molecules are omitted for clarity) [24] suggests that the side chain of the 11f unit in 16f (yellow spheres) is located over the Cu\textsubscript{2}(µ-OH)\textsubscript{2} core, which results in the formation of a hydrophobic pocket similar to the active site of AP, as shown in Figure 11b,c. Therefore, we conclude that the hydrophobicity of supramolecular complexes is important in terms of improving the stabilization of the supermolecule–MNP complexes (i.e., the ES complexes) that have smaller K\textsubscript{m} values and the effective extraction of MNP from the aqueous layer. Namely, the hydrophobic active site of the artificial supramolecular complexes is important for mimicking the hydrophobic active site of natural AP and hydrophobicity/hydrophilicity balance is important for catalytic activity.
In conclusion, we report on the design, and synthesis of Bar building units that are functionalized with amino acids and related units, with which many supramolecular complexes are formed by the 2:2:2 self-assembly of 2 (Zn$_2$L$^2$) and Cu$^{2+}$ in a two-phase solvent system based on the structure of the active site of AP. The ease of functionalizing the barbital units allowed us to construct a series of various supramolecular complexes which were then used to assess their catalytic activity for the hydrolysis of MNP and related studies of the reaction mechanism. A comparison of the hydrolysis of MNP in a single-phase system and that in the two-phase system in the presence of 8, 9, 15, or 16 strongly suggest that two-phase solvent system contributes to the improvement in the activity of MNP hydrolysis by reducing the product inhibition by HPO$_4^{2-}$. In addition, the hydrolysis of MNP by the hydrophobic supramolecular complexes follows Michaelis–Menten kinetics, and functionalization with Boc-protected phenylalanine (16f) results in a higher $V_{\text{max}}$ and a lower $K_{m}$, and a greater $k$ value than the corresponding values for 9a. The $K_m/K_i$ value for 16f (ca. 5.4) is close to that for AP (ca. 2.3) and competitive inhibition by inorganic phosphate was observed, unlike for our previously reported complexes such as 10a–c. We therefore conclude that the distribution of the Cu$_2$(μ-OH)$_2$ active site of the supermolecules in both layers in the two-phase solvent system contributes to the improvement of catalytic turnover, and the formation of hydrophobic Cu$_2$(μ-OH)$_2$ site mainly in organic layer appropriately mimics the binding mode of AP with the substrates (for the E–S complexation) and the

![Figure 11.](image-url)
inhibitors (product inhibition). These findings should be highly useful for the design of more efficient catalysts in reference to biochemistry including enzymatic reactions.

**Author Contributions:** Y.M. carried out the synthesis of the barbital derivatives, UV/Vis titrations of supramolecular complexes, and hydrolysis of MNP by supramolecular complexes. A.B.R., H.I., and Y.H. carried out some of the measurements for the hydrolysis of MNP by the supramolecular complexes. Y.S. synthesized some of the barbital derivatives and carried out the hydrolysis of MNP by the supramolecular complexes. S.A. supervised all experiments and the preparation of the manuscript. All of the authors have read and approved the final version of the manuscript.

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