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Peptide Synthesis in Aqueous Solution. IV. Preparation and Properties of [*p*-(Benzyloxycarbonyloxy)phenyl]dimethylsulfonium Methyl Sulfate (Z-ODSP), [*p*-(*t*-Butoxycarbonyloxy)phenyl]dimethylsulfonium Methyl Sulfate (Boc-ODSP) and [*p*-(9-Fluorenylmethyloxycarbonyloxy)phenyl]dimethylsulfonium Methyl Sulfate (Fmoc-ODSP) as Water-Soluble *N*-Acylating Reagents†

Ikunori Azuse, Masahiro Tamura, Keisuke Kinomura, Hideo Okai,* Katsushige Kouge,†† Fumio Hamatsu,†† and Tatsuya Koizumi††

Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Shitami, Saijo-cho, Higashihiroshima 724

††Sanshin Chemical Industrial Co. Ltd., 531, Nagahama, Hirao-cho, Kumage-gun, Yamaguchi 742-11

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In order to increase the applications of the water-soluble active ester method, water-soluble acylating reagents, [*p*-(benzyloxycarbonyloxy)phenyl]dimethylsulfonium methyl sulfate, [*p*-(*t*-butoxycarbonyloxy)-phenyl]dimethylsulfonium methyl sulfate and [*p*-(9-fluorenylmethyloxycarbonyloxy)phenyl]dimethyl-sulfonium methyl sulfate, were synthesized from of *p*-hydroxyphenyldimethylsulfonium methyl sulfate. These compounds had high solubility (more than 30%) in water and were found to act as excellent water-soluble acylating reagents for the synthesis of *N*-benzyloxycarbonyl, *N*-*t*-butoxycarbonyl, *N*-9-fluorenylmethyl-oxycarbonyl amino acid derivatives in aqueous solution. They converted dipeptides to *N*-acyl-dipeptides in aqueous media as well as amino acids. In addition, they were found to be efficient reagents for protecting the side chains of lysine, ornithine, and cysteine.

In 1987, we reported that *p*-hydroxyphenyldi-methylsulfonium methyl sulfate (HODSP) was found to be an excellent reagent for peptide synthesis in aqueous solution.[1] We also pointed out that HODSP has a high reactivity, as the same as that of conventional active ester reagents, such as *p*-nitrophenol or *N*-hydroxysuccinimide. By carrying out peptide synthesis in aqueous solution we can strictly control the pH during the reaction. This means that arginine residue, which has a guanide group in the side chain, can be treated while unprotected. By employing the HODSP active ester method, FMRFamide (Phe-Met-Arg-Phe-NH_2), a molluscan neuropeptide from the ganglia of the clam *Macrocallisa nimbosa*,[2] was synthesized.[3] The water-soluble active ester method is an ideal method for both Arg- and Met-containing peptide, such as FMRFamide. The peptide can be prepared without hydrogenation or HF deprotection, which is known to sometime severely damage the peptide during the final step. We prepared several FMRFamide analogs by using this water-soluble active ester method as well as naturally occurring FMRFamide.[4]

After we established peptide synthesis using water-soluble active ester, we then set out to develop new types of water-soluble acylating reagents. We selected three amino protecting groups (benzyl-oxycarbonyl-, *t*-butoxycarbonyl, and 9-fluorenyl-methyloxycarbonyl groups) which are widely used in peptide synthesis; their deprotecting conditions are all different. [*p*-(Benzyloxycarbonyloxy)phenyl]dimethyl-sulfonium methyl sulfate (**1**), [*p*-(*t*-butoxycarbonyloxy)-phenyl]dimethylsulfonium methyl sulfate (**2**), and [*p*-(9-fluorenylmethyloxycarbonyloxy)phenyl]dimethyl-sulfonium methyl sulfate (**3**) were prepared and practically allowed to react with amino acids in aqueous media. Furthermore, we applied these water-soluble acylating reagents to the selective side-chain protection of amino acids (lysine, ornithine, and cysteine). In this paper we discuss the utility and applicability of water-soluble acylating reagents in peptide synthesis.

Results and Discussion

Preparation of [*p*-Benzyloxycarbonyloxy)phenyl]dimethylsulfonium Methyl Sulfate (1), [*p*-(*t*-Butoxycarbonyloxy)phenyl]dimethylsulfonium Methyl Sulfate (2), and [*p*-(9-Fluorenylmethyloxycarbonyloxy)-phenyl]dimethylsulfonium Methyl Sulfate (3). Compounds **1**, **2**, and **3** were all easily synthesized from the reaction of HODSP with benzyloxycarbonyl chloride, di-*t*-butyl dicarbonate, and 9-fluorenylmethyloxy-carbonyl chloride, respectively, in CH_3CN. These compounds were stable and did not undergo decomposition when stored below 10 °C. Compounds **1**, **2**, and **3** were soluble in water (solubility:>30 wt%) and very soluble in DMF, alcohol, CH_3CN, and $CHCl_3$.

Preparation of Benzyloxycarbonyl, *t*-Butoxycarbonyl, 9-Fluorenylmethyloxycarbonyl Amino Acids Derivatives. In order to establish optimal conditions for the reaction between amino acids and **1**, **2**, or **3** in aqueous solution, the effect of five bases (NaOH, Na_2CO_3,

† This is one part of studies on the water-soluble active ester method for peptide synthesis. The previous studies (part I, II, and III) appeared in this journal. See Refs. 1, 3, and 4.

$NaHCO_3$, triethylamine, and pyridine) in acylation of alanine were first studied. It was found that all, except pyridine, promoted the reaction and sodium hydroxide and triethylamine gave the highest yield (83%) of *N*-(benzyloxycarbonyl)-L-alanine.

The reaction rate of **1** and **2** with glycine in aqueous solution at pH 7—10 was measured by HPLC. The rate of **1** and **2** with glycine in several concentrations was also measured. It was found that **1** and **2** gave Z-Gly and Boc-Gly in highest yield when they were treated at a pH concentration greater than 10 and 0.25 M (1 M=1 mol dm^{-3}), respectively. The benzyloxycarbonylation of glycine with **1** (0.25 M) came to completion within 2 h at pH 8. In the case of **2**, the reaction was over within 2 h at pH 10. These results showed that the standard conditions for the reaction involve using triethylamine as a base in aqueous solution at a pH and concentration greater than 10 and 0.25 M, respectively, at room temperature. The yield of *N*-acyl amino acid derivatives, prepared in aqueous solution, are shown in Tables 1, 2, and 3. *N*-Acyl amino acid derivatives were obtained in good yields from **1**, **2**, and **3** only in aqueous media. Even if an aqueous-acetonitrile system was employed for the solvent, the yields of *N*-aryl amino acid derivatives were still good. This result definitely shows that these water-soluble acylating reagents could be used as well as conventional *N*-acylating reagents. It is another merit of water-soluble acylating reagents that the unreacted reagents and HODSP, which forms during the reaction as a by-product, can easily be removed because of their high solubility in water.

We studied a possible side reaction, *N*-methylation, because HODSP has methyl sulfate as a counter anion. We collected some of the reaction mixture of the water-soluble acylating reagent and amino acid, and then carried out the deprotection procedure on that mixture; the resultant mixture was applied to HPLC. No *N*-methyl amino acid was found.

***N*-Acylation of Dipeptides in Aqueous Solution.** The active ester method is generally carried out under mild conditions rather than acid halides, acid anhy-

Table 1. Preparation of Z-Amino Acids in Aqueous Solution[a)]

Z-amino acids	Yield(%)	Mp/°C (lit)	$[\alpha]_D^{25}$ (*c*, solvent) (lit)	Ref.
Ala	83	84—86 (87)	−12.8° (*c* 2.0, AcOH) (−13.9°)	10
Arg(NO_2)	88	130—132 (132—134)	−3.0° (*c* 2.0, MeOH) (−3.5°)	11
Asp	85	115—116 (116)	+7.9° (*c* 7.0, AcOH) (+9.6°)	12
Asn	95	162—163 (165)	+7.3° (*c* 1.6, AcOH) (+7.6°)	13
Glu	82	118—121 (120—121)	−6.8° (*c* 10.0, AcOH) (−7.9°)	14
Gly	90	119—120 (120)	—	12
His	51	165—167 (166—167)	−23.0° (*c* 6.0, 6M HCl) (−25.0°)	15
Ile	85	42—45 (44—46)	+5.9° (*c* 6.0, EtOH) (+6.5°)	16
Leu[b)]	78	149—150 (151.5—152)	−7.3° (*c* 3.05, MeOH) (−7.86°)	17
Met	81	66—67 (68—69)	−14.3° (*c* 2.47, EtOH) (−16.6°)	18
Phe	91	87—89 (88—89)	+5.0° (*c* 2.0, AcOH) (+5.1°)	19
Pro	86	77—80 (77)	−59.0° (*c* 2.0, AcOH) (−60.5°)	19
Ser	85	119—122 (121)	+4.9° (*c* 6.0, AcOH) (+5.6°)	20
Thr	82	97—102 (101—102)	−5.0° (*c* 2.0, AcOH) (−5.6°)	21
Trp	71	123—128 (126)	+2.9° (*c* 1.0, AcOH)	22
Tyr	70	94—98 (101)	+9.7° (*c* 3.0, AcOH) (+11.1°)	12
Val	82	63—66 (66—67)	+0.1° (*c* 10.0, AcOH) (+0.1°)	19
Gly[c)]	88	119—120 (120)		12

a) The reactions were carried out using water as solvent and triethylamine as a base.
b) Dicyclohexylammonium salt. c) This reaction was carried out in water-acetonitrile.

Table 2. Preparation of Boc-Amino Acids in Aqueous Solution[a)]

Boc-amino acids	Yield (%)	Mp/°C (lit)	$[\alpha]_D^{25}$ (*c*, solvent) (lit)	Ref.
Ala	82	82—84 (83—84)	−1.5° (*c* 2.0, DMF) (−13.6°)	23
Arg(NO_2)	78	111—113 (111—114)	−4.8° (*c* 1.0, AcOH) (−6.2°)	24
Asp	75	110—116 (118—119)	−3.0° (*c* 1.0, DMF) (−3.9°)	25
Asn	70	179—183 (181—182)	−8.2° (*c* 1.0, AcOH) (−8.96°)	25
Glu	70	108—109 (110—112)	−16.0° (*c* 1.0, MeOH) (−16.1°)	26
Gly	90	88—89 (88.5—89)	—	26
Ile[b)]	80	47—55 (49—57)	+3.0° (*c* 2.0, AcOH) (+3.0°)	23
Leu	75	72—74 (70—74)	−27.0° (*c* 1.0, AcOH) (−27.0°)	27
Met[c)]	77	120—123	−0.99° (*c* 1.0, AcOH)	
Phe	84	84—87 (85—87)	−3.7° (*c* 1.0, AcOH) (−4.1°)	28
Pro	75	134—136 (136—137)	−57.2° (*c* 2.0, AcOH) (−59.5°)	23
Ser[c)]	74	143—144	−0.9° (*c* 1.0, AcOH)	
Thr[c)]	77	151—152	−3.0° (*c* 1.0, AcOH)	
Tyr[c)]	70	199—203 (205—208)	−5.5° (*c* 1.0, AcOH) (−6.3°)	29
Val[c)]	78	139—140 (140.5)	+0.01° (*c* 1.0, AcOH) (+0.01°)	30

a) The reactions were carried out using water as solvent and triethylamine as a base. b) Hemihydrate. c) Dicyclohexylammonium salt.

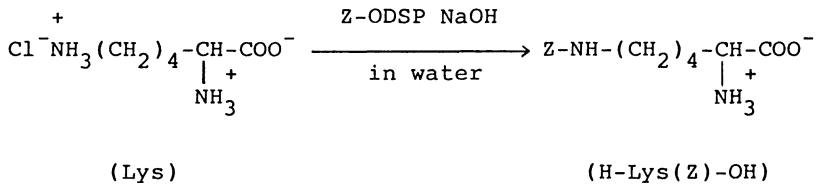
Table 3. Preparation of Fmoc-Amino Acids in Aqueous Solution[a)]

Fmoc-amino acids	Yield (%)	Mp/°C (lit)	$[\alpha]_D^{25}$ (*c*, solvent) (lit)	Ref.
Ala	86	141—143 (143—144)	−19.1° (*c* 1.0, DMF) (−18.6)	31
β-Ala	82	144—146 (145—147)		32
Gln	78	220—222 (221—223)	−18.4° (*c* 1.0, DMF) (−17.0°)	31
Gly	94	172—173 (173—174)		31
Leu	76	151—153 (153—154)	−26.1° (*c* 1.0, DMF) (−24.1°)	31
Met	80	127—131 (129—132)	−29.1° (*c* 1.0, DMF) (−28.3°)	31
Phe	83	180.5—183 (181—183)	−35.6° (*c* 1.0, DMF) (−37.6°)	31
Pro	85	112—113 (114—115)	−34.0° (*c* 1.0, DMF) (−33.4°)	31
Val	75	142—144 (143—144)	−17.0° (*c* 1.0, DMF) (−16.1°)	31
Gly[b)]	87	172—173 (173—174)		31

a) The reactions were carried out using water as solvent and triethylamine as a base. b) This reaction was carried out in water-acetonitrile.

drides or other condensing reagents. The water-soluble active ester can form an amide linkage under much milder conditions than other active esters. We can control the reactivity of the active ester by changing the pH.

N-Acylation of dipeptides in aqueous solution using **1**, **2**, or **3** were carried out. *N*-Acyl-dipeptides were obtained (Yield; Z-Phe-Gly-OH, 77%; Boc-Leu-



Scheme 1.

Val-OH, 70%; Fmoc-Gly-Gly-OH, 75%) in aqueous solution without any side reaction because the pH of these reaction mixtures was strictly controlled.

Selective Acylation of Amino Groups on the Side Chain of Lysine and Ornithine. The other merit of the reaction in water is that we could presume selective acylation of the amino group of side chains of lysine and ornithine. Compound **1** was allowed to react with lysine by controlling the pH of the reaction mixture automatically. The synthetic route of N^{ε}-(benzyloxycarbonyl)-L-lysine (H-Lys(Z)-OH) is shown in Scheme 1. We studied the yield of H-Lys(Z)-OH and Z-Lys(Z)-OH by changing the pH of the reaction. The amino group on the side chain of lysine was selectively benzyloxycarbonylated and H-Lys(Z)-OH was obtained only during one step by strictly controlling the pH of the reaction mixture. A small amount of Z-Lys(Z)-OH, which was produced at the same time, was easily washed out with ethyl acetate. Lysine was also treated with two equivalents of **1**. We recognized a selective formation of H-Lys(Z)-OH at pH 8 and 9. The highest yield of H-Lys(Z)-OH was obtained at pH 11.5 (63.6%); that condition kept the yield of Z-Lys(Z)-OH, a by-product, below 5.4%. The reaction with Z-OSu (dioxane-water as a solvent), which is a commercial benzyloxycarbonylating reagent, was then studied. A selective aminolysis reaction was not observed since the reaction requires organic solvents and the pH of the reaction mixture can not be accurately controlled.

Ornithine was treated with **1** under the same conditions. The same principle can also be applied to the selective benzyloxycarbonylation of the ω-amino group of ornithine. In the case of the reaction in two equal quantities of **1**, we recognized a selective benzyloxycarbonylation of δ-amino group as well as lysine. Presently, the synthetic route of H-Lys(Z)-(OH) or H-Orn(Z)-OH is first to form a Cu(II) complex of lysine or ornithine; they are then allowed to react with benzyloxycarbonyl chloride.[5-9] The Cu(II) complex is removed by hydrogen sulfide. This procedure requires three steps and decreases the yield of the product. By using of this water-soluble acylating reagent, H-Lys(Z)-OH or H-Orn(Z)-OH can be prepared in one step.

Preparation of *S*-(Benzyloxycarbonyl)-L-cysteine. The mercapto group in cysteine is too reactive to be left unprotected, since it is a stronger nucleophilic group than the amino group and is easily converted to disulfides by air oxidation as well. We investigated the reactivity of the mercapto group of cysteine at various pH's in aqueous media using **1**. *S*-(Benzyloxycarbonyl)-L-cysteine was obtained in one step at pH 6.0 or 6.5 (61%), as well as the case of selective acylation of lysine and ornithine, which have amino groups in their side chains.

We dealt with three typical *N*-protecting groups in peptide synthesis. We also showed that the introduction of those groups using the water-soluble active ester could be carried out successfully. It is clear that the water-soluble active ester method can be applied to other urethane-type amino-protecting groups. Additionally, side-chain-protected amino acids, such as H-Lys(Z)-OH, H-Orn(Z)-OH, and H-Cys(Z)-OH were prepared using the water-soluble active esters very easily. We believe that the water-soluble acylating reagent will also be a powerful tool for the chemical modification of proteins in aqueous solution without causing any conformational changing. We plan to show this elsewhere shortly.

Experimental

All melting points were uncorrected. IR spectra were recorded on a Hitachi 269-30 infrared spectrometer. NMR spectra were measured on a Hitachi R-24B (60 MHz) spectrometer. The chemical shifts are given in δ scale with tetramethylsilane as an internal standard. Amino acid analysis and detection of substances were performed by means of HPLC using JASCO TRI ROTER-V and UNIDEC-100V apparatus.

Synthesis of Z-ODSP(1), Boc-ODSP(2), and Fmoc-ODSP (3). HODSP (13.3 g, 50 mmol) was dissolved in CH_3CN (200 ml), and Et_3N (7.0 ml, 50 mmol) was added to the solution below 5 °C with stirring. After 10 min, Z-Cl, $(Boc)_2O$, or Fmoc-Cl (50 mmol) was added. The reaction mixture was stirred below 5 °C for 2—5 h and then the precipitated triethylammonium chloride or unreacted reagents was removed from the reaction mixture by filtration. The filter cake washed with CH_3CN, and combined filtrates were concentrated in vacuo to give products as crystalline solid, which was readily recrystallized from ethyl acetate.

Z-ODSP (1). Yield 90.0%; mp 78—80 °C. IR data ν_{max}^{KBr} 1760 cm^{-1} (ester C=O). ^1H NMR data (DMSO-d_6); δ=3.30 (s, 6H, $-S^+(CH_3)_2$), 3.42 (s, 3H, $CH_3SO_4^-$), 5.35 (s, 2H, benzyl CH_2), 7.41 (2.5H, C_6H_5), 7.92 (dd, 4H, J_1=31 Hz, J_2=6 Hz, $-O-C_6H_4-S<$). Found: C, 51.42; H, 4.58%. Calcd for $C_{17}H_{20}O_7S_2$: C, 51.51; H, 4.99%.

Boc-ODSP (2). Yield 80.0%; mp 118.5—121 °C. IR data ν_{max}^{KBr} 1760 cm^{-1} (ester C=O). ^1H NMR (DMSO-d_6); δ= 1.50 (s, 9H, *t*-butyl), 3.30 (s, 3H, $CH_3SO_4^-$), 3.40 (s, 6H,

$-S^+-(CH_3)_2$), 7.85 (dd, 4H, J_1=33 Hz, J_2=8 Hz, $-O-C_6H_4-S<$). Found: C, 45.78; H, 6.31%. Calcd for $C_{14}H_{22}O_7S_2$: C, 45.92; H, 6.00%.

Fmoc-ODSP (3). Yield 85.0%; mp 90.5—92 °C. IR data ν_{max}^{KBr} 1760 cm^{-1} ster C=O). ^1H NMR ($CDCl_3$); δ=3.41 (s, 6H, $-S^+(CH_3)_2$), 3.68 (s, 3H, $CH_3SO_4^-$), 4.30 (t, 1H, J=7 Hz, $>CH-CH_2-OCO_2-$), 4.54 (d, 2H, J=7 Hz, $>CH-CH_2-OCO_2-$), 7.24—7.81 (m, 10H, aromatic protons of Fmoc+right peaks of dd of $-O-C_6H_4-S<$), 8.15 (d, 2H, J=11 Hz, left peaks of dd of $-O-C_6H_4-S<$). Found: C, 60.96; H, 5.07%. Calcd for $C_{24}H_{24}O_7S_2$: C, 61.02; H, 5.12%.

***N*-Benzyloxycarbonyl Amino Acids, *N-t*-Butoxycarbonyl Amino Acids and *N*-(9-Fluorenylmethyloxycarbonyl) Amino Acids (General Method).** L-Amino acid (10 mmol) and Et_3N (12 mmol) were dissolved in water (10 ml) and Z-ODSP, Boc-ODSP, or Fmoc-ODSP (12 mmol) was added to the solution at room temperature with stirring. After 8—24 h, 1 M HCl or 20% citric acid was added to the solution and the pH of the solution was adjusted to 3.0. In the case of Fmoc-amino acids, water was added to the reaction mixture, then extracted with ether; the aqueous phase was acidified with 1 M HCl. The mixture was extracted with ethyl acetate. The solution was washed with water and then dried over anhydrous sodium sulfate. The filtrate was concentrated in vacuo. The residue was recrystallized from the solvents indicated. Side chain amino acid derivatives were prepared using different procedures.

Synthesis of *N*-Acyl Dipeptides. Z-Phe-Gly-OH. H-Phe-Gly-OH (1.07 g, 3 mmol) was adjusted to 10.0 by 1 M NaOH. Z-ODSP (1.44 g, 3.3 mmol) was added to a stirring solution and the reaction mixture was acidified with 20% citric acid. The mixture was extracted with ethyl acetate. The solution was then washed with water and dried over anhydrous sodium sulfate. The solution was concentrated and crystallized from ether; Yield 77%; mp 158—160 °C [lit, 159—161 °C].[33] Prepared similarly were the following dipeptide derivatives:

Z-Ile-Phe-OH. Yield 72%; mp 174—176 °C [lit, 175—176 °C].[34]

Boc-Leu-Val-OH. Yield 70%; mp 108—110 °C [lit, 109—111 °C].[35]

Fmoc-Gly-Gly-OH. Yield 75%; mp 176—177 °C [lit, 176—177 °C].[32]

Preparation of H-Lys(Z)-OH and H-Orn(Z)-OH. Lys·HCl (1.83 g, 10 mmol) and 2 M NaOH (5 ml) were dissolved in water (50 ml) and the pH of the solution was adjusted to 11.3 by 1 M HCl. Z-ODSP (6.4 g, 16 mmol) was added to a stirring solution below 20 °C, the pH being automatically maintained at the starting pH with 2 M NaOH for 30 min. The precipitate was filtered and washed with water and ethanol. By a similar procedure, H-Orn(Z)-OH (at pH 11.5) was obtained.

H-Lys(Z)-OH. Yield 63.6%; mp 250—253 °C; $[\alpha]_D^{25}$ +16.0° (*c* 1.0, 6 M HCl). Found: C, 59.92; H, 7.12; N, 9.94%. Calcd for $C_{14}H_{20}N_2O_4$: C, 59.98; H, 7.19; N, 9.99%.

H-Orn(Z)-OH. Yield 80%; mp 254—255 °C; $[\alpha]_D^{25}$+17.3° (*c* 0.7, 6 M HCl). Found: C, 58.57; H, 6.84; N, 10.42%. Calcd for $C_{13}H_{18}N_2O_4$: C, 58.63; H, 6.81; N, 10.52%.

Preparation of H-Cys(Z)-OH. Cys·HCl (10 mmol) and Et_3N (1.4 ml) were dissolved in water (50 ml) and pH of the solution was adjusted to 6.0 by 1 M HCl. Z-ODSP (10 mmol) was added to a stirred solution below 20 °C, the pH being automatically maintained at the starting pH with 2 M NaOH for 5 h. The precipitate was filtered and washed with water and ethanol. Yield 61%, mp 162—164 °C, $[\alpha]_D^{25}$—122° (*c* 1.0, DMF). Found: C, 51.72; H, 5.15; N, 5.41%. Calcd for $C_{11}H_{13}NO_4S$: C, 51.75; H, 5.13; N, 5.49%.

Determination of the Rate of Aminolysis. pH Effect: Gly or Val (5 mmol) was dissolved in water (10 ml) and the pH of the solution was adjusted to 7—10 by 2 M HCl. Z-ODSP (**1**, 5 mmol) was added to the solution and the total volume of the solution was increased up to 20 mol by water and stirred. The pH was automatically maintained at the starting pH with 2 M NaOH. At predetermined time intervals, 0.1 ml aliquots of the solution were withdrawn and diluted with 4% citric acid (1 ml) and distilled water (total volume, 100 ml). The consumption of Gly or Val was measured using HPLC (column, AApack Na; eluent, 0.1 M citrate buffer; pH 4.25; 60 °C).

Concentration Effect: Gly or Val (5 mmol) was dissolved in water and the concentration of the solution was adjusted to various concentrations (0.01—1 M). Z-ODSP (**1**, 5 mmol) was added to a solution and stirred. The pH was automatically maintained at the starting pH with 2 M NaOH. At determined time intervals, the same procedures were carried out as described above.

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