Amino Acids and Peptides. XL. Synthesis of Ac–Tyr–Val–Ala–Asp–MCA Using Newly Developed Acetylating Reagent[1,2]

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2-Acetoxy-3-benzyl-5-methyl-6-isobutylpyrazine was prepared by cyclization of H–Phe–Leu–CH_2Cl, followed by acetylation with acetic anhydride. This pyrazine derivative can react with amino groups of amino acids or peptides to produce acetyl amino acids or acetyl peptides without acetylation of hydroxy group of Tyr, Ser and Thr. Using this acetylating reagent, Ac–Tyr–Val–Ala–Asp–MCA, which is a specific substrate of the interleukin-I (IL-I) processing enzyme, was prepared.

Key words acetylating reagent; pyrazinone derivative; acetoxypyrazine derivative; Ac–Tyr–Val–Ala–Asp–MCA; specific substrate; interleukin-I processing enzyme

For the synthesis of biologically active peptides or specific substrates for measurement of enzyme activities, acetylation of an amino group is sometimes required. Various kinds of acetylating reagents such as acetyl chloride, acetyl imidazole and acetic anhydride, have been employed for the purpose. 2-Acetoxy-3,6-dialkyl pyrazines have also been applied to acetylation of amines with good results.[3] However, synthesis of 2(1*H*)-pyrazinone derivatives is not simple.[4–6] This paper deals with a novel synthetic procedure for 2-acetoxypyrazine derivatives and evaluation of their acetylation reactivity towards amino acids or peptides and their utility for the synthesis of Ac–Tyr–Val–Ala–Asp–MCA, which is a specific substrate of the IL-I processing enzyme.[7]

Previously, we reported a convenient synthetic procedure for 2(1*H*)-pyrazinone derivatives.[8,9] 2-Acetoxypyrazine derivatives were prepared, according to Fig. 1. Boc–Phe–Leu–CH_2Cl[10] was prepared by the coupling of Boc–Phe–OH and H–Leu–CH_2Cl by the mixed anhydride method.[11] After removal of the Boc group with HCl/dioxane, HCl·H–Phe–Leu–CH_2Cl in MeOH was refluxed for a short time to afford 3-benzyl-5-methyl-6-isobutyl-2(1*H*)-pyrazinone, which was treated with acetic anhydride to give the desired compound, 2-acetoxy-3-benzyl-5-methyl-6-isobutylpyrazine (**1**). 2-Acetoxy-3,5-dimethyl-6-isobutylpyrazine (**2**) was also prepared similary, starting with Boc–Ala–Leu–CH_2Cl.

First of all, the reactivity of the above two compounds (**1**, **2**) with aniline was examined in various solvents (DMF, dioxane, DCM, benzene), because a suitable acylating method of aniline was required for the preparation of acylanilide derivatives. As an example, the method used to determine the reaction rate between compound (**1**) and aniline in benzene will be described. The decrease of **1** and increase of acetanilide and liberated 3-benzyl-5-methyl-6-isobutyl-2(1*H*)-pyrazinone were monitored by HPLC. The peak areas corresponding to **1** and acetanilide were plotted as a function of time, as shown in Fig. 2, and the half time ($t_{1/2}$) was determined. The half times of the reaction of **1** with aniline in various solvents are summarized in Table 1 in comparison with those of compound **2**.

The reactions of the 2-acetoxypyrazine derivatives (**1**, **2**) with aniline proceeded slightly in DMF, dioxane or DCM [120 h (2—7%)], but in benzene, the reactions were dramatically accelerated (half times were 7.5 and 12 h, respectively). It is possible that the activation of 2-acetoxypyrazine derivatives occurs by intramolecular general base catalysis and thus is favored in a non-polar solvent, benzene.[12] However, the above reactions are very slow in DCM and dioxane. Benzene might have the effect of increasing the electron density at the amino group of aniline. These results showed that our acetylating reagents were not suitable for acetylation of the amino group of

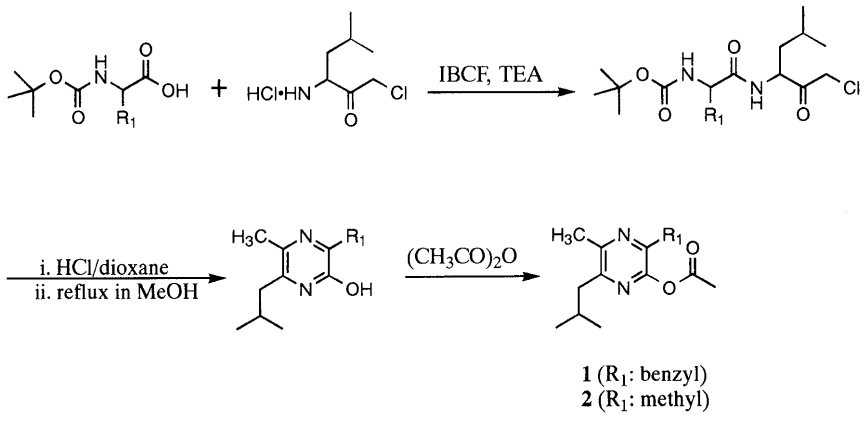

Fig. 1. Synthetic Route to 2-Acetoxypyrazine Derivatives

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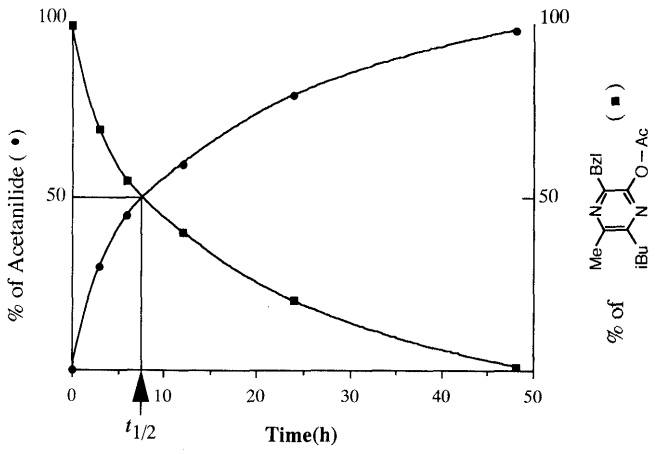

Fig. 2. Reactivity of 2-Acetoxypyrazine Derivative with Aniline in Benzene

Table 1. Half Time ($t_{1/2}$) of Reaction of Acetoxypyrazine Derivatives with Aniline

Solvent	$t_{1/2(h)}$	
	Me, Bzl, iBu, O-Ac	Me, Me, iBu, O-Ac
DMF	120 (7%)[a]	120 (3%)[a]
Dioxane	120 (5%)[a]	120 (4%)[a]
DCM	120 (3%)[a]	120 (2%)[a]
Benzene	7.5	12

a) % of acetanilide.

aromatic derivatives.

Next, the reactivity of **1** with primary amines of H–Phe–OMe, H–Tyr–OMe and H–Val–Thr–OBzl was examined in DMF (polar solvent) and DCM (non polar solvent). As shown in Fig. 3, the decrease of peak areas of **1** and the increase of peak areas of Ac–Val–Thr–OBzl were plotted as a function of time. The half times of the reactions of **1** with primary amines in DMF or DCM are summarized in Table 2 in comparison with those of **2**. Both **1** and **2** reacted rapidly with the amino group of amino acids or peptide in DMF, but were not so reactive in DCM. Compound **1** is a crystalline compound while **2** is an oily material and **1** reacted with amino groups faster than **2**. Therefore, **1** seems more suitable for use as an acetylating reagent in DMF. Moreover, the reaction of **1** or **2** with Boc–Tyr–OMe[13] or Boc–Val–Thr–OBzl[14] for 5 d did not afford the *O*-Ac derivatives, although both amino and hydroxyl functions were acetylated with acetic anhydride, as summarized in Table 2. These results suggest that protection of the hydroxyl group of an amino acid is not required when 2-acetoxypyrazines are employed for *N*-acetylation of peptides.

Next, Ac–Tyr–Val–Ala–Asp–MCA, which is a specific substrate of the IL-I processing enzyme,[7] was prepared by a conventional solution method according to the route shown in Fig. 4.

First of all, 7-amino-4-methylcoumarin, which was prepared as reported by Zimmerman *et al.*,[15] was coupled with Boc–Asp(OBzl)–OH by a mixed anhydride method

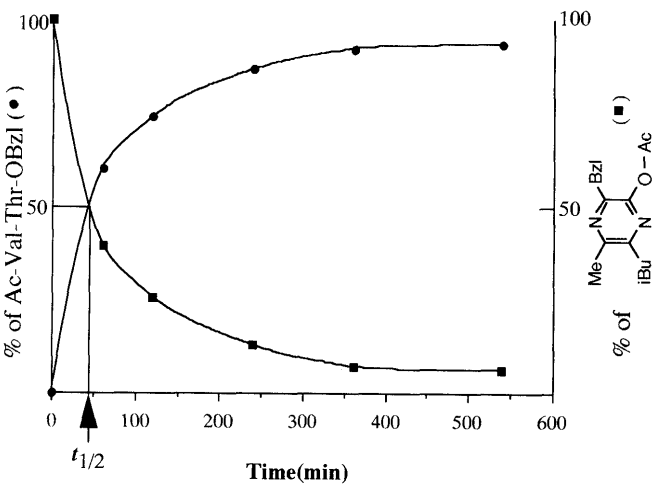

Fig. 3. Reactivity of 2-Acetoxypyrazine Derivative with H–Val–Thr–OBzl in DMF

Table 2. Half Time ($t_{1/2}$) of Reaction of Acetoxypyrazine Derivatives with Amino and Hydroxyl Component in DMF

Amino and hydroxyl component	$t_{1/2(min)}$	
	Me, Bzl, iBu, O-Ac	Me, Me, iBu, O-Ac
H–Phe–OMe	76	98
H–Tyr–OMe	82 [28 h (13.8%)][a]	179
H–Val–Thr–OBzl	48 [8 h (43.8%)][a]	101
Boc–Tyr–OMe	— [24 h (57.0%)][b]	—
Boc–Val–Thr–OBzl	— [24 h (9.1%)][b]	—

—: No reaction with hydroxyl group after 5 d. *a*) Reacted in DCM, % of *N*-acetyl derivative. *b*) Reacted with $(CH_3CO)_2O$ in DMF, % of *O*-acetyl derivative.

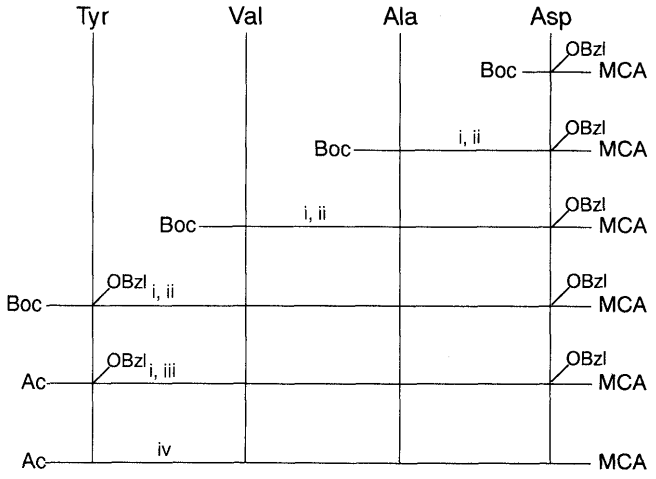

Fig. 4. Synthetic Scheme for Ac–Tyr–Val–Ala–Asp–MCA

i, TFA, anisole; ii, IBCF, TEA; iii, 2-acetoxy-3-benzyl-5-methyl-6-isobutylpyrazine; iv, H_2, Pd/C.

to afford Boc–Asp(OBzl)–MCA. After removal of the Boc group by TFA treatment, Boc–Ala–OH, Boc–Val–OH and Boc–Tyr(OBzl)–OH were coupled successively by the mixed anhydride method to afford Boc–Tyr(OBzl)–Val–Ala–Asp(OBzl)–MCA. After removal of the Boc group, acetylation was carried out by using **1** to afford Ac–Tyr(OBzl)–Val–Ala–Asp(OBzl)–MCA quantitatively. The

protected acetyl tetrapeptide was hydrogenated over a palladium catalyst to give the desired peptide, Ac–Tyr–Val–Ala–Asp–MCA. Homogeneity of this peptide was ascertained by HPLC, elemental analysis and amino acid analysis. Ac–Tyr–Val–Ala–Asp–MCA was successfully employed for measurement of the enzymatic activity of the IL–I processing enzyme.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.) and the $[\alpha]_D$ values are given in 10^{-1} deg·cm^2·g^{-1}. ^{1}H (400, 500 MHz) and ^{13}C (100, 125 MHz) NMR spectra were recorded on either a Bruker AM400 or ARX500 spectrometer. Chemical shift values are expressed as ppm downfield from tetramethylsilane used as an internal standard (δ-value). The J values are given in Hz. Mass spectra were determined on a Hitachi M-2000 mass spectrometer using the electron impact (EI) technique. Amino acid composition of an acid hydrolysate (6 N HCl, 110 °C, 18 h) of a peptide was determined with an amino acid analyzer, K-202 SN (Kyowa Seimitsu Co.). On TLC (Kiesegel G, Merck), Rf^1, Rf^2, Rf^3, and Rf^4 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$ and MeOH (1 : 1), AcOEt and hexane (1 : 2) and $CHCl_3$, AcOEt and MeOH (30 : 19 : 1).

Boc–Ala–Leu–CH_2Cl A mixed anhydride [prepared from Boc–Ala–OH (0.69 g, 3.70 mmol), NMM (0.44 ml, 4.0 mmol) and isobutyl chloroformate (0.48 ml, 3.7 mmol) as usual] in THF (30 ml) was added to an ice-cold solution of H–Leu–CH_2Cl·HCl [prepared from Boc–Leu–CH_2Cl[10] (0.97 g, 3.7 mmol) and 5.1 N HCl/dioxane (3.6 ml, 18.5 mmol) as usual] in DMF (30 ml) containing NMM (0.44 ml, 4.0 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 0.83 g (66.7%), mp 79—80 °C, $[\alpha]_D^{25}$ −60.0° (c=1.0, MeOH), Rf^1 0.7. ^{1}H-NMR ($CDCl_3$ 500 MHz) δ: ppm 6.79 (1H, br s, NH–Leu), 5.01 (1H, d, J=5.9, NH–Ala), 4.77 (1H, m, H_α-Leu), 4.26 (1H, d, J=15.9, H_γ-Leu), 4.17 (1H, br s, H_α-Ala), 1.66 (1H, m, H_β-Leu), 1.53—1.45 (10H, m, *tert*-Bu + H_β-Leu), 1.35 (3H, d, J=7.1, H_β–Ala), 0.94 (6H, m, H_δ–Leu), ^{13}C-NMR ($CDCl_3$ 125 MHz) δ: ppm 201.4 (*quart*, COCH$_2$Cl), 172.9 (*quart*, CHCONH), 155.7 (*quart*, OCONH), 80.5 (*quart*, *tert*-Bu), 54.6 (*tert*, C_α–Ala), 50.0 (*tert*, C_α–Leu), 46.7 (*sec*, COCH$_2$Cl), 40.0 (*tert*, C_γ-Leu), 28.3 (*prim*, *tert*-Bu), 24.9 (*sec*, C_β-Leu), 23.2 (*prim*, C_α-Leu), 21.5 (*prim*, C_α-Leu), 17.5 (*prim*, C_β-Ala). *Anal.* Calcd for $C_{15}H_{27}ClN_2O_4$: C, 53.8; H, 7.85; N, 8.37. Found: C, 53.6; H, 8.13; N, 8.43.

3-Benzyl-5-methyl-6-isobutyl-2(1*H*)-pyrazinone H–Phe–Leu–CH_2Cl [prepared from Boc–Phe–Leu–CH_2Cl[10] (0.9 g, 2.19 mmol) and 5.1 N HCl/dioxane (2.6 ml, 13.2 mmol) as usual] was dissolved in MeOH (10 ml), and the mixture was refluxed for 2 h. After removal of the solvent, the residue was extracted with $CHCl_3$. The extract was washed with water, dried over $MgSO_4$ and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 393 mg (70.0%), mp 133—135 °C, Rf^2 0.31. MS m/z: 256 M$^+$, ^{1}H-NMR ($CDCl_3$ 500 MHz) δ: ppm 13.5 (1H, br s, NH), 7.41—7.15 (5H, m, 3-CH_2-ph), 4.06 (2H, s, 3-CH_2-ph), 2.40 (2H, d, J=7.4, 6-CH_2CH(CH$_3$)$_2$), 2.28 (3H, s, 5-CH_3), 2.05 (1H, m, 6-CH_2CH(CH$_3$)$_2$), 0.97 (6H, d, J=6.3, 6-CH_2CH(CH$_3$)$_2$), ^{13}C-NMR ($CDCl_3$ 125 MHz) δ: ppm 157.6 (*quart*, C-2), 154.1 (*quart*, C-3), 138.2 (*quart*, C-1′), 135.1 (*quart*, C-6), 130.0 (*quart*, C-5), 129.3 (*tert*, C-3′, 5′), 128.2 (*tert*, C-2′, 6′), 126.2 (*tert*, C-4), 39.5 (*sec*, 3-CH_2-ph), 38.9 (*sec*, 6-CH_2CH(CH$_3$)$_2$), 28.7 (*tert*, 6-CH_2CH(CH$_3$)$_2$), 22.3 (*prim*, 6-CH_2CH(CH$_3$)$_2$), 18.8 (*prim*, 5-CH_3). *Anal.* Calcd for $C_{16}H_{20}N_2O$: C, 61.4; H, 7.60; N, 6.81. Found: C, 61.6; H, 7.61; N, 6.85.

3,5-Dimethyl-6-isobutyl-2(1*H*)-pyrazinone H–Ala–Leu–CH_2Cl [prepared from Boc–Ala–Leu–CH_2Cl (0.67 g, 2.0 mmol) and 5.1 N HCl/dioxane (2.0 ml, 10.0 mmol) as usual] was dissolved in MeOH (15 ml). The reaction mixture was refluxed for 2 h. After removal of the solvent, the residue was extracted with $CHCl_3$. The extract was washed with water, dried over $MgSO_4$ and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 323 mg (89.9%), mp 109—110 °C, Rf^2 0.17. MS m/z: 180 M$^+$, ^{1}H-NMR ($CDCl_3$ 500 MHz) δ: ppm 13.3 (1H, br s, NH), 2.44 (2H, d, J=7.4, 6-CH_2CH(CH$_3$)$_2$), 2.42 (3H, s, 3-CH_3), 2.28 (3H, s, 5-CH_3), 2.03 (1H, m, 6-CH_2CH(CH$_3$)$_2$), 0.98 (6H, d, J=6.6, 6-CH_2CH(CH$_3$)$_2$), ^{13}C-NMR ($CDCl_3$ 125 MHz) δ: ppm 157.9 (*quart*, C-2), 153.1 (*quart*, C-3), 134.3 (*quart*, C-6), 129.6 (*quart*, C-5), 39.0 (*sec*, 6-CH_2CH(CH$_3$)$_2$), 28.9 (*prim*, 6-CH_2CH(CH$_3$)$_2$), 22.3 (*tert*, 6-CH_2CH(CH$_3$)$_2$), 19.6 (*prim*, 3-CH_3), 18.7 (*prim*, 5-CH_3). *Anal.* Calcd for $C_{10}H_{16}N_2O$: C, 66.6; H, 8.95; N, 15.5. Found: C, 66.5; H, 8.89; N, 15.5.

2-Acetoxy-3-benzyl-5-methyl-6-isobutylpyrazine (1) 3-Benzyl-5-methyl-6-isobutyl-2(1*H*)-pyrazinone (323 mg, 1.3 mmol) was refluxed in acetic anhydride (10 ml) for 1 h. After removal of the excess acetic anhydride, the residue was extracted with ether. The extract was washed with water, dried over $MgSO_4$ and evaporated to give the title compound as a yellow crystalline solid, yield 370 mg (95.4%), mp 37—38 °C, Rf^1 0.71, Rf^3 0.79. MS m/z: 298 M$^+$, ^{1}H-NMR ($CDCl_3$ 400 MHz) δ: ppm 7.28—7.20 (5H, m, 3-CH_2-ph), 4.06 (2H, s, 3-CH_2-ph), 2.62 (2H, d, J=7.3, 6-CH_2CH(CH$_3$)$_2$), 2.55 (3H, s, 2-OCOCH$_3$), 2.21 (3H, s, 5-CH_3), 2.11 (1H, nonet, J=6.7, 6-CH_2CH(CH$_3$)$_2$), 0.94 (6H, d, J=6.6, 6-CH_2CH(CH$_3$)$_2$). *Anal.* Calcd for $C_{18}H_{22}N_2O$: C, 72.5; H, 7.43; N, 9.39. Found: C, 72.5; H, 7.49; N, 9.31.

2-Acetoxy-3,5-dimethyl-6-isobutylpyrazine (2) 3,5-Dimethyl-6-isobutyl-2(1*H*)-pyrazinone (314 mg, 1.75 mmol) was heated under reflux in acetic anhydride (20 ml) for 1 h. After removal of the excess acetic anhydride, the residue was extracted with ether. The extract was washed with water, dried over $MgSO_4$ and evaporated to give the title compound as a yellow oily material, yield 314 mg (80.4%), Rf^3 0.3. MS m/z: 222 M$^+$, ^{1}H-NMR ($CDCl_3$ 400 MHz) δ: ppm 2.63 (2H, d, J=7.3, 6-CH_2CH(CH$_3$)$_2$), 2.55 (3H, s, 2-OCOCH$_3$), 2.40 (3H, s, 3-CH_3), 2.37 (3H, s, 5-CH_3), 2.21 (1H, nonet, J=6.7, 6-CH_2CH(CH$_3$)$_2$), 0.95 (6H, d, J=6.6, 6-CH_2CH(CH$_3$)$_2$).

General Procedure for Examination of Reactivity of 2-Acetoxypyrazine Derivatives with Aniline A 2-acetoxypyrazine derivative (0.5 mmol) and aniline (0.5 mmol) were dissolved in benzene (4 ml), DMF (4 ml), DCM (4 ml) or dioxane (4 ml). The reaction mixture was stirred at room temperature. An aliquot (0.05 ml) was taken periodically and diluted with MeOH (0.3 ml), and 0.01 ml of the diluted solution was analyzed with an HPLC apparatus [column, μ bondasphere C18 (3.9 × 150 mm); eluent, A=0.05% TFA/CH_3CN, B=0.05% TFA/H_2O (linear gradient in 25 min) A/B: 90/10—10/90; flow rate, 1.0 ml/min; detection, 220 nm]. The peak areas corresponding to **1** or **2** and acetanilide were plotted as a function of time and the half time ($t_{1/2}$) was determined (see Fig. 2, Table 1).

General Procedure for Examination of Reactivity of 2-Acetoxypyrazine Derivatives with Primary Amine of Peptide 1) Synthesis of standard sample, Ac–Val–Thr–OBzl: H–Val–Thr–OBzl·HCl [prepared from Boc–Val–Thr–OBzl[14] (300 mg, 0.61 mmol) and 5.0 N HCl/dioxane (0.71 ml, 3.65 mmol) as usual] was dissolved in DMF (20 ml) containing TEA (0.085 ml, 0.61 mmol) and acetic anhydride. The reaction mixture was stirred at room temperature for 2 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 190 mg (93.1%), mp 184—185 °C, $[\alpha]_D^{25}$ −49.5° (c=1.0, MeOH), Rf^4 0.71. *Anal.* Calcd for $C_{18}H_{22}N_2O$: C, 61.5; H, 7.74; N, 7.98. Found: C, 61.5; H, 7.51; N, 8.00.

2) Compound **1** (12.8 mg, 0.05 mmol) and an amino acid methyl ester or a dipeptide benzyl ester (0.05 mmol) were dissolved in DMF (4 ml) or DCM (4 ml). The reaction mixture was stirred at room temperature. An aliquot (0.05 ml) was taken periodically and diluted with MeOH (0.3 ml), and 0.01 ml of the diluted solution was analyzed with an HPLC apparatus [column, μbondasphere C18 (3.9 × 150 mm); eluent, A=0.05% TFA/CH_3CN, B=0.05% TFA/H_2O (linear gradient in 25 min) A/B: 90/10—10/90; flow rate, 1.0 ml/min; detection, 220 nm]. The peak areas corresponding to **1** and acetylated amino acid or peptide were plotted as a function of time and the half time ($t_{1/2}$) was determined (see Fig. 3 and Table 2).

General Procedure for Examination of Reactivity of 2-Acetoxypyrazine Derivatives with Hydroxyl Group of Thr and Tyr Compound **1** (26 mg, 0.12 mmol) and Boc–Tyr–OMe (35.4 mg, 0.12 mmol) or Boc–Val–Thr–OBzl (50 mg, 0.12 mmol) were dissolved in DMF (2 ml). The reaction mixture was stirred at room temperature. An aliquot (0.05 ml) was taken

periodically and diluted with MeOH (0.3 ml), and 0.01 ml of the diluted solution was analyzed with an HPLC apparatus [column, μbondasphere C18 (3.9 × 150 mm); eluent, A=0.05% TFA/CH_3CN, B=0.05% TFA/H_2O (linear gradient in 25 min) A/B: 90/10—10/90; flow rate, 1.0 ml/min; detection, 220 nm]. After 5 d, no *O*-acetyl derivative was detected. The examination of the reactivity of acetic anhydride with Boc–Tyr–OMe or Boc–Val–Thr– OBzl in DMF was also carried out in the same manner. The results are summarized in Table 2.

Boc-Asp(OBzl)-MCA A mixed anhydride [prepared from Boc–Asp(OBzl)–OH (10.0 g, 0.03 mol), NMM (3.30 ml, 0.03 mol) and IBCF (4.02 ml, 0.03 mol) as usual] in THF (50 ml) was added to an ice-cold solution of 7-amino-4-methylcoumarin (1.8 g, 0.01 mol) in DMF (50 ml). The reaction mixture was stirred at 0 °C for 1h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and purified by silica-gel chromatography (eluent; 2% MeOH/$CHCl_3$), yield 2.0 g (41.6%), mp 125—127 °C, $[\alpha]_D^{25}$ −33.2° (c=1.0, MeOH), Rf^1 0.55. *Anal.* Calcd for $C_{26}H_{28}N_2O_7$: C, 65.0; H, 5.87; N, 5.82. Found: C, 64.8; H, 5.85; N, 5.80.

Boc-Ala-Asp(OBzl)-MCA A mixed anhydride [prepared from Boc–Ala–OH (344 mg, 1.82 mmol), TEA (0.26 ml, 1.82 mmol) and IBCF (0.24 ml, 1.82 mmol) as usual] in THF (10 ml) was added to an ice-cold solution of H–Asp(OBzl)–MCA·TFA [prepared from Boc–Asp(OBzl)–MCA (0.5 g, 1.04 mmol), TFA (0.7 ml) and anisole (0.12 ml, 11 mmol) as usual] in DMF (10 ml) containing TEA (0.15 ml, 1.04 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 456 mg (79.6%), mp 100—113 °C, $[\alpha]_D^{25}$ −25.0° (c=0.5, MeOH), Rf^1 0.56. *Anal.* Calcd for $C_{29}H_{33}N_3O_8 \cdot 0.75H_2O$: C, 61.6; H, 6.15; N, 7.44. Found: C, 61.7; H, 6.11; N, 7.45.

Boc-Val-Ala-Asp(OBzl)-MCA A mixed anhydride [prepared from Boc–Val–OH (120 mg, 0.55 mmol), TEA (0.08 ml, 0.55 mmol) and IBCF (0.07 ml, 0.55 mmol) as usual] in THF (10 ml) was added to an ice-cold solution of H–Ala–Asp(OBzl)–MCA·TFA [prepared from Boc–Ala–Asp(OBzl)–MCA (0.25 g, 0.46 mmol), TFA (0.5 ml) and anisole (0.06 ml, 0.51 mmol) as usual] in DMF (10 ml) containing TEA (0.07 ml, 0.46 mmol). The reaction mixture was stirred at 0 °C for 1h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 246 mg (82.1%), mp 212—213 °C, $[\alpha]_D^{25}$ −61.0° (c=0.5, MeOH), Rf^1 0.64. *Anal.* Calcd for $C_{34}H_{42}N_4O_9$: C, 62.8; H, 6.51; N, 8.61. Found: C, 62.9; H, 6.58; N, 8.59.

Boc-Tyr(OBzl)-Val-Ala-Asp(OBzl)-MCA A mixed anhydride [prepared from Boc–Tyr(OBzl)–OH (213 mg, 0.58 mmol), TEA (0.08 ml, 0.58 mmol) and IBCF (0.08 ml, 0.58 mmol) as usual] in THF (10 ml) was added to an ice-cold solution of H–Val–Ala–Asp(OBzl)–MCA·TFA [prepared from Boc–Val–Ala–Asp(OBzl)–MCA (0.25 g, 0.38 mmol), TFA (0.7 ml) and anisole (0.05 ml, 0.46 mmol) as usual] in DMF (10 ml) containing TEA (0.05 ml, 0.39 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to afford crystals, which were collected by filtration and recrystallized from MeOH, yield 240 mg (69.0%), mp 210—215 °C, $[\alpha]_D^{25}$ −25.0° (c=0.1, MeOH), Rf^1 0.55. *Anal.* Calcd for $C_{50}H_{57}N_5O_{11} \cdot H_2O$: C, 62.8; H, 6.51; N, 8.61. Found: C, 62.9; H, 6.58; N, 8.59. Amino acid analysis: Tyr (1) 0.6; Val (1) 0.8; Ala (1) 1.0; Asp (1) 0.9 (average recovery 90.0%).

Ac-Tyr(OBzl)-Val-Ala-Asp(OBzl)-MCA 3-Benzyl-5-methyl-6-isobutyl-2-acetoxypyrazine (**1**) (86.0 mg, 0.29 mmol) was added to a solution of H–Tyr(OBzl)–Val–Ala–Asp(OBzl)–MCA·TFA [prepared from Boc–Tyr(OBzl)–Val–Ala–Asp(OBzl)–MCA (219 mg, 0.24 mmol), TFA (0.5 ml) and anisole (0.04 ml, 0.36 mmol) as usual] in DMF (10 ml) containing TEA (0.05 ml, 0.39 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to afford crystals, which were collected by filtration and recrystallized from MeOH, yield 194.7 mg (95.0%), mp 273—278 °C, $[\alpha]_D^{25}$ −21.0° (c=0.1, MeOH), Rf^1 0.73, *Anal.* Calcd for $C_{47}H_{51}N_5O_{10} \cdot H_2O$: C, 65.4; H, 6.18; N, 8.11. Found: C, 65.5; H, 6.06; N, 8.14.

Ac-Tyr-Val-Ala-Asp-MCA The title compound was prepared from Ac–Tyr(OBzl)–Val–Ala–Asp(OBzl)–MCA (101 mg, 0.12 mmol) by catalytic hydrogenation in MeOH (50 ml). After removal of Pd and the solvent, ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 70.7 mg (89.3%), mp 233—235 °C, $[\alpha]_D^{25}$ −54.1° (c=0.1, MeOH), Rf^1 0.08. *Anal.* Calcd for $C_{33}H_{39}N_5O_{10} \cdot H_2O$: C, 57.9; H, 5.99; N, 10.2. Found: C, 57.9; H, 5.91; N, 10.0. Amino acid analysis: Tyr (1) 0.7; Val (1) 0.9; Ala (1) 1.0; Asp (1) 0.9 (average recovery 88.9%), Analytical HPLC: the title compound was observed as a single peak at the retention time of 15.8 min [column, μbondasphere C18 (3.9 × 150 mm); eluent, A=0.05% TFA/CH_3CN, B=0.05% TFA/H_2O (linear gradient in 20 min) A/B: 90/10—40/60; flow rate, 1.0 ml/min; detection, 220 nm].

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References and Notes

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