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Probing Trypsin Specificity with Basic 3-Substituted Glutarate Diesters and Related Monoester Substrate Analogs: Evidence for Allosteric Activation of Trypsin Catalysis

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A series of dimethyl glutarates bearing basic substituents at C-3, and related monoesters, have been evaluated as substrates of trypsin in order to probe the asymmetric synthetic potential of the enzyme with respect to enantiotopic ester group, and enantiomer, discrimination. While none of the mono- or diesters proved to be a trypsin substrate, several of them accelerated trypsin-catalyzed hydrolysis of the standard reference substrate BAEE, in a manner consistent with an allosteric activation process. The results provide the first examples of allosteric activation of trypsin by modifiers that are sterically precluded from interacting effectively at the active site.

Enzymes are now commonly applied in asymmetric synthesis, with hydrolytic enzymes being the most widely applied group (*1*). Trypsin is a serine protease that has been extensively used in protein sequence determination (*2*). However, with a few important exceptions in transpeptidation and related areas (*3*), it has not been exploited to any significant extent in organic synthesis. One reason for this is that trypsin is highly specific for substrates such as lysine or arginine esters (*4*), although unnatural esters with basic side chains, such as **1** (*5*) and **2** (*6*), have been shown to be substrates of the enzyme.

STRUCTURES 1–6

The fact that trypsin is such a readily available and relatively inexpensive enzyme prompted us to examine further its asymmetric synthetic potential. Taking the known substrate activities of **1** and **2** into account, catalysis of enantiotopically selective hydrolysis of symmetrical diesters such as **3–6** to the corresponding acid-ester chirons was selected as our initial benchmark criterion.

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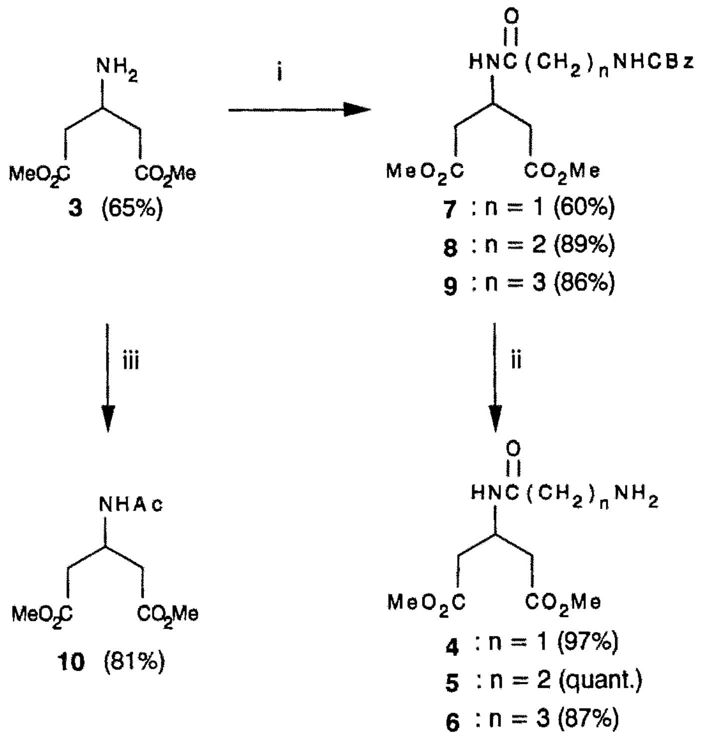


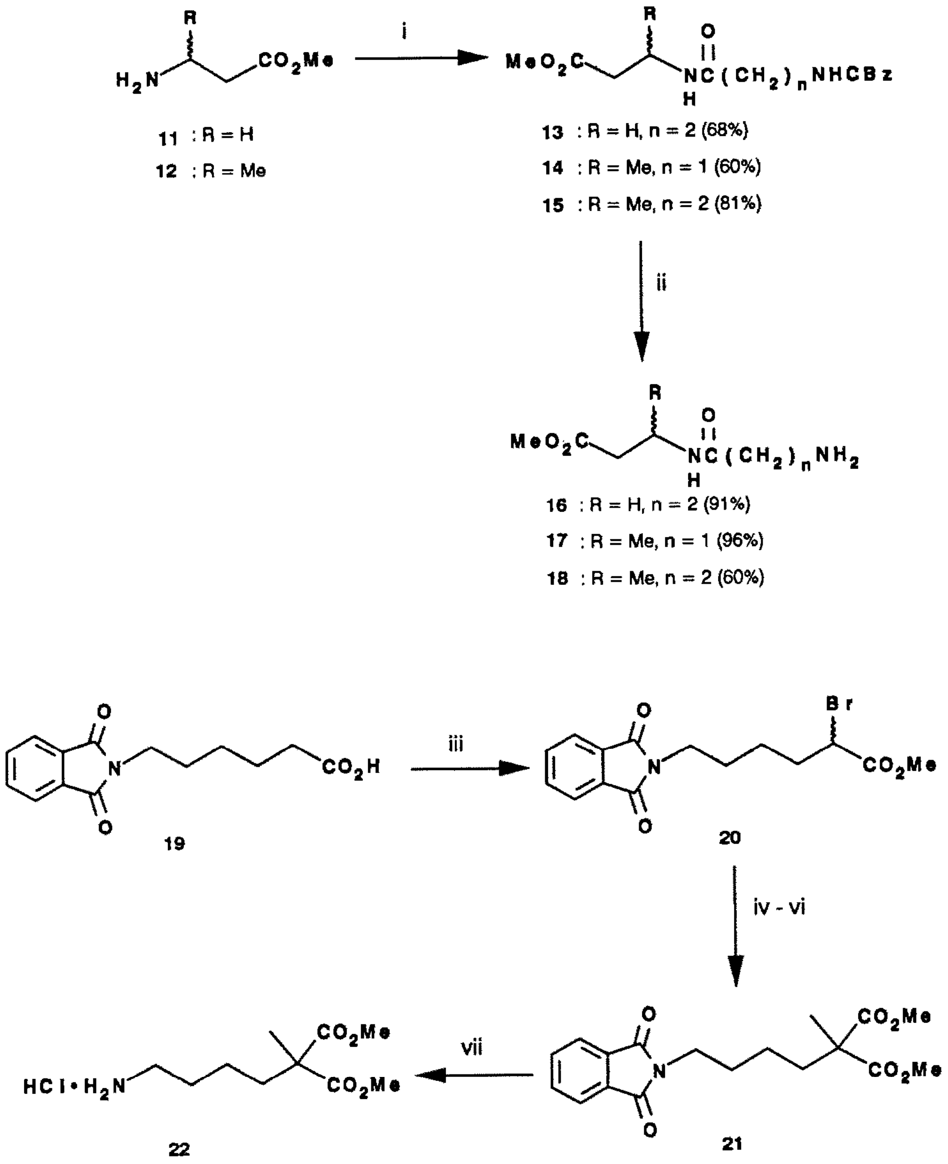
SCHEME 1. Reagents: (i) $CBzNH(CH_2)_nCO_2H$, DCC/DMAP in CH_2Cl_2. (ii) H_2, Pd/C in MeOH/AcOH. (iii) Ac_2O, NaOAc in $CHCl_3$, reflux.

RESULTS

The potential substrates **4–6** were prepared as shown in Scheme 1 from dimethyl 3-aminoglutarate (**3**), itself obtained by the method of Josey and Jenner (*7*). Also, since diethyl 3-*N*-acetylglutarate had been shown to be a good chymotrypsin substrate, its dimethyl analog **10** was made as an additional reference substrate (*8*).

Under the usual pH 7 preparative-scale conditions, none of the diesters **3–6**, nor **10**, proved to be a substrate for trypsin. These results contrasted sharply with the facile trypsin-catalyzed hydrolysis reported for the unbranched monoester **1** (*5*). Accordingly, the racemic monoesters **16–18** were prepared for evaluation as substrates (*9*) as shown in Scheme 2. In addition, in view of the value of malonate diester probes in pig liver esterase specificity studies (*10*), the malonate diester **22** was also included.

Although under preparative-scale conditions, none of **16–18**, nor **22**, was a trypsin substrate, the kinetic behaviors observed were of considerable interest. It was observed that autolysis of trypsin was prevented in the presence of **4–6** and **16–18**, but not when **3**, **10**, or **22** was the potential substrate. The potential of compounds **3–6** as possible inhibitors of trypsin was therefore examined, using α-



SCHEME 2. Reagents: (i) CBzNH(CH$_2$)$_n$CO$_2$H, DCC/DMAP in CH$_2$Cl$_2$. (ii) H$_2$, Pd/C in CH$_2$Cl$_2$/AcOH. (iii) 1. SOCl$_2$ 2. NBS/HBr 3. MeOH. (iv) NaCN, MeOH, reflux. (v) NaH, MeI in THF. (vi) HCl, MeOH/H$_2$O, reflux. (vii) 1. N$_2$H$_4$, MeOH 2. aq. HCl.

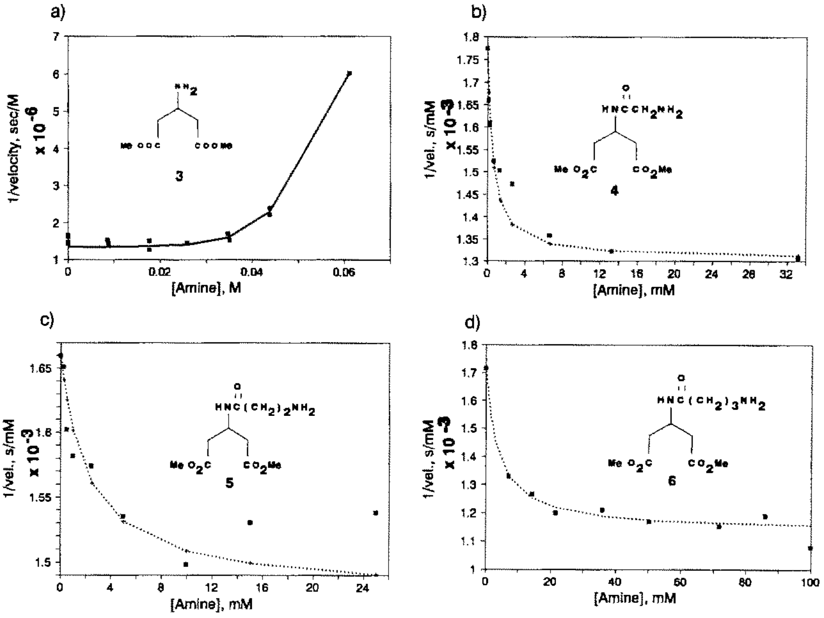


FIG. 1. Dixon plot representations of the effects of **3–6** on the rate of trypsin-catalyzed hydrolysis of BAEE. The experimentally based points are shown as ■. The influence of diester **3** was insignificant at concentrations below 35 mM. The results for the diesters **4–6** are in accord with their activating the enzyme as allosteric modifiers, as indicated by the excellent fit of the experimental plots (■) with the curves (· · ·) calculated on the basis of an allosteric activation model (*16*).

N-benzoyl-L-arginine ethyl ester (BAEE) as the reference substrate. The results are summarized as Dixon plots (*11*) in Fig. 1. Under the kinetic conditions applied, only dimethyl 3-aminoglutarate (**3**) showed any inhibitory activity, and then only at high concentration.

The most unusual effects were exhibited by the diesters **4–6**. These compounds were not only not inhibitors, but exhibited instead quite the opposite effect in that they induced accelerations in the rates of trypsin-catalyzed hydrolysis of BAEE.

DISCUSSION

The preparations of the potential substrates **4–6**, **10**, **16–18**, and **22** were straightforward, except that they proved to be somewhat unstable. Accordingly, they were used immediately. Their structures and purities were confirmed by ^{1}H NMR and GLC or HPLC analysis, with full characterization being carried out on the CBz-precursors **7–9** and **13–15** and on the phthalimido precursor **21** for the malonate **22**.

The glutarate diesters **3–6** were selected for evaluation since they are branched analogs of the known trypsin substrate **1**, with the variations in the lengths of their basic $NHCO(CH_2)_nNH_2$ C-3-function spanning that of the $-(CH_2)_4NH_2$ side chain of the preferred lysine-derived substrates. Despite the inclusion of this amino side chain feature, designed to satisfy the basic substrate proclivity of trypsin, no catalysis of **3–6** hydrolysis was observed. The *N*-acetyl diester **10** was included as a reference compound since the enantiotopically selective hydrolysis of its diethyl ester analog by the closely related serine protease chymotrypsin has been established (*8, 12*). The failure of **10** to undergo trypsin-promoted hydrolysis shows that the binding mode proposed for chymotrypsin with such diesters (*8b*) cannot operate for trypsin.

Since the straight chain monoester **1** is a good trypsin substrate (*5*), the inability of trypsin to catalyze the hydrolyses of **3–6** is at least partly attributable to their branched structures. Molecular graphics analyses (*13, 14*) of projected ES-complexes of trypsin with each of **4–6** indicated that the steric interactions between the S_2-site residue Ser-214 and the branching $-CH_2COOMe$ group would be particularly severe. The proscribing effect of this structural feature on trypsin specificity was therefore explored further using the racemic monoesters **16–18** as probes. The malonate diester **22** was also included in the survey in order to probe steric effects near the hydrolytic center. Once again, none of **16–18**, nor **22**, proved to be a substrate. The fact that the hydrolysis of the straight-chain compound **16** was not catalyzed by trypsin is particularly significant, since it shows that the narrow specificity of the enzyme is not wholly due to its inability to form active ES-complexes with branched mono- or diesters.

Although none of the candidate structures were trypsin substrates, they were not without effect on the enzyme. For example, diesters **4–6** and monoesters **16–18** were effective in preventing the trypsin autolysis observed with solutions of the enzyme alone, or in the presence of **3**, **10**, and **22**. This evidence of possible enzyme inhibition was probed further by evaluating **3–6** as potential inhibitors, using BAEE as the reference substrate. From the Dixon plots obtained (Fig. 1), it is evident that only **3** truly acts as an inhibitor in the hydrolysis of BAEE, and then only at high concentrations. In contrast, in the presence of **4–6**, the rate of trypsin-catalyzed BAEE hydrolysis was enhanced. These data are compatible with binding of **4–6** to an allosteric activator site, the existence of which has been suggested previously (*15*). This interpretation is supported by the similarities of the experimentally derived Dixon plots with those calculated assuming an allosteric activation (*16*) (Fig. 1). To our knowledge, the accelerating effects of **4–6** are the first attributable to trypsin modifiers that are sterically precluded from interacting effectively with the active site (*17*). The conclusion that they are incapable of fitting satisfactorily at the hydrolytic site is supported by the graphics modeling analyses, and by their total resistance to trypsin-catalyzed hydrolysis. However, in the future, protein engineering of trypsin (*18*) could create mutants of broadened specificity that would accept **4–6** as substrates. Interestingly, similar allosteric effects induced by lysine derivatives have recently been observed in other enzyme systems (*19*).

EXPERIMENTAL SECTION

General methods. Chemicals were purchased from Aldrich, or from Caledon Laboratories Ltd. (Georgetown, Ont.). Tetrahydrofuran and ethyl ether were distilled from sodium/benzophenone before use. α-*N*-Benzoyl-L-arginine ethyl ester and bovine trypsin (Type III, dialyzed and lyophilized, salt-free, Lot 16F-0249) were obtained from Sigma and were used as received. NMR spectra were obtained using Varian T-60, XL-200, or XL-400 spectrometers. Infrared spectra were recorded on a Nicolet 5DX FT-IR system. Preparative-scale enzyme assays were performed using a Metrohm 614 Impulsomat, 632 pH meter, and 655 Dosimat. HPLC was performed on a Varian 5500 instrument. Melting points were measured using an Electrothermal apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN). Solutions for kinetics were dispensed using calibrated glass pipettes, or Gilson Pipetman 100-, 1000-, or 5000-μl variable pipettes. Kinetics experiments were performed on a Radiometer RTS-822 pH-stat system, with the data recorded directly by a PC-XT computer.

Preparation of Esters

Dimethyl 3-(2-aminoacetamido)glutarate (*4*). In a modification of the procedure of Wang *et al.* (*20*), diester **3** (340 mg, 1.95 mmol), N-CBz glycine (407 mg, 1.95 mmol), and DMAP (80 mg, 0.66 mmol) were dissolved in CH_2Cl_2 (30 ml). DCC (454 mg, 2.2 mmol) was added, and the mixture was stirred for 3 h at 23°C. AcOH (10 drops) was added, and the solution was filtered. The filtrate was washed with 1 M HCl (3 × 10 Ml), 5% aqueous Na_2CO_3 (3 × 10 ml), and then dried ($MgSO_4$). A solid was obtained on evaporation of the solvent. Recrystallization from $CHCl_3$/Et_2O at 0°C afforded dimethyl 3-(2-*N*-CBz-aminoacetamido)glutarate (**7**, 427 mg, 60%) as fluffy needles, mp 82°C: ir (KBr) ν 3389, 3271, 3096, 1725, 1655 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.63 (2H, *A*BX, $J_{A-B} = -16.5$ Hz, $J_{A-X} = 6$ Hz), 2.71 (2H, A*B*X, $J_{B-X} = 5.3$ Hz), 3.675 (6H, s), 3.839 (2H, d, $J = 5.6$ Hz), 4.59–4.64 (1H, AB*X*), 5.132 (2H, s), 5.459 (1H, br s), 6.916 (1H, d, $J = 8.5$ Hz), 7.27–7.37 (5H, m) ppm; ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.43, 168.37, 156.45, 136.10, 128.45, 128.13, 127.99, 67.045, 51.803, 44.442, 42.926, 37.304 ppm. *Anal.* Calcd for $C_{17}H_{22}N_2O_7$: C, 55.73; H, 6.05; N, 7.65. Found: C, 55.53; H, 5.97; N, 7.61.

Removal of the CBz group was effected by the method of Bergmann and Zervas (*21*). The CBz-glutarate **7** (10.87 g, 29.7 mmol) in MeOH (150 ml) was treated with freshly activated decolorizing carbon (≈1 g) (*22*). The mixture was filtered, and AcOH (3 ml) and 10% Pd/C catalyst (500 mg) were added to the filtrate. The mixture was shaken for 18 h under 50 psi of H_2. The catalyst was removed by filtration through Hyflo Super-Cel, and the solvent was evaporated to leave a waxy mass. This wax was taken up in CH_2Cl_2 (100 ml), and dry NH_3 gas was passed through for 5 min. The precipitated NH_4OAc was removed, and the solvent was evaporated to afford **4** (6.7 g, 97%) as a clear, viscous oil: ir (film) ν 3600–3000, 1732, 1651 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.5 (2H, br s), 2.6 (2H, s), 2.7 (2H, s), 3.3 (2H, br s), 3.65 (6H, s), 4.4–4.8 (1H, m), 7.9 (1H, br s) ppm. These general procedures were applied to the preparations of all the following substrate candidates, with the exception of **22**.

Dimethyl 3-(3-aminopropanoylamido)glutarate (**5**). The glutarate diester **3** (520 mg, 3 mmol) was coupled with N-CBz-β-alanine (737 mg, 3.3. mmol) to yield dimethyl 3-(*N*-CBz-3-aminopropionamido)glutarate (**8**, 1.01 g, 89%), which was recrystallized from Et_2O containing a trace of $CHCl_3$, mp 77–78°C: ir (KBr) ν 3315, 3292, 1727, 1736, 1692, 1640 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.379 (2H, t, $J = 5.8$ Hz), 2.599 (2H, *A*BX, J_{A-B} = −16.1 Hz, J_{A-X} = 6 Hz), 2.699 (2H, A*B*X, J_{B-X} = 5.9 Hz), 3.474 (2H, dt, J_1 = 5.8 Hz, J_2 = 6.5 Hz), 3.667 (6H, s), 4.61 (1H, AB*X*), 5.093 (2H, s), 5.514 (1H, br t), 6.463 (1H, d, J = 8.3 Hz), 7.344 (5H, s) ppm. *Anal.* Calcd for $C_{18}H_{24}N_2O_7$: C, 56.83; H, 6.36; N, 7.36. Found: C, 56.71; H, 6.37; N, 7.57.

Deprotection of **8** (6.4 g, 16.8 mmol) as described above afforded **5** (4.26 g, quant.): ir (film) ν 3600–3000, 1729, 1649, 1556, 1204 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.9 (2H, br s), 2.2–2.5 (2H, m), 2.67 (4H, m), 2.8–3.2 (2H, m), 3.67 (6H, s), 7.4–7.8 (1H, m), 7.2–7.6 (1H, br s) ppm.

Dimethyl 3-(4-aminobutyramido)glutarate (***6***). Coupling of **3** (520 mg, 3 mmol) with N-CBz-τ-aminobutanoic acid (783 mg, 3.3. mmol) gave, after recrystallization from Et_2O, dimethyl 3-(4-*N*-CBz-aminobutyramido)glutarate (**9**, 1.023 g, 86%), mp 66–67°C: ir (KBr) ν 3326, 3283, 1727, 1688, 1647, 1546, 1276, 1265 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 1.787 (2H, tt, J_1 = 7.1 Hz, J_2 = 6.6 Hz), 2.201 (2H, t, J = 7.1 Hz), 2.612 (2H, *A*BX, J_{A-B} = −16.1 Hz, J_{A-X} = 6 Hz), 2.711 (2H, A*B*X, J_{B-X} = 5.9 Hz), 3.227 (2H, dt, J_1 = 6.6 Hz, J_2 = 6.3 Hz), 3.672 (6H, s), 4.57 (1H, AB*X*X′, J'_{X-X} = 8.7 Hz), 5.089 (2H, s), 5.14 (1H, br t, J = 6.3 Hz), 6.607 (1H, d, J'_{X-X} = 8.7 Hz), 7.35 (5H, br s) ppm. *Anal.* Calcd for $C_{19}H_{26}N_2O_7$: C, 57.86; H, 6.64; N, 7.10. Found: C, 57.85; H, 6.55; N, 7.10.

Deprotection of **9** (5.0 g, 12.7 mmol) afforded **6** (2.86 g, 87%) as a waxy solid: ir (film) ν 3674–3137, 1735, 1651, 1556, 1204 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.5–3.0 (8H, m), 2.65 (4H, m), 3.67 (6H, s), 4.3–4.8 (1H, m), 7.0 (1H, br d) ppm.

Dimethyl 3-acetamidoglutarate (***10***). This was prepared by acetylation of **3** as described by Cohen (*8a*), mp 70°C: ^1H NMR ($CDCl_3$) δ 1.95 (3H, s), 3.5–3.9 (4H, m), 3.67 (6H, s), 4.33–4.75 (1H, m), 6.1–6.7 (1H, br s).

Methyl β-alanyl-β-alaninate (***16***) (*23*). Methyl β-alaninate (*23b*) (**11**, 340 mg, 3.3 mmol) and N-CBz-β-alanine (852 mg, 3.8 mmol) were coupled as described above to give, after recrystallization from benzene, methyl (N-CBz-β-alanyl)-β-alaninate (**13**, 795 mg, 68%), mp 109–110°C (lit. (*23*) mp 110–111°C): IR (KBr) ν 3324, 3298, 1735, 1682, 1636, 1546, 1275, 1179 cm^{-1}. Deprotection of **13** (500 mg, 1.62 mmol) afforded **16** (258 mg, 91%), mp > 220°C (dec.): ^1H NMR (D_2O) δ 2.1–2.6 (6H, m), 3.0–3.4 (4H, m), 3.5 (3H, s) ppm.

(±)-*Methyl 3-(2-aminoacetamido)butanoate* (***17***). Methyl 3-aminobutanoate (*24*) (**12**, 500 mg, 4.27 mmol) was coupled with CBz-glycine (982 mg, 4.69 mmol) to give, after chromatography on silica (EtOAc : hexanes, 2 : 1), (±)-methyl 3-(2-*N*-CBz-aminoacetamido)butanoate (**14**, 791 mg, 60%), mp 95–96°C: ir (KBr) ν 3407, 3291, 1737, 1725, 1656, 1282 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.198 (3H, d, J = 6.4 Hz), 2.496 (2H, d, J = 4 Hz), 3.654 (3H, s), 3.812 (2H, d, J = 5.9 Hz), 4.33 (1H, m), 5.112 (2H, s), 5.389 (1H, br s), 6.515 (1H, d, J = 7.7 Hz), 7.29–7.35 (5H, m) ppm. *Anal.* Calcd for $C_{15}H_{20}N_2O_5$: C, 58.43; H, 6.54; N, 9.09. Found: C, 58.84; H, 6.57; N, 9.10.

Deprotection of **14** (350 mg, 1.14 mmol) gave **17** (190 mg, 96%): ir (film) 3309, 3070, 1735, 1659, 1532, 1200 cm^{-1}; ^1H NMR (CD_3CN, 400 MHz) δ 1.145 (3H, d, J = 7.3 Hz), 1.691 (2H, br s), 2.413 (1H, *A*BX, J_{A-B} = −15.3 Hz, J_{A-X} = 6.6 Hz), 2.514 (1H, A*B*X, J_{B-X} = 6.1 Hz), 3.113 (2H, s), 3.609 (3H, s), 4.16–4.24 (1H, AB*X*, m), 7.35 (1H, br s) ppm.

(±)-*Methyl 3-(3-aminopropionamido)butanoate* (***18***). Methyl 3-aminobutanoate

(**12**, 340 mg, 2.9 mmol) and CBz-β-alanine (720 mg, 3.2 mmol) were coupled to yield, after chromatography on silica (2 : 1 EtOAc : hexanes), (±)-methyl 3-(3-*N*-CBz-aminopropionamido)butanoate (**15**, 504 mg, 49%), mp 89.5–91°C: ir (KBr) ν 3296, 1731, 1686, 1635, 1274 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.209 (3H, d, $J = 6.8$ Hz), 2.38 (2H, m), 2.474 (1H, *A*BX, $J_{A-B} = -15.7$ Hz, $J_{A-X} = 5.3$ Hz), 2.536 (1H, A*B*X, $J_{B-X} = 5.5$ Hz), 3.45–3.50 (2H, m), 3.675 (3H, s), 4.31–4.38 (1H, AB*X*, m), 5.093 (2H, s), 5.484 (1H, br s), 6.088 (1H, d, $J = 6.4$ Hz), 7.27–7.38 (5H, m) ppm. *Anal.* Calcd for $C_{16}H_{22}N_2O_5$: C, 59.62; H, 6.88; N, 8.69. Found: C, 59.88; H, 7.11; N, 8.82.

Deprotection of **15** (300 mg, 0.93 mmol) gave **18** (105 mg, 60%) as a waxy solid: ir (film) ν 3362, 3289, 1729, 1642, 1556 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.15 (3H, d, $J = 7$ Hz), 2.0–3.0 (6H, m), 2.55 (2H, d, $J = 6$ Hz), 3.75 (3H, s), 4.0–4.7 (1H, m), 6.9–7.4 (1H, br) ppm.

Dimethyl 2-methyl-2-(4-aminobutyl)malonate hydrochloride (**22**). Using the procedure of Harpp *et al.* (*25*), 6-phthalimidohexanoic acid (*26*) (**19**, 20 g, 76.5 mmol) in CCl_4 (10 ml) was treated with $SOCl_2$ (35 ml, 482 mmol) at 70°C for 30 min, under a $CaCl_2$ drying tube. The mixture was then cooled to 23°C, and *N*-bromosuccinimide (17.78 g, 100 mmol), CCl_4 (50 ml), and 48% aqueous HBr (7 drops) were added. The mixture was heated to 85°C for 1.5 h, and then cooled in an ice bath. MeOH (20 ml) was added, and the reaction was stirred for 10 min at 0°C before being warmed to 25°C. The volatile materials were evaporated, and the residue was taken up in Et_2O. After filtration, the ethereal solution was washed successively with 2 M NaOH (3 × 20 ml), water (1 × 20 ml), and saturated aqueous $NaHSO_3$ (2 × 20 ml). The organic layer was dried ($MgSO_4$) and concentrated to afford (±)-methyl 2-bromo-6-phthalimidohexanoate (**20**, 21.51 g, 79%): ir (film) ν 1771, 1739, 1715, 720 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.2–2.3 (6H, m), 3.63 (2H, t, $J = 4$ Hz), 3.75 (3H, s), 4.2 (1H, t, $J = 7$ Hz), 7.5–7.85 (4H, m) ppm. *Anal.* Calcd for $C_{15}H_{16}NO_4Br$: C, 50.87; H, 4.55; N, 3.95; Br, 22.56. Found: C, 50.33; H, 4.54; N, 3.73; Br, 22.70.

The bromo-ester **20** (19.67 g, 55.5 mmol) was dissolved in MeOH (100 ml). Sodium cyanide (3.0 g, 61 mmol) was added, and the mixture was heated under reflux for 8.5 h. The reaction solution was cooled and filtered through Hyflo Super-Cel, the filtrate was concentrated, and the dark brown residue was taken up in Et_2O (100 ml) and refiltered. The filtrate deposited yellow–tan crystals, which were recrystallized from $Et_2O/CHCl_3$, giving (±)-methyl 2-cyano-6-phthalimidohexanoate (5.5 g, 33%), mp 93–94°C: ir (KBr) ν 2247, 1772, 1731, 1713, 1397, 1270, 723 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.2–2.3 (6H, m), 3.4–3.95 (3H, m), 3.8 (3H, s), 7.6–7.95 (4H, m) ppm. *Anal.* Calcd for $C_{16}H_{16}N_2O_4$: C, 63.99; H, 5.37; N, 9.33. Found: C, 63.69; H, 5.37; N, 9.03.

Sodium hydride (320 mg, 60% in oil, 8 mmol) was washed with pentane (5 ml), and then suspended in dry THF (30 ml). The suspension was heated under reflux and the above cyanoester (2 g, 6.66 mmol) in THF (20 ml) was added dropwise over 30 min. The reaction was refluxed for a further 30 min before being cooled to 23°C. Methyl iodide (4 ml, 64 mmol) was added rapidly, and the mixture was refluxed once again for 1.5 h. After cooling, the solvent was evaporated and the residue was taken up in CH_2Cl_2 (50 ml) and filtered. The filtrate was treated with freshly activated Norit. Evaporation of the solvent afforded an oil that, after chromatography on silica (hexanes : EtOAc 4 : 1), gave (±)-methyl 2-methyl-2-

cyano-6-phthalimidohexanoate (1.19 g, 57%): ir (film) ν 2245 (weak), 1771, 1748, 1715, 1615, 1248, 721 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.5–2.2 (6H, m), 1.6 (3H, s), 3.55–3.8 (2H, m), 3.75 (3H, s), 7.5–7.9 (4H, m) ppm. *Anal.* Calcd for $C_{17}H_{18}N_2O_4$: C, 64.96; H, 5.77; N, 8.91. Found: C, 64.84; H, 5.89; N, 9.09.

Using the method of Nelson and Cretcher (*27*), the above 2-methyl-2-cyanoester (1.14 g, 3.63 mmol) in MeOH (30 ml) containing water (1.5 ml) was heated under reflux, with HCl gas bubbling through, for 45 min. The mixture was cooled to 0°C, and more HCl was passed through until saturation was reached. It was then stirred at 23°C for 18 h and then heated further under reflux for 1 h. After evaporation, the residue was extracted with CH_2Cl_2 (3 × 15 ml), and the combined extracts were dried ($MgSO_4$) and concentrated to give an oil. This was chromatographed on silica (7 : 3 hexanes : EtOAc) to yield dimethyl 2-methyl-2-(4-phthalimidobutyl)-malonate (**21**, 540 mg, 43%): ir (film) ν 1771, 1714, 1615, 1397, 1240, 721 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.2–2.2 (6H, m), 1.4 (3H, s), 3.5–3.8 (2H, m), 3.7 (6H, s), 7.8–7.95 (4H, m) ppm. *Anal.* Calcd for $C_{18}H_{21}NO_4$: C, 62.24; H, 6.09; N, 4.03. Found: C, 62.28; H, 6.13; N, 3.81.

Deprotection of the amino group was effected by the method of King *et al.* (*28*). The malonate **21** (210 mg, 0.6 mmol) in MeOH (10 ml) was treated with 32% aqueous hydrazine (62 μl, 0.63 mmol) under reflux for 1 h. On cooling, the solvent was evaporated, and the oily residue was taken up in 2 M HCl (10 ml), whereupon a white precipitate formed. After 1 h, the suspension was filtered, and the solids were washed with 0.01 M HCl. The combined acidic filtrate was carefully evaporated and then dried *in vacuo* for 18 h. The resulting solid was decolorized by washing with EtOAc. Further drying at 0.1 Torr over KOH afforded dimethyl 2-methyl-2-(4-aminobutyl)malonate hydrochloride (**22**, 120 mg, 79%), mp 105–110°C: ir (KBr) ν 3029, 2958, 1735, 1237 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.17–1.23 (2H, m), 1.310 (3H, s), 1.49–1.57 (2H, m), 1.73–1.77 (2H, m), 2.68–2.74 (2H, m), 3.637 (6H, s), 8.05 (3H, br s) ppm.

Attempted Trypsin-Catalyzed Hydrolyses

Each potential substrate **3–6**, **10**, **16–18**, and **22** (ca. 100 mg) in 0.01 M phosphate buffer at pH 7.0 (10 ml) was treated with trypsin (ca. 0.2 mg/mg ester). The pH was maintained by automatic addition of 0.25 M NaOH as needed. Solutions were stirred for 1–7 days, and TLC or HPLC was used to analyze for any product formation. In the case of dimethyl 3-aminoglutarate (**3**), dimethyl 3-(acetamido)glutarate (**10**), and dimethyl 2-methyl-2-(4-aminobutyl)malonate (**22**) extensive enzyme autolysis occurred. In all other cases neither substrate nor enzyme underwent hydrolysis.

Evaluation of 3–6 as Inhibitors or Accelerators of Trypsin-Catalyzed Hydrolysis of BAEE; General Procedure

All distilled water was freed of CO_2 (*29*) by vigorous boiling while a stream of argon was passed through the liquid. NaOH-titrant solution (0.0022 M) was stored under a soda-lime guard tube. Solutions of the glutarate modifier compounds **3–6**

(0.3 M) and of KCl (0.3 M) were prepared daily. The modifier solutions were adjusted to pH 6.60 with dilute HCl before use. Solutions of α-*N*-benzoyl-L-arginine ethyl ester (0.0041 M) were prepared weekly. Trypsin was dissolved in 0.001 M HCl at a concentration of 1.5 mg/ml. Enzyme and substrate solutions were stored at 0–5°C.

The assays were performed as follows (*30*): in a plastic vessel mounted in the pH-stat, purged with water-saturated argon and thermostatted at 25°C, were mixed aliquots of BAEE (to give final concentrations of 0.273–2.73 × 10^{-4} M), of modifiers **3–6** (in final concentrations of up to 0.1 M), KCl solution to make the final ionic strength 0.1 M, and water to make up the 15.000-ml total volume. This mixture was equilibrated under wet argon for 5 min and the pH was adjusted to 6.60. The catalysis was initiated by the injection of enzyme solution (20 μl). Each run was performed at least in duplicate. The results are recorded as Dixon plots (*11*) in Fig. 1.

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16. The calculations were based on the standard kinetic scheme, shown below, for allosteric activation or inhibition of an enzyme by a nonsubstrate modifier (Ref. (*15c*) and Ref. (*11a*), pp. 440–445).

$$\begin{tikzcd}
E + M + S \arrow[r, shift left, "K_S"] \arrow[d, shift left, "K'"] & ES + M \arrow[l, shift left] \arrow[r, "k_{cat}"] \arrow[d, shift left, "\alpha K'"] & E + M + P \\
EM + S \arrow[u, shift left] \arrow[r, shift left, "\alpha K_S"] & EMS \arrow[l, shift left] \arrow[u, shift left] \arrow[r, "\beta k_{cat}"] & EM + P
\end{tikzcd}$$

$$\frac{1}{v} = \frac{1}{V_{\max}}\left[\left(\frac{1 + \frac{[M]}{\alpha K'}}{1 + \beta\frac{[M]}{\alpha K'}}\right) + K_S\left(\frac{1 + \frac{[M]}{K'}}{1 + \beta\frac{[M]}{\alpha K'}}\right)\frac{1}{[S]}\right]$$

Calculated values for $1/v$ were obtained assuming that K_s approximated our experimental K_M value for BAEE of 0.0169 ± 0.0007 mM and basing $V_{\max}$ on our 9.0 ± 0.1 s^{-1} k_{cat} value (lit. K_M 0.01 mM, k_{cat} 8.4 s^{-1} (see Inagami and Sturtevant (1960) in Ref. (*17*)). The substrate and modifier concentration values employed were the experimental values. The unknown parameters K', $\alpha K'$, and β were adjusted using a spreadsheet to superimpose the Dixon plots of observed and calculated data until a good agreement was obtained by visual inspection. The values used in the Fig. 1 plots were: for **4**, $K' = 0.14$ mM, $\alpha K' = 0.93$ mM, $\beta = 1.8$; for **5**, $K' = 0.47$ mM, $\alpha K' = 3.1$ mM, $\beta = 1.49$; for **6**, $K' = 1.96$ mM, $\alpha K' = 6$ mM, $\beta = 1.69$.
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