MeOH); Rf^{1} 0.49, Rf^{11} 0.72[6] was condensed by the DCC plus HOBT procedure[7] in DMF with the C-terminal tripeptide ester hydrochloride HCl·H–Trp–Ile–Leu–OBzl[II] [*Anal.* Calcd. for $C_{30}H_{41}O_4N_4Cl \cdot H_2O$: C, 62.63; H, 7.55; N, 9.74. Found: C, 62.62; H, 7.26; N, 9.84. mp 219—220°; $[\alpha]_D^{27}$ −25° (c=0.75, 50% MeOH); Rf^{1} 0.83, Rf^{11} 0.87], after neutralization with N-methylmorpholine, to give the fully protected octapeptide ester Z–pyrGlu–Gly–Lys(Z)–Arg-(NO_2)–Pro–Trp–Ile–Leu–OBzl [III] [*Anal.* Calcd. for $C_{70}H_{90}O_{16}N_{14} \cdot H_2O$: C, 59.97; H, 6.62; N, 13.99. Found: C, 59.68; H, 6.49; N, 13.98. mp 155—160° (decomp.), $[\alpha]_D^{23}$ −61° (c=1, EtOH), Rf^{1} 0.82, Rf^{11} 0.88]. This was then dissloved in MeOH containing a little amount of acetic acid and hydrogenated in the presence of Pd-black to remove all the protecting groups. The deblocked octapeptide thus obtained was purified by column chromatography on SP-Sephadex using ammonium formate buffer to give homogeneous pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–Leu–OH [IV] [diformate tetrahydrate, *Anal.* Calcd. for $C_{47}H_{73}O_{10}N_{13} \cdot 2$ HCOOH. 4 H_2O: C, 51.42; H, 7.50; N, 15.91. Found: C, 51.11; H, 7.43; N, 16.30. Amino acid ratios in acid hydrolysate: Lys 0.95, Arg 0.99, Glu 1.06, Pro 1.05, Gly 1.05, Ile 0.96, Leu 0.96: $[\alpha]_D^{27}$ −96° (c=0.5, H_2O); Rf^{1} 0.25, Rf^{11} 0.54].

This synthetic octapeptide was identiacl with the natural origin "Xenopsin" in multiple criteria; chromatographic behaviors, enzymatic digestion patterns and biological activities.

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Spectrofluorometric Titrants for Trypsin and Chymotrypsin[1]

In order to determine the absolute concentration of active sites in a given enzyme solution, one requires a stoichiometric reaction which can be observed simply and accurately. Several spectrophotometric methods for this purpose have been described.[2] However, spectrophotometric titrations with the *p*-nitrophenyl esters so far developed require at least 1—2 nmole of an enzyme. In this respect, spectrofluorometric titration techniques seem fruitful in that the amount of enzyme required for assay should be much less than for conventional spectrophotometric assays. We have previously reported that *p*-nitrophenyl *p*-amidinobenzoate (NPAB) is a good titrant for determining operational normality of trypsin.[3] By changing the leaving group of NPAB from *p*-nitrophenol to 4-methylumbel-

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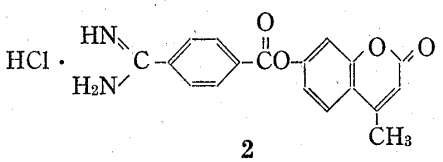
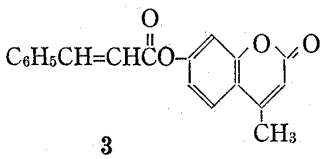
liferone (**1**), a highly fluorescent compound,[4] a design may be made for such a fluorometric titrant. The present communication reports the synthesis and utilization of a new reagent, 4-methylumbelliferyl *p*-amidinobenzoate hydrochloride (**2**) as a titrant for trypsin as well as that of 4-methylumbelliferyl *trans*-cinnamate (**3**), a titrant for chymotrypsin, combination of the same leaving group and a *trans*-cinnamoyl group.[2c]



2



3

2 and **3** were synthesized from the corresponding acid chlorides[3a] and **1** in the presence of pyridine. **2**: colorless fine pillars from DMF–ether, mp 252—254°; *Anal.* Calcd. for $C_{18}H_{15}O_4N_2Cl$: C, 60.26; H, 4.21; N, 7.81; Cl, 9.88. Found: C, 60.37; H, 4.20; N, 8.01; Cl, 9.83. **3**: colorless prisms from EtOAc, mp 149—151°; *Anal.* Calcd. for $C_{19}H_{14}O_4$: C, 74.45; H, 4.60. Found: C, 74.36; H, 4.69.

The "burst" titration and the steady state kinetics[5] revealed that **2** is a good titrant for trypsin ($K_{m(app)}=5.19\times10^{-8}$ M; $k_{cat}=5.13\times10^{-2}$ sec^{-1}; enzyme purity=65.4%) and that, rather than expected, fairly good also for chymotrypsin ($K_{m(app)}=2.25\times10^{-7}$ M; $k_{cat}=2.13\times10^{-2}$ sec^{-1}; enzyme purity=89.4%). **3** is a good titrant for chymotrypsin ($K_{m(app)}=2.84\times10^{-8}$ M; $k_{cat}=7.17\times10^{-3}$ sec^{-1}; enzyme purity=88.8%). These values of purity agree well with those obtained by other methods.[2b,3] Assays were typically performed as follows: 0.8—0.03 mM of **2** in 1 mM HCl containing 5% (v/v) of DMSO (10 μl), 50 mM sodium Veronal-HCl buffer, pH 8.2 (3.0 ml) and a 15 μM trypsin (Worthington, 2X crystallized) solution (10 μl) were mixed at 25°, and the increase in emission at 450 nm (appearance of **1**) was measured (excitation at 360 nm). For titration of chymotrypsin (Worthington, 3X crystallized), 1.4—0.1 mM of **2** was employed. **3** was used in a similar way as above except that 0.2—0.03 mM of **3** in monoglyme (10 μl) and 4 μM chymotrypsin were applied.

Recently Elmore, *et al.*[6] have reported that the analogous esters of **1** of *p*-guanidinobenzoic acid and *p*-trimethylammoniumcinnamic acid are titrants for trypsin and chymotrypsin, respectively. It is interesting that the *p*-guanidinobenzoate[6] is specific to trypsin though **2** is useful also for chymotrypsin. In view of that a number of related enzyme groups have been, and will be found in nature, comparative and quantitative studies of them by means of various sets of titrants will become good criteria to their mechanistic understanding as well as their activity evaluation.

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