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XANTHENE DERIVED POTENT NONPEPTIDIC INHIBITORS OF RECOMBINANT HUMAN CALPAIN I

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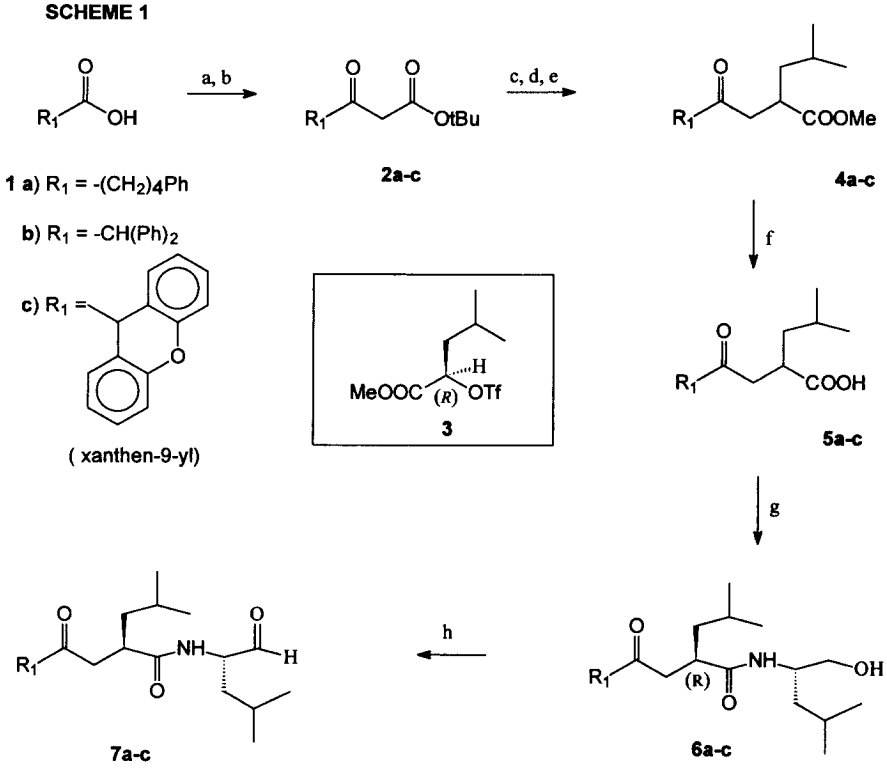
Abstract. Novel and potent, xanthene derived reversible aldehyde (**7c**) and α-ketocarboxamide (**10a**), and irreversible fluoromethyl ketone (**10b**) inhibitors of recombinant human calpain I are described.

Introduction. The possible role of calcium-activated neutral proteases (calpains) in many nervous system diseases and disorders, including stroke, Alzheimer's disease, amyotrophy, motor neuron damage and muscular dystrophy has attracted considerable attention to the discovery of novel inhibitors of this family of cysteine proteases.[1] Two major forms of calpain have been identified: calpain I and calpain II. While calpain II is the predominant form in many tissues, calpain I is thought to be the predominant form activated during the pathological conditions of nervous tissues.[2] Potent peptide-based reversible aldehyde and α-ketocarbonyl,[3] and irreversible halomethyl ketone, diazomethyl ketone, epoxysuccinate, and acyloxymethyl ketone[4] inhibitors of calpains have been reported. Previous studies indicated that calpain prefers Leu or Val at P_2. Takahashi[5] commented that "*... the subsite specificity of calpain at the P_3 position is less rigid than those at the P_2 and P_1 positions. However, an amino acid with an aromatic or a bulky aliphatic side chain at the P_3 position may to some extent increase the susceptibility of the scissile bond to calpain.*" We now report the discovery of a series of novel and potent xanthene (occupying the P_3 position) derived nonpeptidic reversible aldehyde (**7c**) and α-ketocarboxamide (**10a**), and irreversible fluoromethyl ketone (**10b**) inhibitors of human recombinant calpain I.[6]

Chemistry. Commercially available **1a-c** was treated with 1,1'-carbonyldiimidazole, followed by *tert*-butyl lithioacetate to generate the β-ketoester **2a-c** (Scheme 1). Following Hoffman's procedure,[7] treatment of **2a-c** with sodium hydride and (*R*)-triflate-ester (**3**), generated the intermediate diester which on selective hydrolysis by TFA, followed by decarboxylation, produced the γ-ketoester **4a-c**. Basic hydrolysis of **4a-c** produced the corresponding γ-ketoacid **5a-c** which was coupled with (*S*)-leucinol to produce **6a-c** as the major product. Assuming that the alkylation of β-ketoester **2a-c** by (*R*)-triflate-ester **3** took place in an S_N2 fashion as evidenced by Hoffman,[7] the stereochemistry around P_2-site in **6a-c** was assigned as (*R*) (note that the priority of groups around P_2-site in **6a-c** is different than that around chiral center in compound **3**). Oxidation of **6a-c** produced the desired aldehydes **7a-c**. Similarly compound **5c** was coupled with 3-(*S*)-amino-2-hydroxy-5-methyl-hexanoic acid-N-ethylamide, **8a**, (prepared by following the method of Harbeson et al.[3b]) and 3-amino-1-fluoro-2-hydroxy-4-phenylbutane, **8b**, (prepared by following the method of Imperiali et al.[8] modified by Revesz et al.[9]) to generate α-hydroxyamide **9a** and fluorohydroxy compound **9b** respectively (Scheme 2). Dess-Martin oxidation of **9a** and **9b** gave α-ketoamide **10a** and fluoromethyl ketone **10b** (diastereomeric mixture, epimeric at P_1) respectively.

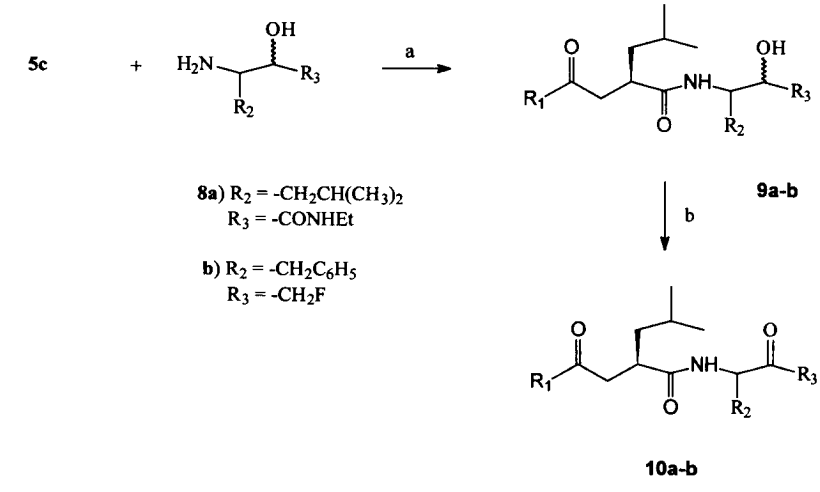
Biology. The inhibitory activities of the compounds **7a-c** and **10a-b** were determined using recombinant human calpain I, prepared as described by Meyer et al.[10] with Suc-Leu-Tyr-MNA, as substrate.[11,12] Inhibition data for **7a-c**, **10a-b** and reference compounds **11** (Cbz-Val-Phe-H, MDL 28170),[13] **12** (Cbz-Leu-Abu-CONHEt),[3b] and **13** (Cbz-Leu-Phe-CH_2F)[14] are shown in Table 1.

SCHEME 1



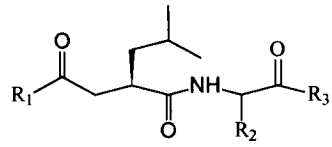
Reagents: (a) 1,1'-carbonyldiimidazole, THF, 0 °C to 23 °C; (b) $Li^+ {}^-CH_2COOtBu$, THF; -78 °C to 0 °C; (c) 60% NaH, THF, **3**, 23 °C; (d) TFA, 23 °C; (e) C_6H_6, reflux; (f) LiOH, MeOH-H_2O; 70-75 °C; (g) (*S*)-leucinol, BOP, HOBt, NMM, DMF, 0 °C to 23 °C; (h) Pyr.SO_3, Et_3N, DMSO-CH_2Cl_2, 0 °C to 23 °C.

SCHEME 2



Reagents: (a) BOP, HOBt, NMM, DMF, 0 °C to 23 °C; (b) Dess-Martin periodinane, CH_2Cl_2, 23 °C.

Table 1. Recombinant Human Calpain I Inhibitory Activity of Compounds **7a-c** and **10a-b**, and **11-13**[a]



Cmpd.	R_1	R_2	R_3	IC_{50}nM	$k_{obs}/I\ M^{-1}s^{-1}$
7a	$-(CH_2)_4Ph$	$-CH_2CH(CH_3)_2$	H	138	-
7b	$-CH(Ph)_2$	$-CH_2CH(CH_3)_2$	H	50	-
7c	-xanthen-9-yl	$-CH_2CH(CH_3)_2$	H	25	-
10a	-xanthen-9-yl	$-CH_2CH(CH_3)_2$	CONHEt	130	-
10b	-xanthen-9-yl	$-CH_2Ph$	CH_2F	-	76,000
11	-	-	-	17	-
12	-	-	-	240	-
13	-	-	-	-	136,000

[a]For compounds **7a-c** and **10a**, the stereochemistry at P_1-site is (*S*); compound **10b** is diasteromeric mixture (at P_1). Compounds **11**, **12**, **13** are reference compounds Cbz-Val-Phe-H, Cbz-Leu-Abu-CONHEt and Cbz-Leu-Phe-CH_2F respectively.

Discussion. Compounds **7a-c** and **10a-b** show good inhibitory activity. However, in the aldehyde series, the presence of two aromatic rings spanning the P_3-P_4 region is preferred over one aromatic ring attached to an alkyl chain (cf. **7b** vs. **7a**). Interestingly, constraining the aromatic rings of **7b** into a xanthene moiety (**7c**), produces the most potent compound of the series. Compound **7c** (IC_{50} 25nM) is comparable to the reference dipeptidyl aldehyde **11** (IC_{50} 17nM in this assay). The corresponding α-ketocarboxamide **10a** (IC_{50} 130nM) also maintains the potency equivalent to the related reference dipeptidyl α-ketocarboxamide **12** (IC_{50} 240nM in this assay). Finally, the irreversible fluoromethyl ketone **10b** (k_{obs}/I 76,000$M^{-1}s^{-1}$) was compared to the corresponding dipeptidyl fluoromethyl ketone **13** (k_{obs}/I 136,300 $M^{-1}s^{-1}$). It should be noted that these inhibitors were also tested for inhibition of cathepsin B, a related cysteine protease; they displayed the following inhibitory activities: **7c** (IC_{50} 440nM), **10a** (IC_{50} 1150nM), and **10b** (k_{obs}/I 1000$M^{-1}s^{-1}$). Thus compounds **7c**, **10a** and **10b** prefer calpain I by >17-fold, approximately 9-fold and 76-fold, respectively over cathepsin B.

Conclusion. We have described a series of novel and potent xanthene derived inhibitors (reversible and irreversible) of recombinant human calpain I. Such inhibitors should provide useful tools for the assessment of the role of calpain in different neurological functions. The outcome of these studies will be the basis of future publications from our laboratories.

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11. For reversible inhibitors, the reaction mixture contained 50mM Tris. HCl (pH 7.5), 50mM NaCl, 0.2 mM Suc-Leu-Tyr-MNA (Enyme Systems Products, Dublin, CA), 1mM EDTA, 1mM EGTA, 5mM β-mercaptoethanol, 10 nM recombinant human calpain I, varying concentrations of inhibitor and 5mM $CaCl_2$ in a final volume of 200 μL in a polystyrene microtiter plate. Assays were initiated by addition of $CaCl_2$ and the increase in fluorescence (λ_{ex} = 340nm, λ_{em} = 430nm) was monitored at ambient temperature using a Fluoroskan II fluorescence plate reader. Values of IC_{50} s were calculated from velocities determined from the linear portion of reaction progress curves. For irreversible inhibitors, reactions were performed at ambient temperature in single cuvettes with the increase in fluorescence (λ_{ex} = 340 nm, λ_{em} = 425 nm) recorded continuously on a Perkin-Elmer LS50B spectrofluorimeter (Norwalk, CT, U.S.A.) and were monitored until there was no further product generated in inhibitor-containing assays. Inhibitor concentrations were at least 10-fold greater than the enzyme concentration in all cases. Values of k_{obs}, the pseudo first-order rate constant for inactivation, were calculated from plots of fluorescence vs. time by non-linear regression (Sigma Plot) to the exponential equation (1)[12]

$$y = Ae^{(-kobs*t)} + B \quad (1)$$

where y is the fluorescence at time t (F_t), A is the amplitude of the reaction (F_0-F_∞), and B is the maximal amount of product formed when the enzyme is completely inactivated (F_∞). The apparent second-order rate constant for inactivation was calculated from the slope of a plot of k_{obs} versus inhibitor concentration as (k_{obs}/I)* (1+S/K_m), correcting for the effect of substrate on the inactivation rate.
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