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Novel Route to the Synthesis of Peptides Containing 2-Amino-1′-hydroxymethyl Ketones and Their Application as Cathepsin K Inhibitors

Rohan V. Mendonca,* Shankar Venkatraman and James T. Palmer

Celera, 180 Kimball Way, South San Francisco, CA 94080, USA

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Abstract—Cathepsin K is highly expressed in human osteoclasts, and is implicated in bone resorption. This makes it an attractive target for the treatment of osteoporosis. Peptides containing 2-amino-1′-hydroxymethyl ketones and 2-amino-1′-alkoxymethyl ketones were discovered as potent inhibitors of cathepsin K. A novel synthetic route was devised to facilitate rapid elucidation of the SAR of these inhibitors. The synthesis and SAR of hydroxymethyl ketones are presented.

Cysteine proteases have been implicated in various diseases such as cancer metastasis,[1] rheumatoid arthritis,[2] Chagas disease,[3] muscular dystrophy,[4] etc. We have been particularly interested in cathepsin K, as it is implicated in osteoporosis. Cathepsin K is a member of papain superfamily of cysteine proteases. Cathepsin K is highly expressed in human osteoclasts and degrades Type I collagen under acidic conditions in osteclast mediated bone resorption.[5,6] In addition, pycnodysostosis a rare sclerosing skeletal dysplasia with low bone turnover or increased bone density, has been shown to be caused by mutations in the cathepsin K gene.[7] Cathepsin K therefore appears to be a novel therapeutic target for various osteoclastic-related bone diseases.

To date a number of reversible and irreversible inhibitors of cathepsins have been reported.[8] The known reversible inhibitors of cysteine proteases are aldehydes,[9] nitriles,[10] α-keto amides,[11] cyclopropenones,[12] diaminoketones,[13] etc. These chemotypes inhibit cysteine proteases by forming a reversible covalent bond between the electophilic functionality of the inhibitor and the nucleophilic sulfur atom of the active site cysteine residue.[14,15] Herein we report hydroxymethyl and alkoxymethyl ketone type inhibitors (**1**, Fig. 1) that inhibit cathepsin K, L, B, and S by the above mentioned mechanism.

We also devised a new synthetic route (Scheme 2) to hydoxymethyl (Fig. 1, R=H) and alkoxymethyl ketones (Fig. 1, R=CH_3, CH_2CH_3, CH_2Ph) which circumvents using a potentially explosive reagent used in prior synthetic methodologies.[16–18] This novel synthetic route (Scheme 2) also eliminates intermediates that are potential irreversible inhibitors of cathepsins and avoids racemization of the product caused by the hydrolysis of the acyloxymethyl ketone to the hydroxymethyl ketone under basic conditions.

Chemistry

The syntheses of hydroxymethyl ketones in our laboratory were carried out via bromomethyl ketones, which were synthesized from commercially available amino acids using literature methods (Scheme 1).[16,17] The bromomethyl ketones **3** were then converted to the 2,5-

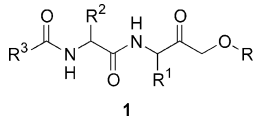

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Figure 1. R=H, CH_3, CH_2CH_3, CH_2Ph.

*Corresponding author. Tel.: +1-650-866-6289; fax: +1-650-866-6654; e-mail: rohan.mendonca@celera.com

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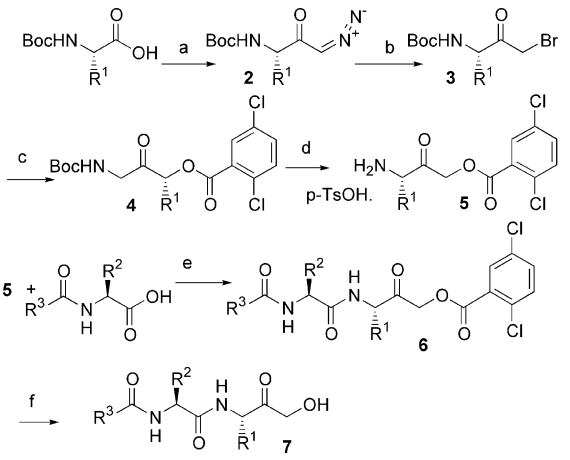
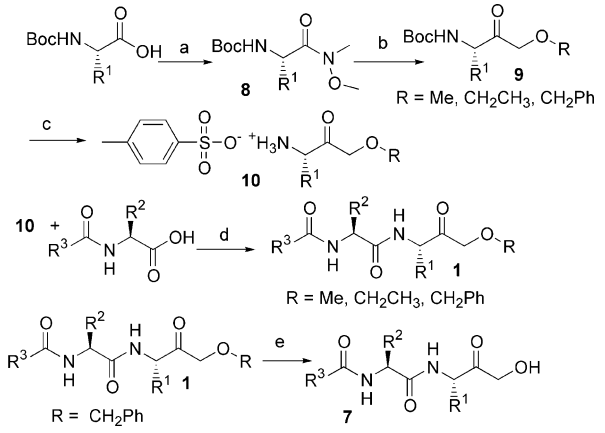


Scheme 1. Synthesis of hydroxymethyl ketones via diazomethyl ketones: (a) 1. IBCF, NMM, CH_2N_2; (b) HBr/HOAc; (c) 2,5-dichlorobenzoic acid, KF, DMF; (d) anhyd *p*-TsOH, CH_2Cl_2; (e) IBCF, NMM, THF; (f) cat. K_2CO_3, CH_3OH.



Scheme 2. Synthesis of hydroxymethyl ketone via benzyloxymethyl ketones: (a) DCC, THF, CH_3NHOCH_3; (b) $ROCH_2Cl$, Mg, $HgCl_2$, THF, −20–0 °C, 24 h; (c) anhyd *p*-TsOH, CH_2Cl_2; (d) IBCF, NMM, THF; (e) 20% $Pd(OH)_2$, cyclohexene, EtOH, 80 °C.

dichloro benzoates **4** by potassium fluoride mediated substitution of the carboxylic acid.[19] The de-protection of ketones **4** were carried out using anhydrous *p*-TsOH to yield amines **5**. Amines **5** were then coupled to N-protected amino acids under typical peptide coupling procedures to give **6**. This was hydrolyzed with catalytic potassium carbonate in methanol to give the hydroxymethyl ketone **7**.[20]

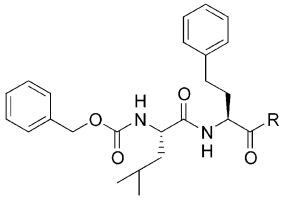
The above mentioned synthetic route (Scheme 1) involves the use of explosive reagent diazomethane, making it unsuitable for large scale synthesis. In addition acyloxymethyl ketones **4** are known irreversible inhibitors of cysteine proteases.[19] This led us to devise an alternative synthetic route to hydroxymethyl ketones.

The syntheses of hydroxymethyl ketones and alkoxymethyl ketones were carried out via Weinreb amide (Scheme 2). Commercially available amino acids were converted to the Weinreb amides **8**.[21] The Weinreb amides were then added to alkoxymethyl Grignard,[22–24] generated in situ at −60 °C and then warmed to room temperature to obtain the alkoxymethyl ketones **9**.[25] Catalytic mercuric (II) chloride is crucial for the generation of the alkoxymethyl Grignard. The alkoxymethyl ketones **9** were de-protected to amines **10** and then coupled further to obtain the final alkoxymethyl ketones **1**. The benzyloxymethyl ketones (**1**, R=CH_2Ph) obtained from the above procedure were reduced to the hydroxymethyl ketones **7** using hydrogen transfer conditions.[26] The added advantage of this method is that it yielded alkoxymethyl ketones that can be used to probe the prime side binding sites of the enzyme cathepsin K.

Results and Discussion

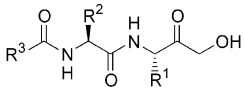
A panel of substituted methyl ketones were screened against cathepsin K, L, B, and S.[27] Table 1 shows various substituted methyl ketones that incorporate the same R^3 and R^2 elements to get a more accurate comparison of the effect of the R substituent on cathepsin binding, potency and selectivity. A similar derivative incorporating a hydroxymethyl ketone **15** and methyl ketone **16** is also included in the table for comparison purposes. It is clear from Table 1 that the hydroxymethyl ketone **15** is the most potent of all these

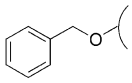
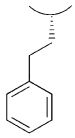
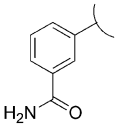
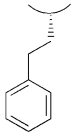
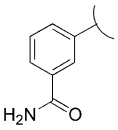
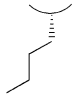
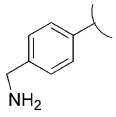
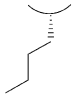
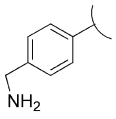
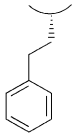
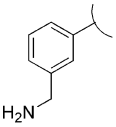
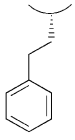
Table 1. SAR of substituted methyl ketones. K_i app. values are in μM



Compd	R	Cat K μM	Cat L μM	Cat S μM	Cat B μM
12	CH_2OCH_3	0.607	22.3	1.96	>150
13	$CH_2OCH_2CH_3$	0.947	16.7	4.5	>150
14	CH_2OCH_2Ph	1.0	6.34	0.451	119
15	CH_2OH	0.025	0.64	0.188	34.7
16	CH_3	1.44			519

Table 2. SAR of hydroxymethyll ketones. K_i app. values are in μM



	R^3	R^2	R^1	Cat K μM	Cat L μM	Cat S μM	Cat B μM
17				0.067	3	0.811	32.2
18				0.111	0.79	0.088	1.44
19				0.11	0.76	0.11	1.14
20				0.020	0.13	0.46	1.25
21				0.003	0.017	0.046	1.95
22				0.004	0.081	0.18	0.27

chemotypes. This is probably due to the stabilization of the enzyme–inhibitor complex via additional hydrogen bonds between the α-hydroxyl group and the active site hydrogen bond acceptors.

Modifications to the side-chain elements were made (R^1 and R^2) to improve their potency towards cathepsin K and selectivity over cathepsin B, L, and S. Based on selectivity issues (**15** and **17**) the isoleucine-homphenylalanine sequence was chosen for R^3 optimization. Table 2 presents the data from the hydroxymethyl ketones.

Comparison of compounds **18** and **22** in Table 2 also suggest that basic groups at R^3 yielded more potent compounds for cat K, as they can interact with asp 61 at the S3 subsite of the enzyme.[28] The substitution pattern of aminomethyl groups (**21** and **22**) on the benzoic acid is also critical for the interaction with aspartic acid 61 of the cathepsin K enzyme.[28] This fact will be further explored to yield more potent compounds in future.

Conclusions

We have shown a novel synthetic route to hydroxymethyl and alkoxymethyl ketones, which, in addition to circumventing various synthetic problems can also be used to facilitate the identification of potent and selective inhibitors of cathepsins K, L, B, and S.

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25. **General procedure for the syntheses of alkoxymethyl ketones**: In a dry flask, Mg turnings (199 mg, 7.44 mmol), previously dried in an oven overnight at 100 °C was weighed out, along with $HgCl_2$ (71 mg, 0.49 mmol). This was then purged with nitrogen for 10–15 min to ensure an anhydrous atmosphere. To this was added anhydrous THF under nitrogen. The temperature was lowered to −40 °C and benzyloxymethyl chloride (1.1 mL, 7.44 mmol) was added via syringe to the stirring suspension. This was then stirred under nitrogen for 6 h. The temperature was allowed to warm up to 3–5 °C during this time. The temperature was then lowered to −60 °C and to this was added 400 mg (1.24 mmol) of **8** ($R + CH_2CH_2Ph$). This was then stirred overnight and allowed to come to room temperature in the process. The reaction is then quenched by adding sat. NH_4Cl solution slowly with stirring. This was then stirred for 15–30 min or till effervescence ceased. This is then extracted with ethyl acetate (3×50 mL), dried with anhydrous Mg_2SO_4, filtered and concentrated. This is then purified by flash column chromatography using silica gel 60 and 15% ethyl acetate in hexane to yield 317.3 mg of product. ^1H NMR (200 MHz, $CDCl_3$) δ 1.43 (s, 9H), 1.74–1.82 (m, 1H), 2.14–2.16 (m, 1H), 2.60–2.67 (m, 2H), 4.13–4.14 (d, 2H), 4.49–4.61 (m, 3H), 5.14–5.17 (m, 1H), 7.1–7.4 (m, 10H). > 99% ee by chiral HPLC (Chiralpak OJ and OD) using a 50% IPA/50% Hexane gradient.

Note: 6 equiv of Mg turnings were used for protected mono peptide, 9 equiv for protected dipetide, etc.)

26. General procedure for the reduction of the benzyloxymethyl ketone to the hydroxymethyl ketone using hydrogen transfer conditions: To a solution of the benzyloxymethyl ketone (214 mg, 0.39 mmol) in 12 mL ethanol was added 5 mL cyclohexene. To this was added 35 mg 20% palladium hydroxide on carbon (0.1 equiv by weight or greater). The reaction was then refluxed for 1–2 h till starting material was consumed. This was followed by TLC analysis. The reaction was then cooled to room temperature and filtered through Celite. The filtrate was then concentrated to give the pure product 18. ^1H NMR (200 MHz, DMSO-d_6): δ 0.8–1.0 (m, 6H), 1.19–1.3 (m, 1H), 1.5–1.6 (m, 1H), 1.7–1.85 (m, 1H), 1.9–2.10 (m, 1H), 3.6–3.7 (m, 1H), 4.17–4.19 (d, 2H), 4.29–4.35 (m, 1H), 4.40–4.45 (t, 1H), 5.0–5.15 (m, 1H), 7.14–7.3 (m, 4H), 7.5–7.6 (m, 1H), 8.0–8.10 (m, 2H), 8.37 (s, 1H), 8.5–8.53 (d, 1H). $M + H^+$ (454.20).

27. **Materials**: Cathepsin B (Calbiochem; San Diego, California) and cathepsin L (Athens Research and Technology; Athens, Georgia) were purified from human liver and purchased from the indicated commercial sources. Human cathepsins S and K were cloned and expressed as described.[29,30] The enzyme active site concentrations were measured by titration with the either E-64 or the vinyl sulfone APC-3316, Me-Pip-Phe-HphVSPh.[31]

Inhibition Assays: Inhibitor potency measurements were performed at room temperature using 96-well kinetic plate readers. Reaction velocities were monitored at varying inhibitor concentrations by following the hydrolysis of aminomethylcoumarin substrates (ex_{355}, em_{460}) as indicated. All substrates were added at a concentration equal to their K_m. Control reactions in the absence of inhibitor were performed in parallel. The K_i apparent (K_i') values were determined by a nonlinear least squares regression fit of the experimentally derived data to the Morrison equation for tight-binding inhibitors as described[32] or by least squared regression fit of the Henderson equation for tight-binding inhibitors.[33] Enzyme and inhibitor were incubated 30-min prior to initiation of reaction by the addition of substrate.

Cathepsin B: Enzyme (5.0 nM) was mixed with inhibitor in 50 mM MES or BES (pH 6.0), 2.5 mM DTT, 2.5 mM EDTA, 0.05% Tween 20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (300 μM).

Cathepsin K: Enzyme (3.6 nM) was mixed with inhibitor in 50 mM MES (pH 5.5), 2.5 mM DTT, 2.5 mM EDTA, 0.05% Tween-20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (40 μM).

Cathepsin L: Enzyme (1.3 nM) was mixed with inhibitor in 50 mM MES (pH 5.5), 2.0 mM EDTA, 2 mM DTT, 0.05% Tween-20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (10 μM).

Cathepsin S: Enzyme (1.0 nM) was mixed with inhibitor in 50 mM MES (pH 6.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM 2-mercaptoenthanol, 0.001% bovine serum albumin and 10% DMSO. The substrate was Z-Val-Val-AMC (10 μM).

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