A New Synthesis of Peptidyl Epoxysuccinates for Probing Cysteine Protease-Inhibitor P_3/S_3 Binding Interactions

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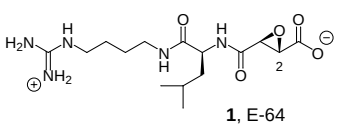
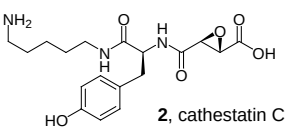
Abstract: A new synthesis of peptidyl epoxysuccinates has been developed involving the *N*-acylation of amino acid benzyl esters (**12**) with the tartrate ester derived epoxy acid **13** followed by deprotection of the benzyl esters and acylation with amines **21–28**. This synthesis is ideally suited for the rapid synthesis of peptidyl epoxysuccinate inhibitors of cysteine proteases, in order to probe selectivity issues concerning inhibitor/enzyme P_3/S_3 binding interactions.

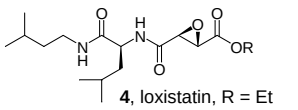
Key words: peptidyl epoxysuccinates, E-64 analogs, cysteine protease inhibitors, peptide synthesis

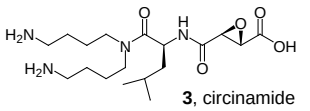
Cysteine proteases are an important class of enzymes involved in the degradative processing of peptides and proteins.[1,2] They are ubiquitous in nature and play vital roles in numerous pathological processes including arthritis, osteoporosis, Alzheimer's disease, cancer cell invasion, and apoptosis.[1–3] Cysteine proteases are also essential to the life cycles of many pathogenic protozoa,[4,5] including *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, and *Plasmodium falciparum*, the most dangerous of the malarial parasites.[6] Cruzain[7] and falcipain,[8] the major cysteine proteases of *T. cruzi* and *P. falciparum*, have been identified as potential therapeutic targets for treatment of Chagas' disease and malaria, respectively.[6,7,9–12]

In connection with ongoing efforts to develop potent and selective inhibitors[13] of cruzain and falcipain,[14–16] we became interested in a class of naturally occurring peptidyl epoxysuccinate derivatives, of which E-64 (**1**),[17–19] cathestatin C (**2**),[20,21] and circinamide (**3**)[22] are representative examples. These compounds are potent, highly selective irreversible inhibitor of cysteine proteases. Considerable effort has been devoted to the development of E-64 analogs as therapeutic agents.[23–27] Loxistatin (**4**),[28] a prodrug analog of E-64, was developed for treatment of muscular dystrophy and was taken into human clinical trials in Japan.[29] Potent and selective inhibitors of cathepsin B also have been developed based on the epoxysuccinyl motif.[26,30]

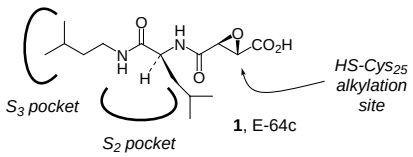




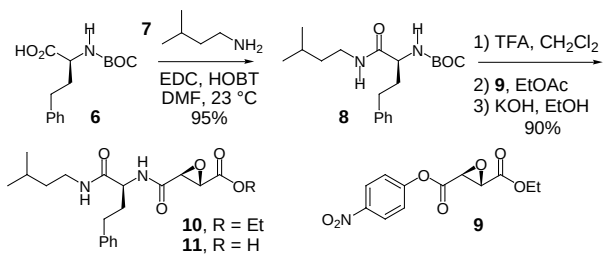




The regiochemistry of the inhibition reaction of papain with E-64 was determined by Rich,[31] who established that substitution of the epoxide by the active site cysteine residue occurs at C(2), adjacent to the negatively charged carboxylate. This conclusion has been verified by three different crystal structures of the papain•E64 or papain•E64c complexes.[32–34] Examination of these X-ray structures reveals that the peptide backbone of E-64 or E-64c (**5**) orients in a direction opposite to that of chloro- or fluoromethyl ketone inhibitors,[7,35,36] which are presumed to bind in a manner analogous to the natural peptide substrates. Consequently, the L-leucyl side chain of **1** does not optimally occupy the enzyme S_2 binding pocket.

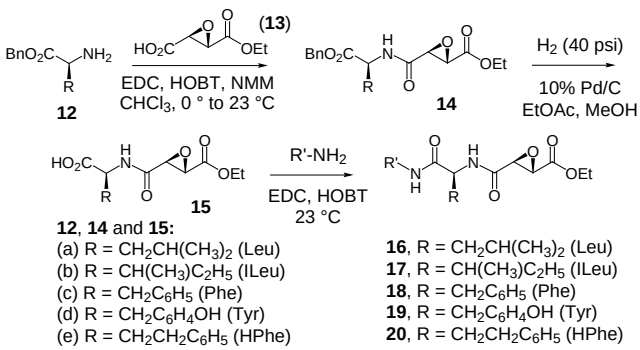


Armed with these insights, we embarked on a program designed to optimize the interactions of the epoxysuccinyl inhibitors with the S_2 and S_3 binding pockets of the targeted cysteine proteases. Initially, we utilized Yokoo's procedure for synthesis of E-64c analogs.[37] As illustrated below by the synthesis of **11** containing a L-homophenylalanine residue at P_2, this route involves the coupling of the BOC protected amino acid (**6**) with an amine (in this case, isopentyl amine, **7**) envisaged to serve as the P_3 substituent. Deprotection of the resulting peptide **8** followed by *N*-acylation with the *p*-nitrophenyl ester **9**[38] and ester hydrolysis then completed the synthesis of **11**.



While this sequence proved extremely useful for the synthesis of a series of peptidyl epoxysuccinates by varying

the amino acid (P_2 residue) at the beginning of the synthesis, this route proved less convenient for synthesis of analogs designed to probe the structural requirements of the P_3/S_3 interactions, since the P_3 substituent (e.g., **7** in the above scheme) is introduced in the very first step. This prompted us to develop a new synthesis of peptidyl succinate derivatives that would permit the P_3 substituent to be introduced at the end of the synthesis. Thus, acylation of L-amino acid benzyl esters **12**[39,40] (typically as the *p*-toluenesulfonate salts) [40] with mono acid **13**[41] provided amides **14** in good to excellent yield (Table 1). The optimized coupling conditions called for use of EDC, HOBT and *N*-methylmorpholine (NMM) in $CHCl_3$ at 0–23 °C. The reaction was very sluggish when performed using Et_3N as the base, as the Et_3N salt of **13** is insoluble in $CHCl_3$. The amount of racemization observed was also somewhat enhanced when Et_3N was used. Chloroform was used as solvent, rather than DMF which is more commonly employed for peptide couplings, [42,43] because product isolation was easier owing to the presence of fewer side products, and consequently the isolated yields were generally better for the reactions performed in $CHCl_3$. The epoxide unit of **14** is susceptible to ring opening by HOBT, and this side reaction was much more serious when the reactions were performed in DMF than in $CHCl_3$. However, it proved necessary to use HOBT in these couplings in order to minimize racemization of the α-amino acid ester. Nevertheless, as shown in the Table, 2–3% racemization was observed during these couplings, as evidenced by the presence of a minor set of epoxide C–H resonances in the ^1H NMR spectrum of the purified reaction product. [44]

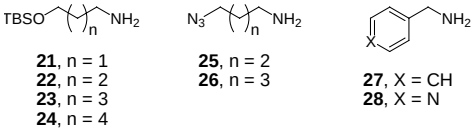


Hydrogenolysis of amides **14** provided carboxylic acids **15** in excellent yield (Table 1), which were used immediately in coupling reactions with amines **7** and **21–28**. Representative results are summarized in Table 2. Many of the acylations gave much better yields in $CHCl_3$ (using EDC and NMM) than in DMF (using EDC and Et_3N). This effect was especially pronounced in the acylations of the leucine derivative **15a**, for which comparative data are shown in the Table. Importantly, no evidence of additional racemization of the α-*N*-acylamino acid was observed (which typically compromises peptide synthesis when performed by activation of the carboxyl end of a growing peptide chain). [42]

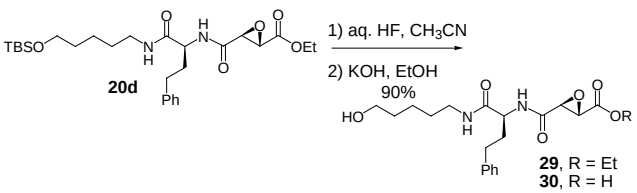
Table 1 Synthesis of Amides **14** and Carboxylic Acids **15**

Amides[a]	Solvent	Yield[b]	% d.e.	Carboxylic Acids[c]	Yield[b]
14a	$CHCl_3$	80–83%	96%	**15a**	94–99%
14a	DMF	77–80%	–		
14b	$CHCl_3$	74–78%	98%	**15b**	94–99%
14c	$CHCl_3$	76–83%	>99%	**15c**	95%
14c	DMF	68%	>98%		
14d	$CHCl_3$	72%	94%	**15d**	96–98%
14d	DMF	56%	92%		
14e	$CHCl_3$	77–80%	96%	**15e**	96%
14e	DMF	54–68%	86–94%		

[a] All acylation reactions of **12a–12e** and **13** were performed using EDC and HOBT in the indicated solvent. *N*-Methylmorpholine (NMM) was used as the base for the reactions performed in $CHCl_3$, whereas Et_3N was used for the couplings performed in DMF.
[b] Yields of product isolated by chromatography.
[c] Deprotection of the benzyl esters of amides **14a–14e** were performed by hydrogenation over 10% Pd/C using 40 psi H_2 in a 1 : 1 mixture of EtOAc and MeOH.



Deprotection of any functional groups introduced in the P_3 amine unit (see **20d** below, for example) and then hydrolysis of the ethyl ester provides the targeted epoxysuccinate derivatives. All of the ethyl esters **16–20** so prepared (Table 2) have been converted into the corresponding carboxylic acids and tested as inhibitors of cysteine proteases. Of the E-64 analogs described herein, **30** is the most potent inhibitor of cruzain, with **11** being the second most potent. [45] Details of the enzyme inhibition studies of these and related inhibitors will be reported in due course. [46]



In summary, we have developed a new synthesis of peptidyl epoxysuccinate derivatives. Although the overall efficiency of this synthesis of peptidyl epoxysuccinate derivatives is somewhat lower than Yokoo's method for synthesis of E-64c analogs, [37] it is a highly attractive alter-

Table 2 Synthesis of Peptidyl Amides by the Coupling of Carboxylic Acids **15** and Primary Amines[a]

Carboxylic Acids	R'NH$_2$	Conditions[a]	Product	Yield[b]
15a	**7**	$CHCl_3$, NMM, 0° to 23°C	**4**	82%
15a	**21**	$CHCl_3$, NMM, 0° to 23°C	**16b**	76%
15a	**21**	DMF, Et_3N, 23°C	**16b**	33%
15a	**22**	$CHCl_3$, NMM, 0° to 23°C	**16c**	65%
15a	**22**	DMF, Et_3N, 23°C	**16c**	28%
15a	**23**	$CHCl_3$, NMM, 0° to 23°C	**16d**	72%
15a	**23**	DMF, Et_3N, 23°C	**16d**	32%
15a	**24**	$CHCl_3$, NMM, 0° to 23°C	**16e**	65%
15a	**24**	DMF, Et_3N, 23°C	**16e**	24%
15b	**7**	$CHCl_3$, NMM, 0° to 23°C	**17a**	70%
15b	**25**	$CHCl_3$, NMM, 0° to 23°C	**17f**	47%
15b	**26**	$CHCl_3$, NMM, 0° to 23°C	**17g**	43%
15d	**22**	DMF, Et_3N, 0° to 23°C	**19c**	63%
15d	**23**	DMF, Et_3N, 0° to 23°C	**19d**	81%
15e	**7**	$CHCl_3$, NMM, 0° to 23°C	**10**	76%
15e	**21**	$CHCl_3$, NMM, 0° to 23°C	**20b**	70%
15e	**22**	$CHCl_3$, NMM, 0° to 23°C	**20c**	64%
15e	**23**	$CHCl_3$, NMM, 0° to 23°C	**20d**	66%
15e	**24**	$CHCl_3$, NMM, 0° to 23°C	**20e**	52%
15e	**27**	DMF, Et_3N, 23°C	**20h**	80%
15e	**28**	DMF, Et_3N, 23°C	**20i**	55%

[a] All acylation reactions summarized in Table 2 were performed using EDC and HOBT in the indicated solvent. *N*-Methylmorpholine (NMM) was used as the base for the reactions performed in $CHCl_3$, whereas Et_3N was used for the couplings performed in DMF.
[b] Yields of product isolated by chromatography.

native particularly for the rapid synthesis of analogs designed to probe selectivity issues concerning inhibitor/enzyme P_3/S_3 binding interactions.

Representative Experimental Procedures

{2*S*-[2*α*,3*β*(*S*)]}-Oxirane Carboxylic Acid, 3[({3-Phenyl-1-[(benzyloxy)carbonyl]propyl}amino)carbonyl] Ethyl Ester (14e)

To a solution of the *p*-toluenesulfonic acid salt of L-homophenylalanine benzyl ester[40] (0.44 g, 1.0 mmol), monoethyl epoxysuccinate **13**[41] (0.16 g, 1 mmol), HOBT (0.13 g, 1 mmol) and *N*-methylmorpholine (0.11 mL, 1 mmol) in $CHCl_3$ (5 mL) under nitrogen at 0 °C was added EDC (0.21 g, 1.1 mmol). The resulting mixture was stirred for 1 h at this temperature then at 23 °C for 9 h. The mixture was partitioned between EtOAc (30 mL) and H_2O (30 mL). The organic portion was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (elution with hexane–EtOAc 7–3 then 5:5) to afford a white solid (0.32 g, 77%). mp 102 °C.

$[\alpha]^{25}_D$ = +50.8 (*c* = 2.5, $CHCl_3$).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.33 (t, *J* = 7.0 Hz, 3 H), 2.06 (m, 1 H), 2.22 (m, 1 H), 2.56 (m, 2 H), 3.21 (d, *J* = 2.0 Hz, 1 H), 3.65 (d, *J* = 2.0 Hz, 1 H), 4.27 (m, 2 H), 4.69 (m, 1 H), 5.17 (d, *J* = 12.0 Hz, 1 H), 5.22 (d, *J* = 12.0 Hz, 1 H), 6.53 (d, *J* = 80 Hz, 1 H), 7.08–7.41 (m, 10 H).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.02, 31.51, 33.51, 51.78, 52.84, 53.77, 62.29, 64.49, 126.38, 128.27, 128.45, 128.65, 128.69, 134.97, 140.28, 165.74, 166.39, 171.13.

IR ($CHCl_3$) 3684, 3400, 3025, 3016, 1745, 1689, 1523, 1426, 1227, 1205, 1028 cm^{-1}.

HRMS (CI, CH_4) calcd. for $C_{23}H_{25}NO_6$ 411.1675, found 411.1672.

Anal. $C_{23}H_{25}NO_6$ (411.1) calcd C 67.14, H 6.12, N 3.42; found C 67.17, H 6.49, N 3.40.

[2*S*-[2*α*,3*β*(*S*)]}-3({[3-Phenyl-1-(carboxyl)propyl]amino}carbonyl)oxirane Carboxylic Acid Ethyl Ester (15e)

A mixture of benzyl ester **14e** (0.30 g, 0.7 mmol) and 10% Pd/C (150 mg) in 40 mL of EtOAc and MeOH (40 mL) was shaken in a Parr–Knorr apparatus under hydrogen (40 psi) for 10 min. The catalyst was removed by filtration through a pad of Celite and the solution concentrated in vacuo to afford a solid (0.22 g, 96%), mp 110–112 °C. $[\alpha]^{25}_D$ = +74.7 (*c* = 3.0, CH_3OH).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.33 (t, *J* = 7 Hz, 3 H), 2.11 (m, 1 H), 2.29 (m, 1 H), 2.68 (t, *J* = 7 Hz, 2 H), 3.23 (s, 1 H), 3.69 (s, 1 H), 4.29 (m, 2H), 4.66 (m, 1 H), 6.53 (d, J = 8 Hz, 1H), 7.24 (m, 5H), 10.10 (br, 1 H).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 14.02, 31.70, 33.14, 51.62, 53.69, 62.40, 126.52, 128.30, 128.70, 140.08, 166.29, 166.35, 175.52.

IR (KBr) 3403, 3057, 30,30, 2982, 2932, 1751, 1742, 1648, 1551, 1498, 1442, 1395, 1370, 1346, 1239, 1214, 1169, 1028, 898 cm^{-1}.

HRMS (CI, CH_4), calcd for $C_{16}H_{19}NO_6$ 321.1212, found 321.1203.

{2*S*-[2*α*,3*β*(*S*)]}-3-{[(3-Phenyl-1-{[(5-*t*-butyldimethylsilanoxylpentyl)amino]carbonyl} propyl)amino]carbonyl}oxirane Carboxylic Acid Ethyl Ester (20d)

To a solution of amine **23**[47] (0.44 g, 2.0 mmol) acid **15e** (0.64 g, 2 mmol), HOBT (0.26 g, 2 mmol) and *N*-methylmorpholine (0.22 mL, 2 mmol) in $CHCl_3$ (10 mL) under N_2 at 0 °C was added EDC (0.42 g, 2.2 mmol). The resulting mixture was stirred for 1 h at this temperature then at 23 °C for 9h. The mixture was then partitioned between EtOAc (30 mL) and H_2O (30 mL). The organic portion was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (elution with hexane–EtOAc 7–3) to afford **20d** (0.42 g, 66%) which was used immediately in the next step.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.04 (s, 6 H), 0.88 (s, 9 H), 1.31 (t, *J* = 7.6 Hz, 3 H), 1.36 (m, 2 H), 1.51 (m, 4 H), 1.97 (m, 1 H), 2.16 (m, 2 H), 2.62 (m, 2 H), 3.25 (m, 2 H), 3.27 (d, *J* = 1.6 Hz, 1 H), 3.60 (t, *J* = 6 Hz, 2 H), 3.63 (d, *J* = 1.6 Hz, 1 H), 3.63 (d, J_{ab} = 1.6 Hz, 1 H), 4.26 (m, 2 H), 4.34 (m, 1 H), 5.78 (t, *J* = 5.6 Hz, 1 H), 6.20 (d, *J* = 8 Hz, 1 H), 7.23 (m, 5H).

{2*S*-[2*α*,3*β*(*S*)]}-3-{[(3-Phenyl-1-{[(5-hydroxylpentyl)amino]carbonyl}propyl)amino] carbonyl}oxirane Carboxylic Acid Ethyl Ester (29)

A 48% solution of HF (0.31 mL, 0.84 mmol) was added to a suspension of **20d** (0.29g, 0.56 mmol) in MeCN (5 mL) and CH_2Cl_2 (5 mL) at 0 °C and the mixture was then allowed to warm to 23 °C and stirred for 15 min. The mixture was diluted with CH_2Cl_2 (30 mL) and washed with concentrated $NaHCO_3$ (2 x 20 mL), brine (20 mL), dried (Na_2SO_4) and concentrated in vacuo. The residue was redissolved in CH_2Cl_2 and MeOH and passed through a short pad of silica gel to give, after concentration in vacuo, a solid (0.21 g, 95%) mp 157 °C; $[\alpha]^{25}_D$ = +31.6 (*c* = 1.2, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.32 (t, *J* = 7.2 Hz, 3 H), 1.40 (m, 2 H), 1.54 (m, 4 H), 1.84 (br s, 1H), 1.97 (m, 1 H), 2.15 (m, 1 H), 2.62 (m, 2 H), 3.26 (m, 2 H), 3,32 (d, *J* = 2 Hz, 1 H), 3.63 (m, 2 H),

3.65 (d, *J* = 2 Hz, 1 H), 4.26 (m, 2 H), 4.38 (m, 1 H), 6.28 (br s, 1 H), 6.85 (br s, 1 H), 7.23 (m, 5 H).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 14.02, 22.95, 28.99, 31.75, 31.98, 33.75, 39.50, 52.62, 52.80, 53.72, 62.34, 62.43, 126.37, 128.28, 128.63, 140.46, 166.10, 166.49, 170.33.

IR ($CHCl_3$) 3440, 3390, 1755, 1680, 1525, 1460, 1375, 1310, 1080, 1030, 935, 905 cm^{-1}.

HRMS (CI, NH_3), calcd for $C_{21}H_{31}N_2O_6$ (M+1)+407.2182, found 407.2162.

{2*S*-[2*α*,3β*(S)*]}-3-{[(3-Phenyl-1-{[(5-hydroxylpentyl)amino]carbonyl}propyl)amino]carbonyl}oxirane Carboxylic Acid (30)

A 1N solution of KOH in EtOH (0.5 mL, 0.50 mmol) was added to a solution of **29** (0.20g, 0.50 mmol) in THF (5 mL) and EtOH (5 mL) at 0 °C and stirred for 30 min. The solvent was then evaporated in vacuo and the residue dissolved in cold water (5 mL), acidified to pH 2 with 10% $KHSO_4$ and extracted with EtOAc (5 × 10 mL). The combined organic portions were washed with brine (20 mL), dried (Na_2SO_4) and concentrated in vacuo to obtain **30** as a syrup which was crystallized with EtOAc and hexane to give a white solid (0.18 g, 95%). mp 133 °C. $[\alpha]_D^{25}$ = +56.0 (c = 2.5, CH_3OH).

^{1}H NMR ($CDCl_3$ and CD_3OD 400 MHz): δ = 1.40 (m, 2 H), 1.54 (m, 4 H), 1.84 (br s, 1H), 1.97 (m, 1 H), 2.15 (m, 1 H), 2.62 (m, 2 H), 3.26 (m, 2 H), 3,32 (d, *J* = 2 Hz, 1 H), 3.63 (m, 2 H), 3.65 (d, *J* = 2 Hz, 1 H), 4.38 (m, 1 H), 6.29 (br s, 1 H), 6.87 (br d, *J* = 8 Hz, 1 H), 7.23 (m, 5 H).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 23.07, 29.08, 31.66, 32.31, 34.22, 51.61, 52.92, 53.04, 60.90, 79.32, 126.10, 128.45, 128.56, 141.40. 165.52, 169.07, 170.79

IR (KBr) ν = 3300, 3088, 2941, 2861, 1742, 1666, 1644, 1556, 1497, 1455, 1250 cm^{-1}.

HRMS (CI, CH_4), calcd for $C_{19}H_{27}N_2O_6$ (M+H)+379.1869, found 379.1868.

Anal. $C_{19}H_{26}N_2O_6$ (378.43) calcd C 60.31, H 6.93, N 7.40; found C 60.43, H 7.32, N 7.09.

Acknowledgement

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