

# An Improved Preparation of the Activity-Based Probe JPM-OEt and In Situ Applications

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**Abstract:** A short, stereoselective synthesis of the general, cell permeable cathepsin probe JPM-OEt, is presented. The synthetic route is improved and described in more detail than previous reports for related compounds. This serves as a facile method for the synthesis of multi-gram quantities of activity-based probes utilizing an epoxide-succinyl scaffold. Additionally, JPM-OEt is shown to be cell permeable, allowing in vivo characterization of cysteine proteases. More importantly, this reagent has recently been shown to be an effective general inhibitor of papain family cysteine proteases in animal models of cancer. For this reason the outlined synthesis method will enable future in vivo studies using this reagent.

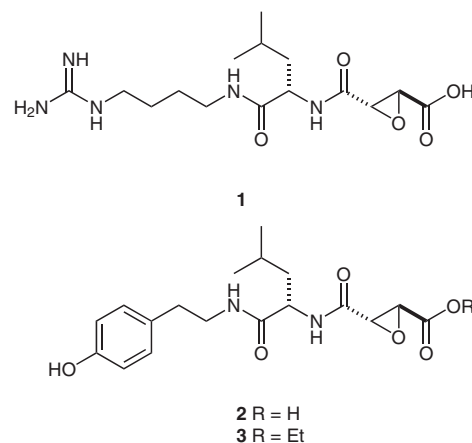
**Key words:** epoxides, medicinal chemistry, inhibitors, cysteine proteases, activity based probes

The lysosomal cysteine proteases of the papain family are among the most extensively studied of all proteolytic enzymes. In particular, cathepsins B, L, H, S, Z and K have received much attention due to their potential role in disease states such as cancer invasion and metastasis,<sup>2</sup> rheumatoid arthritis,<sup>3</sup> asthma,<sup>4</sup> osteoporosis,<sup>5</sup> and Alzheimer's disease.<sup>6,7</sup> Specific affinity labels that covalently tag the papain family in the process of inhibition have been developed.<sup>8</sup> These reagents are designed to carry a range of tagging groups that allow visualization of covalently modified target proteases. Already these types of activity based probes have been applied to study protease function in a number of important biological systems.<sup>9</sup> Such work has led to an understanding of the importance of cathepsin cysteine proteases in diseases such as cancer and malaria.<sup>10</sup>

The natural product E-64 (**1**; Figure 1) is an epoxy-succinyl based inhibitor identified over 20 years ago that targets papain family cysteine proteases. This class of inhibitors has been shown to react with a broad panel of cysteine proteases by covalently-modifying the active-site sulfhydryl nucleophile.<sup>11</sup> In the past, a range of synthetic analogs of E-64 have been prepared in which modifications to the pseudo-peptide portion of the molecule were made.<sup>12–14</sup> Replacement of the agmatine group with a tyramine yields the inhibitor JPM-565 (**2**)<sup>14</sup> (Figure 1).

Although JPM-565 is an attractive activity-based affinity probe due to its broad reactivity with cysteine proteases and its ability to be radioactively labeled with <sup>125</sup>I at the phenol ring, it is a relatively poor reagent for use in intact cells due to its negatively charged carboxylic acid function at cellular pH.

JPM-OEt (**3**), the ethyl ester and synthetic precursor of JPM-565, shows efficient labeling of papain family cysteine proteases when added to intact cells growing in culture, while the related JPM-565 is only able to label these proteases targets after disruption of the cell membrane.<sup>15</sup> Thus, esters of inhibitors such as JPM-565 render otherwise ineffective compounds useful for in vivo studies. This attribute allows active proteases to be labeled in their natural environment and therefore provides a more physiologically relevant profile of protease activity. However, apart from the aforementioned studies, relatively little has been reported regarding the synthesis and application of ethyl ester 'pro-drug' strategies for in vivo application of activity based probes.



**Figure 1** The epoxysuccinyl containing cysteine protease modifying probes E-64, JPM-565 and JPM-OEt.

The broad, class-specific reactivity of JPM-OEt makes it an ideal tool to evaluate the potency and selectivity of various small molecule inhibitors. Furthermore, its ability to label papain family cysteine proteases in intact cells has been critical for the study of protease function in various cellular models. Recently, this reagent has been applied in vivo to animal models of cancer and was found to have

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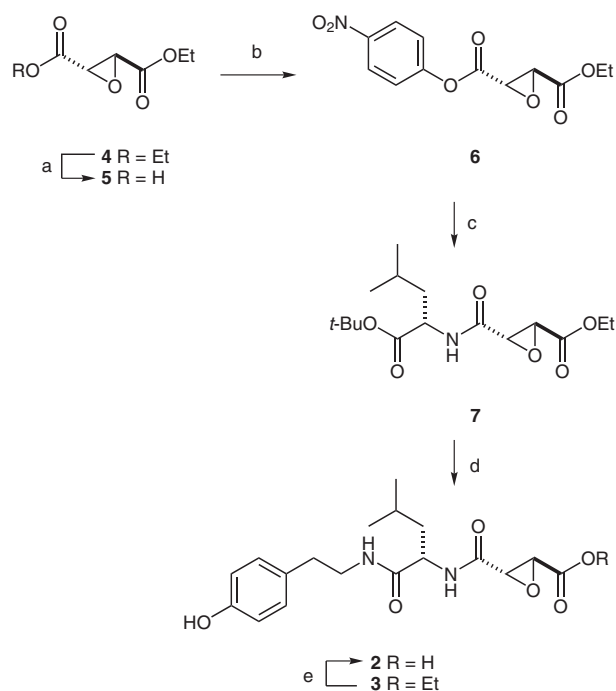
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dramatic effects on various stages of disease progression. As a result, the compound is currently in early stage toxicity and pharmacodynamics studies at the National Cancer Institute with the hope of it reaching human clinical trial in the near future. These extensive *in vivo* studies require the ability to synthesize large quantities of JPM-OEt. However, the previous synthesis reported by Shi et al.<sup>14</sup> lacked specific experimental conditions and details for synthesis. Here we report an efficient synthesis of JPM-OEt using the crystalline nitrophenyl ester **6** as an ethyl (2*S*,3*S*)-(+)-2,3-epoxysuccinate synthon. This synthesis affords highly pure material in multi-gram quantities with only minimal column chromatography steps. This synthesis method is also amenable to further scale-up and GMP production. Moreover, we show that JPM-OEt is an effective activity based probe that targets cysteine proteases of the papain family *in vivo* as well as *in vitro*.

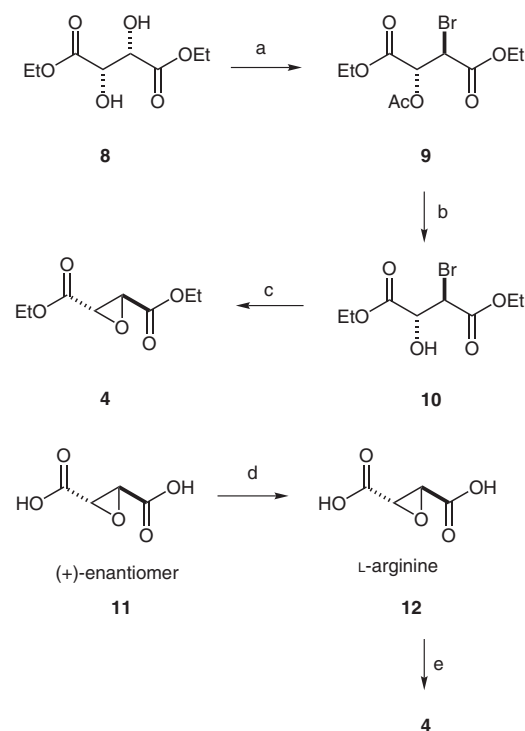
The preparation of JPM-OEt (**3**), as illustrated in Scheme 1, first required the synthesis of ethyl-*L*-*trans*-epoxysuccinic acid (**5**) which was obtained through the parent compound diethyl (2*S*,3*S*)-(+)-2,3-epoxysuccinate (**4**).



**Scheme 1** Reagents and conditions: (a) KOH (1.0 equiv). (b) DCC (1.03 equiv), *p*-nitrophenol (1.0 equiv), 66% (2 steps). (c) H-Leu-*O*-*t*-Bu (1.0 equiv), Et<sub>3</sub>N (1.0 equiv). (d) 1) 2.5% TIS, 2.5% water, TFA-CH<sub>2</sub>Cl<sub>2</sub> (1:1), 2) isobutylchloroformate (1.0 equiv), NMM (1.0 equiv), THF, then tyramine hydrochloride (1.0 equiv), Et<sub>3</sub>N (1.0 equiv), DMF, 76% (3 steps). (e) NaOH (1.0 equiv), 81%.

The diethyl epoxy ester **4** was synthesized by a slight modification of published procedures.<sup>13,16–23</sup> Commercially available diethyl-D-tartrate (**8**) was converted into the bromo ester **9** utilizing HBr–HOAc as described in Scheme 2. Further hydrolysis of ester **9** to yield the bromohydrin **10** was accomplished with HCl–EtOH rather than HBr–HOAc.<sup>24</sup> Ring closure of the bromohydrin **10**

by slow addition of DBU in Et<sub>2</sub>O at 4 °C afforded the epoxide **4** in 79% yield after distillation. We, along with Meara and Rich,<sup>13</sup> have found DBU to give more improved and reproducible yields over the NaOEt method. The diethyl ester **4** may also be obtained through the optical resolution of commercially available (±)-*trans*-epoxysuccinic acid (**11**) by diastereomeric salt formation with *L*-arginine as shown in Scheme 2.<sup>17,23</sup> Recrystallization followed by esterification with absolute EtOH and H<sub>2</sub>SO<sub>4</sub> afforded the epoxy ester **4** in 78% yield based on single enantiomeric isolation. No additional purification was necessary. This method offers yields comparable to those obtained through the bromohydrin and is slightly more cost-effective in obtaining the diethyl-(2*S*,3*S*)-(+)-epoxysuccinate (**4**).



**Scheme 2** Reagents and conditions: (a) HBr–HOAc, r.t., 16 h. (b) HCl–EtOH, reflux, 5 h. (c) DBU (1 equiv), Et<sub>2</sub>O, 0 °C, 79% (3 steps). (d) *L*-arginine, water, MeOH, 78%. (e) H<sub>2</sub>SO<sub>4</sub>, EtOH, 78% (2 steps, based on single enantiomer).

Diester **4** was saponified with 1 equivalent of KOH in EtOH affording, after work-up with dilute HCl, the desired mono-acid **5** (Scheme 1). No additional effort was made to recycle unconsumed starting material.

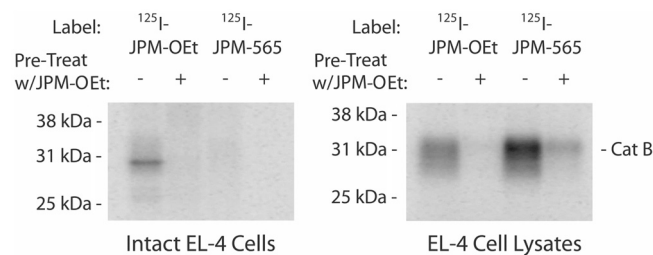
The key step in the synthesis was activation of the ethyl epoxysuccinate carboxylic acid. The first and most popular approach employs the mixed anhydride method that activates the carboxylic acid through a carbonate intermediate by applying *N*-methyl morpholine and isobutyl chloroformate at low temperature, followed by addition of the peptide moiety as a free amine. While this method generally affords good to excellent results, the desired tyramine compound, i.e. JPM-OEt, was isolated in moderate yield from an intractable mixture of products. This problem ap-

appears to only be affiliated with tyramine containing moieties as evidenced by the successful coupling of a variety of amines in high yield to the epoxy succinate **5** through the mixed anhydride approach.<sup>13</sup> This suggests that this phenomenon could be a result of reaction on the phenolic hydroxyl on the tyramine residue. Other coupling methods have been used to activate acid **5**, such as DCC–HOBt,<sup>25,26</sup> synthesis of the NHS or pentafluorophenyl esters,<sup>27</sup> or the use of phosphorus based reagents such as BOP. In agreement with findings by Meara and Rich, we obtained lower yields using the NHS or pentafluorophenyl esters and epoxide decomposition using BOP or PyBOP.<sup>13</sup> However, the acid **5** could be successfully activated with DCC and *p*-nitrophenol providing compound **6** in 66% yield after recrystallization.<sup>17</sup> Nitrophenyl ester **6** has several advantages over previously described activation methods. Given that the ester is a solid, it can be conveniently handled, especially when applied to solid phase reaction conditions.<sup>28</sup> In addition, the formation of by-products during reaction with an amino function appeared to be low. Furthermore, activated ester **6** is a stable compound, with a shelf-life of at least two years when stored at –20 °C under Ar.

Once the activated ester **6** was available, the synthesis of JPM-OEt required only three steps with no purification of intermediates until final product isolation. Briefly, compound **6** was reacted with L-leucine *t*-butyl ester to form amide **7**. Deprotection of the *t*-butyl ester was effected with trifluoroacetic acid. The corresponding free acid was activated by the mixed anhydride method using *N*-methyl morpholine and isobutylchloroformate. Subsequent reaction with a DMF solution of tyramine,<sup>29</sup> followed by flash chromatography and crystallization, afforded JPM-OEt (**3**) as a white solid in 76% overall yield starting from the nitrophenyl ester **6**. It should be noted that a convergent strategy, in which a tyramine–leucine dimer was reacted with activated ester **6**, resulted in the formation of a significant amount of a by-product containing an epoxysuccinyl on the phenolic hydroxyl. This observation further illustrates the incompatibility of a tyramine residue with an activated epoxysuccinyl moiety, as observed in other activation methods. Saponification of JPM-OEt with one equivalent of NaOH in EtOH and subsequent precipitation with Et<sub>2</sub>O afforded the sodium salt of JPM-565 (**2**) as a cream-colored solid in 81% yield.

To examine the efficacy of JPM-565 and its ethyl ester JPM-OEt in the *in vivo* labeling of cysteine proteases of the papain family, EL4 mouse lymphoma cells and lysates thereof were incubated with <sup>125</sup>I radioactively labeled forms of **2** and **3** and analyzed by SDS gel-electrophoresis (Figure 2, left panel). Whereas free acid **2** results in no labeling *in vivo*, its ethyl ester derivative **3** shows an intense reactivity against the cysteine protease cathepsin B. For comparison, EL4 lysates were treated with the same compounds (Figure 2, right panel), showing that both are capable of labeling cathepsin B *in vitro*. These results confirm that JPM-565 (**2**) is an effective and specific label of papain family cysteine proteases and that its ethyl ester

provides a pro-drug strategy for delivery of the probe across cell membranes.



**Figure 2** JPM-OEt but not JPM-565 labels papain family cysteine proteases in intact cells. Left panel: intact EL4 cells were treated with equivalent amounts of <sup>125</sup>I-labeled JPM-OEt or JPM-565, either with or without pre-treatment of 50 μM JPM-OEt. Right panel: EL4 lysates show papain family cysteine protease labeling for both JPM-OEt and JPM-565.

In summary, a short and efficient route towards the general cathepsin inhibitor JPM-OEt is presented. Key feature in the synthesis is the use of nitrophenyl activated epoxysuccinate synthon **6**. JPM-OEt was shown to be an effective inhibitor and activity based probe, both *in vitro* and *in vivo*.

<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Varian spectrometer (<sup>1</sup>H NMR: 400 MHz; <sup>13</sup>C NMR: 100.6 MHz). <sup>1</sup>H chemical shifts are reported in ppm relative to the solvent resonance (7.27 ppm for CDCl<sub>3</sub>, 2.49 ppm for DMSO-*d*<sub>6</sub>), and <sup>13</sup>C chemical shifts are reported relative to the central solvent peak (77.4 ppm for CDCl<sub>3</sub>, 49.5 ppm for DMSO-*d*<sub>6</sub>). All solvents were purchased from EM Science and were of HPLC or DriSolv<sup>®</sup> grade. L-Leucine-*t*-butyl ester hydrochloride was purchased from Bachem. All other reagents were purchased from Aldrich unless otherwise noted and used without further purification. Flash chromatography was conducted on Merck silica gel 60 (230–400 mesh). TLC was performed using Merck 60 F-254 silica gel plates; visualization was either by UV illumination or ceric ammonium molybdate (CAM) dip and heat.

#### Synthesis of Diethyl (2*S*,3*S*)-(+)-2,3-Epoxysuccinate (**4**) via Bromohydrin Method

A 33% solution of HBr (200 mL) in HOAc was added at 0 °C to neat D-diethyl tartrate (49.12 g, 0.238 mol) over a period of 30 min. Fifteen min after the final HBr addition, the solution was allowed to warm to r.t. and was stirred overnight. The reaction mixture was poured onto crushed ice (500 g), and the resulting mixture was extracted with Et<sub>2</sub>O (4 ×). Combined organic layers were washed with H<sub>2</sub>O (3 ×) and brine, dried (MgSO<sub>4</sub>) and concentrated under reduced pressure, affording a mixture of **9** and **10** (70.66 g) as a pale yellow oil. To a solution of this oil in EtOH (300 mL) was added acetyl chloride (8.0 mL, 0.113 mol) and the solution was heated under gentle reflux until TLC analysis indicated complete conversion to the lower running deacetylated product (ca 5 h). After cooling to r.t., the solution was concentrated at a temperature below 40 °C to give bromohydrin **10** as a pale-yellow oil. A solution of DBU (35.5 mL, 0.238 mol) in Et<sub>2</sub>O (150 mL) was added dropwise over a 2 h period to a solution of the crude bromohydrin **10** in Et<sub>2</sub>O (300 mL), cooled to 0 °C. After the final addition of DBU, the mixture was stirred for an additional 0.5 h. H<sub>2</sub>O (50 mL) was added and the mixture was washed with 1 M KHSO<sub>4</sub>, H<sub>2</sub>O, and concentrated under reduced pressure. Vacuum distillation (68–70 °C/0.5 mmHg) [lit. 78–80 °C/0.9 mmHg<sup>17</sup>] gave title compound **4** (35.4 g, 188 mmol, 79%) as a colorless oil; R<sub>f</sub> 0.39 (EtOAc–hexanes, 1:4).

$^1\text{H NMR}$  ( $\text{CDCl}_3$ ):  $\delta = 4.28$  (dq,  $J = 7.0$  Hz, 2.1 Hz, 4 H), 3.67 (s, 2 H), 1.32 (t,  $J = 7.1$  Hz, 6 H).

$^{13}\text{C NMR}$  ( $\text{CDCl}_3$ ):  $\delta = 166.56, 62.28, 52.08, 14.18$ .

HRMS:  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_8\text{H}_{13}\text{O}_5^+$ : 189.0763; found: 189.0648.

#### Synthesis of Diethyl (2*S*,3*S*)-(+)-2,3-Epoxy succinate (4) via L-Arginine Method

A solution of L-arginine (33.24 g, 0.1908 mol) in warm water (50–60 °C; 92 mL) was added gradually over a 45 min period to a stirred solution of ( $\pm$ )-*trans*-epoxy succinic acid (**11**) (1 equiv) in MeOH (368 mL). The mixture was then allowed to stand at r.t. for 5 h before standing overnight at 4 °C. The precipitate was filtered, washed with MeOH–H<sub>2</sub>O (4:1, 75 mL) and then recrystallized from MeOH–H<sub>2</sub>O (425 mL, 2:1) to give **12** (26.0 g) as fine, brilliant crystals after drying under vacuum overnight. Subsequently, 95% H<sub>2</sub>SO<sub>4</sub> (25.0 g) was added dropwise to **12** in absolute EtOH (300 mL). The mixture was refluxed for 5 h, during which it turned into a colorless solution. The solvent was evaporated to a minimum (ca 50 mL remaining) and the colorless residue was poured into ice-water (200 mL). The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (4 × 100 mL), and the combined organics were washed with sat. NaHCO<sub>3</sub>, dried (MgSO<sub>4</sub>), and evaporated, giving the title compound (13.9 g, 73.9 mmol, 78% yield based on single enantiomeric isolation) as a colorless oil that required no further purification.

#### Synthesis of Ethyl (2*S*,3*S*)-(*p*-Nitrophenyl)-oxirane-2,3-dicarboxylate (6)

Diethyl epoxy succinate **4** (6.95 g, 0.0369 mol) was dissolved in absolute EtOH (100 mL) and placed in an ice-bath. A solution of KOH (2.44 g, 0.0369 mol) in absolute EtOH (50 mL) was added dropwise over 15 min to the colorless solution. The reaction mixture was then allowed to stir at 0 °C for 3 h, and then 2 h at r.t. After evaporation of solvent in vacuo, H<sub>2</sub>O (100 mL) was added to the resulting white solid and the solution was washed once with CH<sub>2</sub>Cl<sub>2</sub> (30 mL). The aqueous layer was acidified with concd HCl (3.5 mL) and NaCl (30 g) was added. After extraction with EtOAc (4 × 100 mL), the organic layers were collected, dried (MgSO<sub>4</sub>), and concentrated to afford the crude half ester (**5**) as a colorless oil. Next, a solution of DCC (7.84 g, 0.038 mmol) in EtOAc (20 mL) was added dropwise to a solution of crude compound **5** and *p*-nitrophenol (5.14 g, 0.0369 mol) at 0 °C. During addition, the solution developed a white precipitate. After the reaction was stirred at r.t. overnight, the mixture was filtered and the precipitate was washed with EtOAc (2 ×). The filtrate was concentrated and the resulting residue was recrystallized from EtOAc–hexanes to afford **6** (6.85 g, 66%) as off-white crystals;  $R_f$  0.24 (EtOAc–hexanes, 1:4).

$^1\text{H NMR}$  ( $\text{CDCl}_3$ ):  $\delta = 8.31$  (d,  $J = 9.4$  Hz, 2 H), 7.37 (d,  $J = 9.4$  Hz, 2 H), 4.34 (dq,  $J = 7.1, 4.3$  Hz, 2 H), 3.96 (d,  $J = 1.6$  Hz, 1 H), 3.88 (d,  $J = 1.6$  Hz, 1 H), 1.38 (t,  $J = 7.1$  Hz, 3 H).

$^{13}\text{C NMR}$  ( $\text{CDCl}_3$ ):  $\delta = 166.3, 164.8, 154.5, 146.0, 125.7, 122.3, 63.1, 53.0, 52.2, 14.6$ .

#### *N*-{[*L-trans*-3-(Ethoxycarbonyl)oxiran-2-yl]carbonyl}-*L*-leucyl-3-(*p*-hydroxyphenyl)ethylamide [JPM-OEt (3)]

A solution of leucine *t*-butyl ester hydrochloride (3.98 g, 17.78 mmol) and diisopropylethylamine (3.1 mL, 17.78 mmol, 1.0 equiv) in CH<sub>2</sub>Cl<sub>2</sub> (40 mL) was added dropwise to a solution of nitrophenyl ester **6** (5.0 g, 17.78 mmol) in EtOAc (50 mL). After stirring overnight, the reaction mixture was diluted with Et<sub>2</sub>O (200 mL), subsequently washed with H<sub>2</sub>O, 2% NaOH (6 × 50 mL), H<sub>2</sub>O (3 × 100 mL) and brine, dried (MgSO<sub>4</sub>) and concentrated under reduced pressure to afford **7** as a colorless oil. The *t*-butyl ester **7** was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and triisopropylsilane (2.5 mL), H<sub>2</sub>O (2.5 mL) and trifluoroacetic acid (50 mL) were added. The colorless solution was stirred for 4 h at r.t. and then concentrated under reduced

pressure. Traces of trifluoroacetic acid were removed by repeated co-evaporation with toluene, affording the free carboxylic acid as a viscous pale yellow oil. Neat isobutylchloroformate (2.32 mL, 17.89 mmol) was added at –40 °C to a solution of the carboxylic acid and *N*-methyl morpholine (1.95 mL, 17.78 mmol) in anhyd THF (100 mL). One minute after addition, a solution of tyramine hydrochloride (3.09 g, 17.78 mmol) and Et<sub>3</sub>N (2.47 mL, 17.78 mmol) in DMF (40 mL) was added dropwise over a period of 2 min. Then, the cooling bath was removed and the reaction mixture was allowed to stir for 4 h, at which time it was filtered and evaporated. The residue was dissolved in EtOAc (300 mL), washed with 1 N HCl (3 × 100 mL), sat. NaHCO<sub>3</sub> (3 × 100 mL), H<sub>2</sub>O, and concentrated to afford a golden yellow oil (6.9 g). After initial purification by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>–MeOH, 9:1), further purification was performed with a second flash column, eluting first with CH<sub>2</sub>Cl<sub>2</sub> and then 3% MeOH in CH<sub>2</sub>Cl<sub>2</sub>. The appropriate fractions were collected and concentrated to yield a white foam which upon recrystallization from CH<sub>2</sub>Cl<sub>2</sub>–Et<sub>2</sub>O–petroleum ether (bp 40–60 °C) afforded JPM-OEt (5.30 g, 13.5 mmol, 76%) as a white solid;  $R_f$  0.35 (CH<sub>2</sub>Cl<sub>2</sub>–MeOH, 9:1).

$^1\text{H NMR}$  (DMSO- $d_6$ ):  $\delta = 9.12$  (s, 1 H), 8.57 (d,  $J = 8.2$  Hz, 1 H), 8.10 (t,  $J = 5.9$  Hz, 1 H), 6.95 (d,  $J = 8.2$  Hz, 2 H), 6.63 (d,  $J = 8.6$  Hz, 2 H), 4.31–4.23 (m, 1 H), 4.22–4.12 (m, 2 H), 3.70 (d,  $J = 1.7$  Hz, 1 H), 3.58 (d,  $J = 1.5$  Hz, 1 H), 3.28–3.09 (m, 2 H), 2.56 (t,  $J = 7.0$  Hz, 2 H), 1.55–1.44 (m, 1 H), 1.44–1.35 (m, 2 H), 1.23 (t,  $J = 7.0$  Hz, 3 H), 0.86 (d,  $J = 6.4$  Hz, 3 H), 0.82 (d,  $J = 6.4$  Hz, 3 H).

$^{13}\text{C NMR}$  (DMSO- $d_6$ ):  $\delta = 170.70, 166.93, 164.20, 155.35, 129.29, 129.10, 114.82, 61.47, 52.88, 51.19, 51.12, 41.09, 40.50, 34.17, 24.24, 22.92, 21.69, 13.97$ .

ESI-MS:  $m/z = 393.1$  [ $\text{M} + \text{H}$ ] $^+$ , 415.2 [ $\text{M} + \text{Na}$ ] $^+$ .

HRMS:  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_{20}\text{H}_{29}\text{N}_2\text{O}_6^+$ : 393.2026; found: 393.1947.

#### *N*-{[*L-trans*-3-Carboxyoxiran-2-yl]carbonyl}-*L*-leucyl-3-(*p*-hydroxyphenyl)ethylamide, Sodium Salt [JPM-565 Na $^+$ Salt (2)]

NaOH (1 N, 3.9 mL) was added to a solution of JPM-OEt (1.50 g, 3.82 mmol) in EtOH (20 mL). The solution was stirred at r.t. for 4 h and concentrated under reduced pressure. The solid was redissolved in MeOH (20 mL) and ice-cold Et<sub>2</sub>O was added to precipitate the product. The sodium salt was filtered, washed twice with Et<sub>2</sub>O and dried in vacuo to afford the sodium salt of JPM-565, **2** (1.20 g, 3.11 mmol, 81%) as a cream-colored solid.

$^1\text{H NMR}$  (DMSO- $d_6$ ):  $\delta = 8.54$  (d,  $J = 8.6$  Hz, 1 H), 8.20 (t,  $J = 5.1$  Hz, 1 H), 6.86 (d,  $J = 8.6$  Hz, 2 H), 6.56 (d,  $J = 8.6$  Hz, 2 H), 4.29–4.20 (m, 1 H), 3.27 (d,  $J = 2.0$  Hz, 1 H), 3.25–3.05 (m, 2 H), 2.97 (d,  $J = 2.0$  Hz, 1 H), 2.54 (q,  $J = 6.7$  Hz, 2 H), 1.54–1.31 (m, 3 H), 0.84 (d,  $J = 6.3$  Hz, 3 H), 0.80 (d,  $J = 6.3$  Hz, 3 H).

$^{13}\text{C NMR}$  (DMSO- $d_6$ ):  $\delta = 171.13, 169.01, 166.95, 157.30, 129.15, 127.44, 115.25, 54.86, 52.19, 51.01, 41.12, 40.40, 34.04, 24.30, 22.95, 21.71$ .

HRMS:  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_{18}\text{H}_{25}\text{N}_2\text{O}_6^+$ : 365.1713; found: 365.1634.

#### Labeling Experiments

Cells of the EL4 mouse lymphoma cell line were treated for 30 min with equal amounts of I-125 labeled compound **2** and **3**, either with or without a 30 min pretreatment with 50  $\mu\text{M}$  JPM-OEt (**3**). Cell lysates were prepared using glass beads in a buffer of pH 5.5 (50 mM acetate buffer, 2 mM DTT and 5 mM MgCl<sub>2</sub>), and treated in similar fashion as the whole cells. Samples were quenched by dilution with 4 × SDS sample buffer, boiled and run on a 12.5% SDS-PAGE gel. Labeled proteins were visualized by exposure of the gel to a phosphorimaging screen and scanning on a typhoon scanner.

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