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Stereoselective enzymatic reduction of keto-salinosporamide to (−)-salinosporamide A (NPI-0052)

Rama Rao Manam, Venkat R. Macherla* and Barbara C. M. Potts

Nereus Pharmaceuticals, Inc., 10480 Wateridge Circle, San Diego, CA 92121, USA

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Abstract—Salinosporamide A (NPI-0052, **1**), a highly potent 20S proteasome inhibitor, has been prepared from its ketone precursor (**2**) by asymmetric enzymatic reduction. The yields are quantitative with complete stereoselective conversion to the desired product, with no evidence for the undesired diastereomer. This process should lead to new synthetic strategies for the total synthesis of **1**.

Salinosporamide A (NPI-0052; **1**) is a potent inhibitor of the 20S proteasome, that is currently in clinical trials for the treatment of cancer.[1–3] Structurally, **1** comprises a β-lactone-γ-lactam bicyclic ring system substituted with methyl, cyclohex-2-enylcarbinol, and chloroethyl substituents that give rise to specific and mechanistically important interactions within the proteasome active site.[4] This dense functionality, which includes 5 contiguous chiral centers, makes **1** an extremely attractive and challenging synthetic target. The first enantioselective total synthesis of **1** was reported by Corey and co-workers in 2004 starting from *S*-threonine.[5] Several alternative strategies have been published, with all routes converging upon a common penultimate compound that bears a hydroxyl group in place of chlorine.[6–8] We envisioned a new strategy to complete the total synthesis of **1** through its ketone precursor, 'keto-salinosporamide' (**2**), and developed a suitable method for this transformation. Using ketoreductase enzymes, the ketone group of **2** can be stereoselectively reduced to produce **1** in quantitative yields. Here we report the details of this reaction.

Earlier, we reported the semi-synthesis of keto-salinosporamide (**2**) from **1** and the diastereoselective $NaBH_4$ reduction of **2** to produce **3**, the C-5 epimer of **1**, with about 90% de.[3] The present study was designed to selectively generate **1** from **2**. We evaluated many commercially available reagents for this stereoselective reduction under a variety of reaction conditions (Table 1), none of which gave **1** as a single product. Most reagents gave little to no product or resulted in degradation, except for $NaBH_4$, which still offered no stereoselectivity for **1**, and in some cases concomitantly reduced the β-lactone ring to give compound **4**. At reduced temperatures, C-5 epimer **3** was favored, while increased temperatures generally resulted in over-reduction to **4**; the latter could usually be minimized by decreasing the number of equivalents of reducing agent to <1. Given the lack of selectivity for **1**, we explored the potential of ketoreductase enzymes to execute the required stereoselective reduction.

Enzymes are well known catalysts for stereoselective reductions. There are many ketoreductases available for screening in an attempt to identify a suitable enzyme for a desired transformation.[9] Keto-salinosporamide (**2**) was screened against a panel of 98 NAD(P)H-dependent ketoreductase enzymes using a spectrophometric assay to indicate consumption of NAD(P)H cofactor as a result of enzymatic reduction of the ketone.[9b] Through this screening process, two NADH-dependent ketoreductases, KRED-EXP-C1A and KRED-EXP-B1Y, were shown to be catalytically active against our substrate, with specific activities of 0.28 and 0.26 U/mg, respectively. Small scale (1–2 mg) reactions were performed on these two active enzymes[10] using sodium formate and formate dehydrogenase (FDH) as a cofactor recycler. The products were extracted with EtOAc after 1 h and analyzed by HPLC, which indicated that both enzymes selectively produced the desired product **1** (KRED-EXP-C1A: 68% conversion; KRED-EXP-B1Y: 66% conversion) with about 20% starting material remaining. These reactions were subsequently performed at a 60 mg scale using the same FDH recycler,

* Corresponding author. Tel.: +1 858 200 8334; fax: +1 858 587 4088; e-mail: vmacherla@nereuspharm.com

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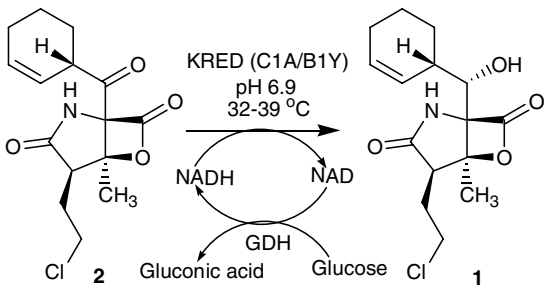
Table 1. Reduction of **2**[a] with $NaBH_4$

$NaBH_4$ (# equiv)	Reaction conditions			Product ratio		
	Solvent[b]	Temperature (°C)	Time (min)	**1**	**3**	**4**
1	Monoglyme + 1% water	−78	14	5	95	0
1	Monoglyme + 1% water	−10	14	30	50	20
1	Monoglyme + 1% water	0	14	33.3	33.3	33.3
2	Monoglyme + 1% water	rt	8	50	0	50
1	Monoglyme + 1% water	rt	8	45	10	45
0.5	Monoglyme + 1% water	rt	8	50	50	0
0.25	Monoglyme + 1% water	rt	8	50	50	0
1	IPA + 1% water	rt	10	50	0	50
0.5	IPA + 1% water	rt	12	60	10	30
0.25	IPA + 1% water	rt	8	50	40	10
0.25	IPA + 5% water	rt	8	10	0	10
0.5	IPA	rt	8	40	50	10
0.5	IPA	0	8	30	70	0
0.25	IPA	rt	8		No reaction	
1	THF + 1% water	rt	10	50	0	50
0.5	THF + 1% water	rt	12	50	50	0
0.25	THF + 1% water	rt	8	30	70	0
1 + LiCl	Monoglyme + 1% water	−78	10	5	95	0
1 + LiCl	Monoglyme + 1% water	0	10	27.2	36.4	36.4
1 + LiCl	Monoglyme + 1% water	10	10	10	30	60
1 + $CeCl_3$	Monoglyme + 1% water	−78	10	5	95	0
1 + $CeCl_3$	Monoglyme + 1% water	0	10	25	50	25
1 + $CeCl_3$	Monoglyme + 1% water	10	10	20	60	20

[a] Degradation or little to no product was observed using the following reagents. (1) $NaBH_4$ on 10% basic Al_2O_3, (2) K-Selectride, (3) KS-Selectride, (4) BTHF-(*R*)-CBS, (5) BTHF-(*S*)-CBS, (6) $NaBH(OAc)_3$, (7) $(CH_3)_4NBH(OAc)_3$, and (8) *i*PrMgCl.
[b] Methyl and ethyl ester derivatives were formed when MeOH and EtOH were used, respectively.

which gave product **1** after 1 h with about 80% conversion. The products were extracted with EtOAc and characterized by LC–MS and NMR to confirm the structure of **1**, albeit with only 50–60% recovery under these exploratory conditions.

To optimize the reaction conditions, these transformations (Scheme 1) were performed on 10–100 mg scale using KRED-EXP-C1A or KRED-EXP-B1Y ketoreductases and glucose dehydrogenase (GDH) recycler in place of FDH; all other reaction conditions were similar to those used previously. The reactions were monitored by an analytical HPLC method in which the two possible products, **1** and **3**, were completely resolved. The results are summarized in Table 2. When KRED-EXP-C1A ketoreductase was used, the conversion from **2** to **1** was 70% complete after 1 h on a 10 mg scale. Based on HPLC analysis of the organic extract, the conversion was 90% complete when the reaction time was increased to 3 h, but subsequent evaluation of the aqueous extract revealed that a portion of the product had decomposed to **5**, which is an expected hydrolysis product that forms in aqueous solution.[3,11] Furthermore, the isolated yields on a 50–100 mg scale were only 40%, further suggesting decomposition. In a similar way, when KRED-EXP-B1Y ketoreductase was used on 10 mg scale, the conversion from **2** to **1** was about 90% complete after 1 h based on organic extract analysis, but the analysis of the mixture of aqueous and organic extracts (1:1) indicated the presence of degradation product **5** in about 20%. When the concentrations of the BIY enzyme and GDH were doubled, the conversion was 90–95% after 40 min, with minimal decomposition (2–5%) and isolated yields of 85–90%.[12] Decomposition was also minimized when biphasic solutions (50% aqueous *t*-BuOAc, *n*-BuOAc, TBME) were used, but the percent conversion was generally very low even with longer reaction times (20–24 h), except in 50% aqueous TBME (80% conversion; Table 2). We note that both KRED-EXP-C1A and KRED-EXP-B1Y were used as lyophilized powders from crude cell lysates, suggesting the potential for improved specific



Scheme 1. Enzymatic reduction of **2** to **1** with NADH dependent ketoreductases.

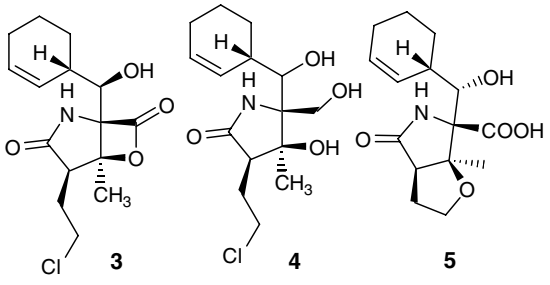


Table 2. Conversion of **2** to **1**[a]

2 (mg)	KRED # equiv (w/w)		GDH # equiv (w/w)	% Solvent in water	Time (h)	% Conversion to **1**[b]
10	C1A	1	0.5	~20% DMSO	1	70
10	C1A	1	0.1	~20% DMSO	1	70
10	C1A	1	0.1	~20% DMSO	2	85
10	C1A	1	0.1	~20% DMSO	3	90
100	C1A	1	0.1	~20% DMSO	1	70
100	C1A	1	0.1	~20% DMSO	3	80[c]
50	C1A	1	0.1	~20% DMSO	4	90[c]
10	B1Y	1	0.1	~20% DMSO	1	90
10	B1Y	1	0.1	50% *t*-BuOAc	1	40
					20	50
10	B1Y	1	0.1	50% *n*-BuOAc	1	0
					24	20
10	B1Y	1	0.1	50% TBME	1	5
					24	80
10	B1Y	2	0.2	~20% DMSO	0.67	95
10	C1A	2	0.2	~20% DMSO	0.67	70
20	B1Y	2	0.2	~20% DMSO	0.67	95[d]
50	B1Y	2	0.2	~20% DMSO	0.67	90[e]

[a] At pH 6.9 using GDH, NAD, glucose.
[b] Based on HPLC analysis of organic extract.
[c] Recovered yield 40% after purification by flash column chromatography. Some decomposition product (**5**) was detected in aqueous layer.
[d] Recovered yield 90% after purification by flash column chromatography.
[e] Recovered yield 85% after purification by crystallization.

activities for these enzymes in their more highly purified forms (BioCatalytics, personal communication).

In conclusion, the keto-salinosporamide (**2**) may serve as a useful precursor in the total synthesis of **1**. We explored a variety of chemical reagents and reaction conditions for the stereoselective conversion of **2** to **1** but were unable to achieve results that strongly favored the desired product. We subsequently screened a library of ketoreductases and identified two enzymes that can be utilized to convert **2** to **1** with complete stereoselective conversion to the desired product, with no evidence for the undesired diastereomer. The KRED-EXP-B1Y ketoreductase is superior to KRED-EXP-C1A in the conversion of **2** to **1**. Doubling the concentrations of B1Y and GDH and decreasing the reaction time resulted in better yields and minimal decomposition of product (2–5%). Interestingly, while chemical reagents stereoselectively reduced **2** to **3**, two ketoreductase enzymes stereoselectively reduced **2** to the desired product **1**.

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10. Protocol for small scale enzymatic reduction: Keto-salinosporamide (**2**, 10 mM in DMSO) was mixed with NAD+ (2 mM), ketoreductase (10 mg/mL, KRED-EXP-C1A or KRED-EXP-B1Y), sodium formate (20 mM), FDH-101 (2 mg/mL) for cofactor recycling, and sodium phosphate buffer (1 mL, 150 mM, pH 6.9). The reaction mixtures were incubated at 33 °C for an hour, extracted with ethyl acetate, evaporated to dryness in a speed vacuum, redissolved in acetonitrile, and analyzed by HPLC for product formation.
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12. The optimized procedure for enzymatic transformation of **2** to **1**: To a solution of **2** (50 mg, 0.16 mmol) in DMSO (1 mL) in a round bottom flask (25 mL), 5 mL of potassium phosphate buffer (150 mM, pH 6.9), 100 mg of ketoreductase EXP-B1Y, 10 mg of GDH-103, 2.5 mL of glucose (50 mM), and 2.5 mL of NAD (1 mM) were added. The above reaction mixture was stirred at 37–39 °C

for 40 min and then extracted with EtOAc (2 × 25 mL); the combined organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to yield a crude product, which was crystallized in 1:1 acetone: heptane (6 mL) in a 20 mL scintillation vial (by slow evaporation under nitrogen gas) to afford **1** as a white crystalline solid (42 mg, 85% yield). The structure of **1** was confirmed by comparison of its mp, specific rotation, and ^1H and ^{13}C NMR spectra with those of an authentic sample. Ketoreductases KRED-EXP-C1A and KRED-EXP-B1Y, GDH-103, D-glucose, and NAD grade II free acid were obtained from BioCatalytics, Pasadena, CA.