Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: http://www.tandfonline.com/loi/tbbb20

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To cite this article: Mitsutaka KINO, Kenzo SHONO, Tetsuo NISHIMURA & Satoru NAGAMURA (1998) Practical Preparation of K-252a from a Fermentation Solution, Bioscience, Biotechnology, and Biochemistry, 62:8, 1627-1629, DOI: 10.1271/bbb.62.1627

To link to this article: http://dx.doi.org/10.1271/bbb.62.1627

Biosci. Biotechnol. Biochem., **62** (8), 1627–1629, 1998

Note

Practical Preparation of K-252a from a Fermentation Solution

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Received March 19, 1998

We developed a practical preparation procedure for K-252a by methylating K-252b on an industrial scale. The water-insoluble K-252a, which was present in the cell mass, was converted to the water-soluble K-252b Na salt in an alkaline solution. The obtained K-252b was methylated with dimethylsulfate in the presence of potassium carbonate in dimethylacetamide. We have already used this method to manufacture 90 kg of K-252b from the fermentation broth, and regenerated 65 kg of K-252a from K-252b.

Key words: K-252a; purification; methylation

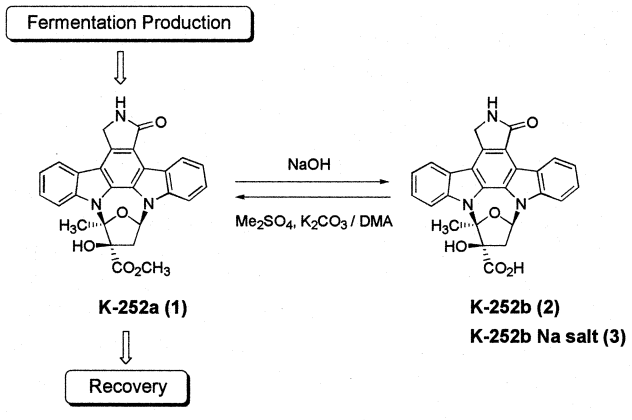
K-252a (**1**), a representative indolocarbazole alkaloid, was originally isolated from *Nocardiopsis* sp. in 1986.[1] It was initially identified as a protein kinase C (PKC) inhibitor and was subsequently found to inhibit a number of serine/threonine protein kinases.[2] More recently, it has also been reported to possess neurotrophic-like properties.[3] The neurotrophic activity demonstrated by **1** for enhancing choline acetyltransferase (ChAT) activity was comparable to the response elicited by the brain-derived neurotrophic factor (BDNF) and insulin-like growth factor I (IGF-I).[4] In addition, recent studies have shown that **1** protected neurons against glucose deprivation,[5] free-radical-mediated injury and amyloid β-peptide toxicity.[6] Moreover, the derivative of **1** has been shown to inhibit the enzymatic activity of neurotrophine receptor trk both *in vitro* and in intact cells, and the compound has also exhibited *in vivo* antitumor efficacy against tumors derived from NIH3T3 cells transfected with NGF (nerve growth factor) receptor trkA.[7] These results suggest that the development of derivatives of **1** could provide effective therapy for treating neurodegenerative diseases and cancer.[8] Therefore, a practical preparation of **1** is critical for the development of novel drugs. As reported elsewhere,[9] the fermentation titer has recently been improved to 2 g/l in a fermentation tank.[9] In this paper, we will describe a practical preparation of **1** by the method of methylating K-252b (**2**) on an industrial scale.

Compound **1** is not particularly soluble in water or other typical organic solvents, the solubility being <1 g/l in MeOH, 6 g/l in acetone and 8 g/l in ethyl acetate.[10] On the other hand, **1** is freely soluble (>100 g/l) in dimethylsulfoxide (DMSO) and dimethylformamide (DMF), but its solubility is drastically reduced if a trace of water is present. In 50% aqueous DMSO or DMF, the solubility of **1** is below 2 g/l, which was the concentration achieved by fermentation.[9] This insolubility and poor partitioning ability to organic solvents make it extremely difficult to effectively extract **1** from the fermentation broth on an industrial scale.

Therefore, we searched for a more efficient recovery method for **1** on an industrial scale. Our search also focused on methods for minimizing the volume of organic solvents. We considered the possibility of converting water-insoluble K-252a to a soluble K-252b alkaline salt in the cell suspension under alkaline conditions, and then extracting it from the broth. Since **1** is present almost entirely in the cell mass, the supernatant does not contain any appreciable amount of **1**. After such extraction, the obtained compound **2** can be converted to **1** by esterification (Scheme 1).

The sodium salt (**3**) of K-252b is soluble to the extent of 14 g/l, but the solubility of the NH_4 salt is below 1 g/l. The K-252b free acid, on the other hand, is essentially insoluble in water. Based on these considerations, we selected NaOH as a base for hydrolysis. Moreover, it was found that the solubility in water of **3** was greatly influenced by the accompanying salt concentration as shown in Fig. 1. The fermentation broth of **1** contained approximately 10 mM KH_2PO_4, 30 mM $CaCO_3$ and additional inorganic salts in smaller proportions.[9] The hydrolysis of **1** required a base concentration of 40 mM NaOH (pH 12), the presence of these salts decreasing the solubility of **3** from 14 g/l to 0.4 g/l. Therefore, in order to keep **3** in solution after its formation, the fermentation broth would require several-fold dilution with water, or removal of the salts by desalting. We tried to desalt the fermentation broth containing **1**, because dilution of the fermentation broth would lead to

Scheme 1



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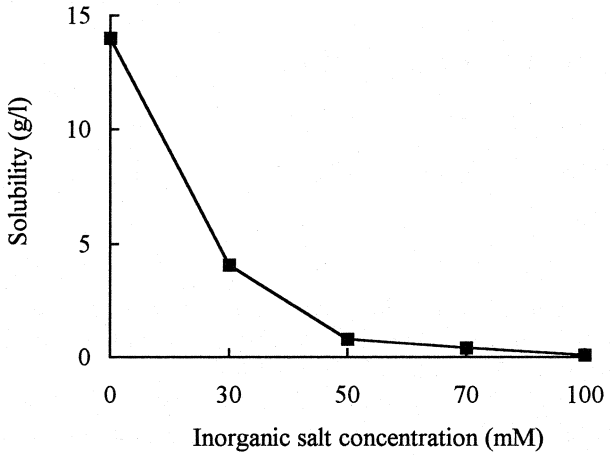

Fig. 1. Solubility of the K-252b Na Salt in an Aqueous Solution at 20°C.

The solubility of the K-252b Na salt was measured in an aqueous solution containing KH_2PO_4 and $CaCO_3$ (1:4) at 20°C.

an increase in the liquid operating volume. It was found that ultrafiltration (UF) was the most effective and convenient method for desalting. The desalted cell suspension was diluted to the initial volume, and then **1** was hydrolyzed to **3** at 80°C by adding NaOH. The hydrolyzed cell suspension contained **3** in the supernatant at the level of 2 g/l. The hydrolyzed cell suspension was then filtered by using UF again, and the filtrate was applied to an adsorption resin. From the eluant, **2** was recovered as a crystalline material in a 75% yield by adjusting the pH level to 3 and concentrating the obtained free base.

Obtained compound **2** was methylated with dimethylsulfate in the presence of potassium carbonate as a base at 30°C to quantitatively give **1**. We found that this methylation was best carried out in dimethylacetamide (DMA) as the reaction solvent at a high concentration of **2** (70 g/l). Moreover, when an equivolume of water was added to the reaction solution at the end of the reaction, **1** was easily recovered as a crystalline material in a 75% yield.

As already mentioned, this procedure offers a practical process for recovering **1** via **2**, and can provide easy access to **1** which is amenable to commercial preparation; for example, by using this method, we have already manufactured 90 kg of **2** from the fermentation broth, and regenerated 65 kg of **1** from **2**.

Experimental

General methods. ^{1}H-NMR spectra were measured with a Jeol JNM-A400 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Mass spectra were measured with a Jeol JMS-HX110/110A instrument. All chemicals and solvents were purchased commercially and used without any further purification. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck).

Preparation of K-252b (**2**) from the K-252a fermentation broth. The K-252a fermentation broth produced by *Nocardiopsis* sp. was adjusted to pH 3 with concentrated sulfuric acid, and the microorganism cells were collected by using an ultrafiltration membrane (UF membrane). 120 kg of K-252a (**1**) was contained in these cells. The cells were washed 4 times by dilution and concentration, and to these washed cells was added water to obtain a cell suspension. The resulting cell suspension was adjusted to pH 12 by adding sodium hydroxide, and then heated at 80°C for 6 h to extract K-252b as the Na salt (**3**). The cell suspension treated in this way was adjusted to pH 10.5 with concentrated sulfuric acid, and then the cell debris was removed by the ultrafiltration membrane (UF). The filtrate was applied to adsorption resin (SP-207, Mitsubishi Chemical Corporation) which was successively washed with 0.1 N NaOH and 20% MeOH, and then subjected to elution with 80% MeOH. A fraction containing **3** was neutralized with concentrated sulfuric acid, and then concentrated *in vacuo*. The resulting solution was cooled and filtered to obtain 90 kg (75%) of **2** as a crystalline material: mp 266°C (dec.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.22 (1H, d, J=7.8 Hz), 8.61 (1H, br s), 8.05 (1H, d, J=7.3 Hz), 7.98 (1H, d, J=8.5 Hz), 7.90 (1H, d, J=8.3 Hz), 7.48 (1H, ddd, J=1.2, 7.1, 8.5 Hz), 7.47 (1H, ddd, J=1.2, 7.1, 8.3 Hz), 7.35 (1H, t, J=7.4 Hz), 7.28 (1H, t, J=7.1 Hz), 7.10 (1H, dd, J=4.9, 7.4 Hz), 6.13 (1H, br s), 5.01 (2H, J=21.8 Hz), 3.36 (1H, dd, J=7.4, 13.8 Hz), 2.22 (3H, s), 1.98 (1H, dd, J=4.9, 13.8 Hz); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 174.1, 171.8, 139.9, 136.8, 132.9, 128.3, 125.6, 125.4, 125.0, 124.1, 124.0, 122.6, 121.2, 120.3, 119.5, 119.4, 115.7, 114.9, 114.5, 109.1, 99.3, 85.0, 84.5, 45.4, 42.5, 22.8; HRFAB-MS m/z $(M+H)^+$: calcd. for $C_{26}H_{20}N_3O_5$, 454.1403; found, 454.1429.

Conversion from K-252b (**2**) to K-252a (**1**). Me_2SO_4 (11.0 l, 116 mol) and K_2CO_3 (16 kg, 116 mol) were added to a solution of **2** (35 kg, 77 mol) in dimethylacetamide (500 l), and the mixture was stirred at 30°C for 6 h. Then, H_2O (500 l) was added, and the resulting solution was stirred at 50°C. The solution was cooled and filtered to obtain 27 kg (75%) of **1** as a crystalline material: mp 265°C (dec.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.20 (1H, d, J=7.8 Hz), 8.63 (1H, br s), 8.05 (1H, d, J=7.7 Hz), 7.93 (1H, d, J=8.5 Hz), 7.89 (1H, d, J=8.3 Hz), 7.47 (1H, ddd, J=1.2, 7.1, 8.5 Hz), 7.47 (1H, ddd, J=1.2, 7.1, 8.3 Hz), 7.35 (1H, t, J=7.4 Hz), 7.28 (1H, t, J=7.1 Hz), 7.14 (1H, dd, J=4.9, 7.4 Hz), 6.34 (1H, br s), 5.02 (2H, J=21.8 Hz), 3.92 (3H, s), 3.38 (1H, dd, J=7.4, 13.8 Hz), 2.14 (3H, s), 2.01 (1H, dd, J=4.9, 13.8 Hz); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 172.9, 171.8, 139.9, 136.8, 133.0, 128.3, 125.6, 125.4, 125.1, 124.2, 123.9, 122.6, 121.3, 120.4, 119.6, 119.5, 115.8, 114.8, 114.6, 109.1, 99.4, 85.0, 85.0, 52.7, 45.5, 42.5, 22.8; HRMS (FAB) m/z $(M+H)^+$: calcd. for $C_{27}H_{22}N_3O_5$, 468.1559; found, 468.1561.

Acknowledgments

The authors thank Dr. Mayumi Yoshida and her staff for recording the NMR and mass spectra. We also thank Torcan Chemical Ltd. for carrying out the methylation reaction on an industrial scale.

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