SYNTHESSES OF INDIRUBINS BY ALDOL CONDENSATION OF ISATINS WITH INDOXYL ANION GENERATED IN SITU BY LIPASE-CATALYZED DEACETYLATION OF INDOXYL ACETATE

Takeshi Sugai,* Kengo Hanaya, and Shuhei Higashibayashi

Department of Pharmaceutical Sciences, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. E-mail: sugai-tk@pha.keio.ac.jp

Abstract – The syntheses of indirubin (76% yield), 6-bromoindirubin (82% yield), and 6-bromoindirubin-3′-oxime (78% yield in two steps) were achieved via the lipase-triggered aldol condensation between isatins and an indoxyl anion in tetrahydrofuran under anhydrous and anaerobic conditions as the key step. The aldol donor was generated in situ by Burkholderia cepacia lipase (Amano PS-IM)-catalyzed deacetylation of commercially available and stable indoxyl acetate in the presence of triethylamine and with 2-propanol as the transesterification reagent. The scale-up of the presently developed reactions is easier than that in the previously reported chemical aldol condensations, because of the simplicity of the isolation procedure and suppression of the oxidative byproduct formation from indoxyl acetate.

Historically, interest in and studies on naturally occurring dyes have been underlying origins of the chemistry of heterocyclic organic compounds. The development of indigo1 is unambiguously one of the most famous examples. Natural indigo is manufactured from indican (1a), which originates from plants, through a fermentation process via the oxidative dimerization of indoxyl (1b), as shown in Scheme 1. This process is accompanied by the formation of a deep-purple dye, indirubin (2a), through another oxidized intermediate, isatin (3a), as also shown in Scheme 1. Indirubin itself has been isolated from plants2 and is known to be a physiologically active3 ingredient in the Chinese medicine “Danggui Longhui Wan.” Also, the effect of 2a toward murine colitis4–6 in “Qing-Dai” is attractive.

As suggested by the pioneering works of von Baeyer,7 indirubin can be formed via aldol condensation between isatin and indoxyl anion, as shown in Scheme 2. Indoxyl anion (1d) is unstable under aerobic
conditions; indoxyl acetate (1c) has therefore been practically used as a precursor of 1d under basic hydrolytic conditions to yield 2a itself and a wide range of non-natural derivatives.\textsuperscript{8-13}

\begin{figure}
\begin{center}
\includegraphics[width=\textwidth]{scheme1.png}
\end{center}
\caption{Pathways for the formation of indigo and indirubin (2a) from indican (1a)}
\end{figure}

\begin{figure}
\begin{center}
\includegraphics[width=\textwidth]{scheme2.png}
\end{center}
\caption{Formation of 2a by way of the aldol condensation between 3a and 1d}
\end{figure}

In recent studies on the lipase-catalyzed deacetylation of aryl acetates under transesterification conditions,\textsuperscript{14-20} we demonstrated the advantages of our approach over conventional chemical reactions. The only byproducts are acetate esters derived from alcohol for transesterification and can very easily be removed from the desired products. We envisaged the aldol condensation between 3a and 1d, which is generated from 1c by lipase-catalyzed deacetylation, in the presence of a proper base, as shown in Scheme 3. The serine hydroxy group located in the catalytic site of lipases attacks the acetyl group in 1c to liberate 1b. Under basic conditions, the resulting \textit{in situ}-formed 1d would function as an aldol donor to 3a. Such “lipase-triggered” aldol reactions have seldom been reported. Acetaldehyde, the tautomer of vinyl alcohol, which is resulted from the transesterification on vinyl acetate, has been shown to function as an aldol donor on an aromatic aldehyde under basic conditions.\textsuperscript{21}

We chose triethylamine rather than an inorganic salt such as potassium carbonate because the addition of triethylamine has been shown in some cases to promote lipase-catalyzed reactions.\textsuperscript{22} The acidity of the
hydrogen triethylammonium ion (pKₐ 10.75), compared with that of indoxyl (pKₐ 10.46), is sufficient to generate 1d in an equilibrium. For the regeneration of free enzyme from the acetylated form, a secondary alcohol such as 2-propanol¹⁷,²³ is added, as shown in Scheme 3. The acidity of the aliphatic alcohol, however, does not affect the aldol reaction.

First, we compared two commercially available lipases that could deacetylate aryl acetates on 1c as the substrate. A solution of 1c (10 mg), 3a, 2-propanol as nucleophile, and triethylamine in tetrahydrofuran (THF) was divided into two test tubes under an argon atmosphere. To each tube, *Burkholderia cepacia* lipase¹⁶,¹⁹,²⁰ (Amano PS-IM, 5 mg) or *Candida antarctica* lipase B¹⁴-²⁰ (Novozyme 435, 5 mg) was added. The tubes were occasionally shaken to promote the reaction by mixing. The initial color of both tubes was orange because of 3a; the color gradually darkened with the formation of deep-purple indirubin. The reaction did not proceed in the absence of lipases. As judged by the change rate of the colors of the mixtures, the reaction mediated by *B. cepacia* lipase appeared faster than that by *C. antarctica* lipase B. In the absence of 3a, the reactions solutions with both of lipases became dark-green. Such color was probably attributed to 1d in THF. Because of the oxidation by trace dissolved oxygen in the solvent, only minute amounts of indigo and indirubin were detected by thin-layer chromatographic analysis of the reaction mixture.

**Scheme 3.** Lipase-mediated synthesis of indirubins 2a and 2b and derivatization to 4
On the basis of the aforementioned information, we proceeded to a preparative-scale reaction. With 10 mmol of 1c, an aldol condensation was performed by stirring 3a (1.5 equiv.), triethylamine (1.0 equiv.), and B. cepacia lipase (5% w/w of substrate) at 30 °C for 51 h. The addition of anhydrous sodium sulfate was necessary to maintain a low water content, which is required for lipase to exert its catalytic activity for transesterification in an organic solvent. In this case, 200 mg of sodium sulfate was added. This amount is plenty for the trapping of water (max. 10 mmol) which would be generated accompanied with the progress of aldol condensation, by forming the hydrated form. After workup, indirubin (2a, 1.98 g, 7.6 mmol) was obtained in 76% yield. Its spectral data were in good agreement with those reported previously.\textsuperscript{10,24,25} As we previously noted, the byproduct in lipase-catalyzed transesterification is isopropyl acetate. Taking advantage of a simple procedure at the stage of workup and purification, we easily scaled the lipase-mediated aldol reactions compared with previously reported chemical processes. Next, we extended our newly developed aldol condensation to the synthesis of an indirubin derivative whose aromatic substitution pattern differed between two dihydro-1H-indole components. In a 10 mmol-scale reaction, 6-bromoindirubin (2b)\textsuperscript{10,13} could be prepared in 82% yield between 1c and 6-bromoisatin (3b).\textsuperscript{9,23,24}

The aforementioned bromine-atom-substituted 2b is the precursor of 6-bromoindirubin 3'-oxime (4), which is a well-known inhibitor for glycogen synthase kinase-3β.\textsuperscript{26-28} Toward the synthesis of 4, crude materials involving 2b (ca. 59% w/w content, see experimental) in the aforementioned aldol condensation could be submitted to the next oxime formation.\textsuperscript{10,13} Fortunately, 4 was highly soluble in pyridine, the solvent of oxime formation reaction. Insoluble impurities such as inorganic salts and the powder of lipase in immobilized form, which were transferred from the previous step, could be easily removed through simple filtration. Desired 4\textsuperscript{10,13} was obtained in 78% yield in two steps from 1c.

In conclusion, we have demonstrated the lipase-catalyzed deacetylation of a heteroaromatic acetate 1c and the aldol condensation of the resultant indoxyl anion 1d with isatins 3a and 3b under basic conditions. So far, a technical problem lied in the difficulty for the removal of oxidative byproducts, indigos from indirubins, due to the scarce solubility of both the desired products and byproducts. Our presently developed lipase-mediated syntheses enabled the suppression of byproduct formation under exhaustive degassed conditions. Indirubin (2a), 6-bromoindirubin (2b), and its 3'-oxime (4) were obtained in preparative-scale reactions.

**EXPERIMENTAL**

\textsuperscript{1}H NMR spectra were measured at 500 MHz and \textsuperscript{13}C NMR spectra were measured at 125 MHz on a VARIAN 500-MR spectrometer and a Bruker AVANCE III HD 500 MHz NMR spectrometer. DMSO-\textit{d}_6 was used as solvent and the residual peak was used as an internal standard (\textsuperscript{1}H NMR: 2.48, \textsuperscript{13}C
NMR: 39.9. IR spectra were measured as ATR on a Jasco FT/IR-4700 spectrometer. High resolution mass spectra were recorded on JEOL JMS-T100LP AccuTOF.

**Starting Materials.** Indoxyl acetate (A0068, 1c), isatin (I0080, 3a) and 6-bromoisatin (B2424, 3b) were purchased from Tokyo Chemical Industry Co., Ltd.

(Z)-3-(3’-Oxo-2’,3’-dihydro-1H-indol-2’-ylidene)-2,3-dihydro-1H-indol-2-one (indirubin, 2a). To mixture of 1c (1.75 g, 10 mmol), 3a (2.21 g, 15 mmol, 1.5 eq.), triethylamine (1.01 g, 10 mmol), and anhydrous Na2SO4 (2.0 g) in THF and 2-propanol (1:5, total 50 mL), B. cepacia lipase (Amano PS-IM, 87.5 mg) was added, and the mixture was stirred under argon atmosphere at 30 °C for 51 h. During the reaction, part of 2a precipitated in the mixture. The insoluble materials and precipitates were separated by filtration. The combined filtrate and washings were concentrated *in vacuo*, and the residue was boiled with hot 2-propanol so that the remaining 3a was removed. The mixture was filtered and washed with 2-propanol until the color of the washings changed from deep red-orange into faint pink. The solids on the filter paper were then combined with the insoluble materials which were obtained at the initial workup procedure, and those were extracted for several days with hot THF using a Soxhlet extractor for several days. Evaporation of the solvent furnished 2a (1.98 g, 76%), IR 3342, 3161, 1659, 1608, 1594, 1459, 1300, 1292, 1205, 1175, 1142, 1095, 1001, 745 cm−1; 1H NMR δ: 6.89 (d, J = 7.5 Hz, 1H), 7.01 (dd, J = 7.5, 7.6 Hz, 1H), 7.01 (dd, J = 7.5, 7.7 Hz, 1H), 7.24 (ddd, J = 1.1, 7.5, 7.7 Hz, 1H), 7.40 (d, J = 8.1 Hz, 1H), 7.58 (ddd, J = 1.1, 7.5, 8.1 Hz, 1H), 7.64 (d, J = 7.6 Hz, 1H), 8.76 (d, J = 7.5 Hz, 1H), 10.88 (s, 1H), 11.01 (s, 1H); 13C NMR δ: 107.1, 111.1, 114.0, 119.5, 121.8, 122.0, 124.9, 125.2, 129.8, 137.6, 138.8, 141.4, 153.0, 171.4, 189.1. Its spectral data were in good accordance with those reported previously.13,24

The identity of the present sample was further confirmed as follows. According to the reported procedure,25 a small portion of 2a was treated with acetic anhydride, 4-(N,N-dimethylamino)pyridine, and pyridine at 85 °C to give the corresponding N(1)-acetyl derivative. IR 3333, 3308, 1683, 1599, 1457, 1366, 1300, 1173, 1054, 744 cm−1; 1H NMR δ: 2.50 (s, 3H), 7.05 (ddd, J = 0.5, 7.5, 7.7 Hz, 1H), 7.26 (ddd, J = 1.0, 7.8, 7.8 Hz, 1H), 7.37 (ddd, J = 1.0, 7.8, 7.8 Hz, 1H), 7.44 (broad d, J = 8.1 Hz, 1H), 7.59 (ddd, J = 1.2, 7.7, 8.1 Hz, 1H), 7.66 (broad d, J = 7.5 Hz, 1H), 8.21 (broad d, J = 7.8 Hz, 1H), 8.96 (dd, J = 1.0, 7.8 Hz, 1H), 11.34 (s, 1H); 13C NMR δ: 27.2, 104.7, 114.3, 115.7, 119.5, 122.6, 122.7, 124.3, 124.9, 125.1, 129.4, 137.9, 138.1, 140.3, 152.6, 170.1, 170.8, 188.9. Its NMR spectra were in good accordance with those reported previously.25 HRMS (ESI) calcd for C18H12N2NaO3 [M+Na+] 327.0750, found 327.0780.

(Z)-3-(3’-Oxo-2’,3’-dihydro-1H-indol-2’-ylidene)-2,3-dihydro-1H-indol-2-one (6-bromoisindirubin, 2b). In a similar manner as described for the synthesis of 2a, 1c (1.75 g, 10 mmol) and 3b (3.39 g, 15
mol) were reacted with the catalysis of *B. cepacia* lipase at 30 °C for 60 h. The insoluble materials and precipitates were separated by filtration. The combined filtrate and washings were concentrated *in vacuo*, and the residue was washed with hot EtOH to remove remaining 3b as mentioned in the previous section. The solids on the filter paper were combined with the insoluble materials which were obtained at the initial workup procedure to give the crude 2b (4.74 g), which still involved Na₂SO₄ and the powder of lipase in immobilized form. A portion (1.0 g) of this crude product was repeatedly extracted with hot pyridine until the insoluble material on the filter paper no more showed pink color. Evaporation of the solvent furnished 2b (590 mg, 82%); ¹H NMR δ: 7.05 (d, *J* = 1.7 Hz, 1H), 7.05 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.21 (dd, *J* = 1.7, 8.1 Hz, 1H), 7.42 (d, *J* = 7.5 Hz, 1H), 7.59 (dd, 1H, *J* = 7.5, 7.5 Hz, 1H), 7.65 (d, *J* = 7.5 Hz, 1H), 8.67 (d, *J* = 8.1 Hz, 1H), 11.01 (s, 1H), 11.06 (s, 1H); ¹³C NMR δ: 105.7, 112.8, 114.1, 119.5, 121.2, 121.9, 122.1, 124.3, 125.0, 126.5, 137.8, 139.4, 142.6, 153.0, 171.2, 189.2. Its NMR spectra were in good accordance with those reported previously. ² HRMS (ESI) calcd for C₁₆H₁₀BrN₃NaO₂ [M+Na⁺] 377.9854, found 377.9833.

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