HETEROCYCLES, Vol. 100, No. 1, 2020, pp. 129 - 136. © 2020 The Japan Institute of Heterocyclic Chemistry
Received, 26th June, 2019, Accepted, 11th September, 2019, Published online, 20th September, 2019
DOI: 10.3987/COM-19-14118

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

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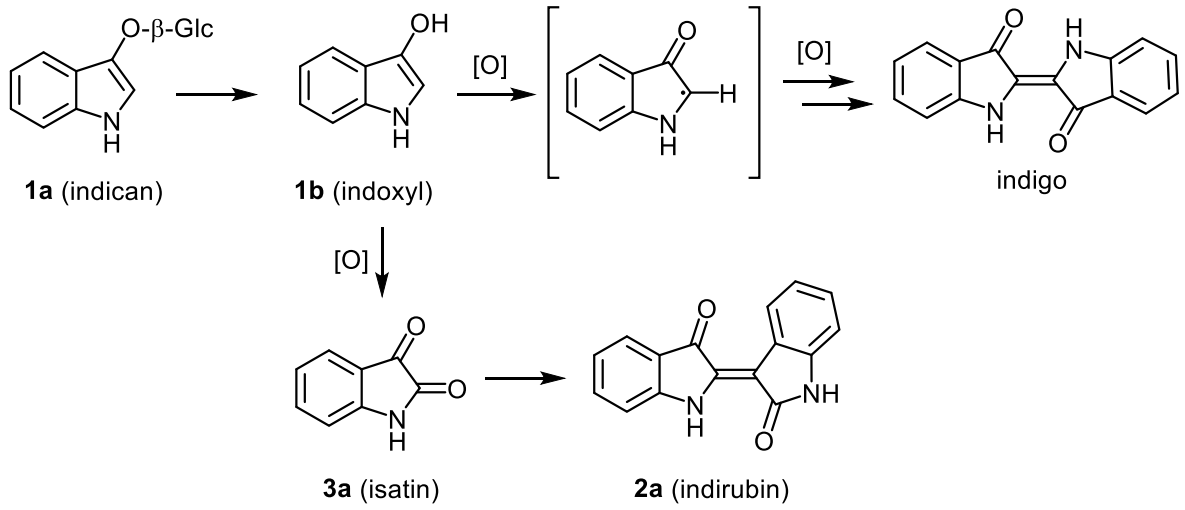
Abstract – The syntheses of indirubin (76% yield), 6-bromoindirubin (82% yield), and 6-bromoindirubin-3′-oxime (78% yield in two steps) were achieved *via* the lipase-triggered aldol condensation between isatins and an indoxyl anion in tetrahydrofuran under anhydrous and anaerobic conditions as the key 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.

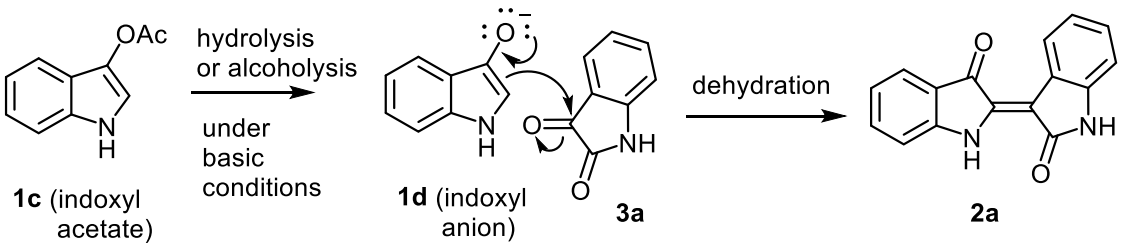
Historically, interest in and studies on naturally occurring dyes have been underlying origins of the chemistry of heterocyclic organic compounds. The development of indigo[1] is unambiguously one of the most famous examples. Natural indigo is manufactured from indican (**1a**), which originates from 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 plants[2] and is known to be a physiologically active[3] ingredient in the Chinese medicine "Danggui Longhui Wan." Also, the effect of **2a** toward murine colitis[4-6] 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.[8-13]



Scheme 1. Pathways for the formation of indigo and indirubin (**2a**) from indican (**1a**)

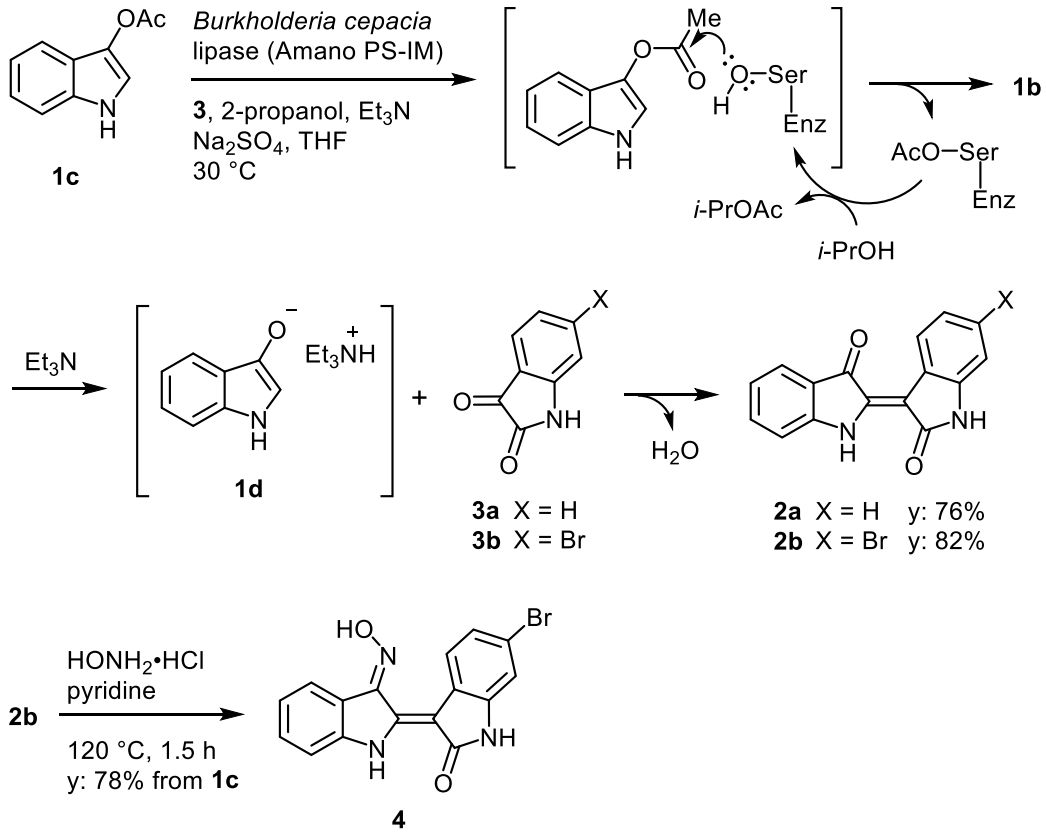


Scheme 2. Formation of **2a** by way of the aldol condensation between **3a** and **1d**

In recent studies on the lipase-catalyzed deacetylation of aryl acetates under transesterification conditions,[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 *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.[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.[22] The acidity of the

hydrogen triethylammonium ion (pK_a 10.75), compared with that of indoxyl (pK_a 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-propanol[17,23] is added, as shown in Scheme 3. The acidity of the aliphatic alcohol, however, does not affect the aldol reaction.



Scheme 3. Lipase-mediated synthesis of indirubins **2a** and **2b** and derivatization to **4**

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* lipase[16,19,20] (Amano PS-IM, 5 mg) or *Candida antarctica* lipase B[14-20] (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.

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.[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-1*H*-indole components. In a 10 mmol-scale reaction, 6-bromoindirubin (**2b**)[10,13] could be prepared in 82% yield between **1c** and 6-bromoisatin (**3b**).[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.[10,26,27] 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.[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**[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

^{1}H NMR spectra were measured at 500 MHz and ^{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-d_6 was used as solvent and the residual peak was used as an internal standard (^{1}H NMR: 2.48, ^{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-1*H*-indol-2'-ylidene)-2,3-dihydro-1*H*-indol-2-one (indirubin, 2a). To a 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 Na_2SO_4 (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); ^{13}C 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 (dd, *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); ^{13}C 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 $C_{18}H_{12}N_2NaO_3$ [M+Na^+] 327.0750, found 327.0780.

(*Z*)-3-(3'-Oxo-2',3'-dihydro-1*H*-indol-2'-ylidene)-2,3-dihydro-1*H*-indol-2-one (6-bromoindirubin, 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 Na_2SO_4 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%), IR 3304, 3157, 1661, 1604, 1587, 1475, 1466, 1302, 1206, 1143, 1005, 960, 909, 808, 754 cm^{-1}; ^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); ^{13}C 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.[10,13] The content of **2b** in crude product was estimated be *ca*. 59% w/w, by comparing the weight before and after the extraction with hot pyridine.

(3*Z*,3'*E*)-6-Bromo-3-(3'-hydroxyimino-2',3'-dihydro-1*H*-indol-2'-ylidene)-2,3-dihydro-1*H*-indol-2-one (6-bromoindirubin-3'-oxime, 4). A portion (3.27 g) of crude materials (4.74 g) involving **2b** (*ca*. 59% w/w content, as mentioned in the previous section) was directly applied to the oxime formation. According to the reported procedure,[10] crude **2b** (*ca*. 6.4 mmol) was treated with hydroxylamine hydrochloride (4.5 g, 64.8 mmol) in pyridine (64 mL) at 120 °C for 3 h. After cooling, the mixture was filtered to remove off-white insoluble materials. The combined filtrate and washings were concentrated *in vacuo*, and the residue was successively washed with $KHSO_4$ aq. solution (10%) and water to give **4** (2.17 g, 78% from **1c**), IR 3168, 1655, 1606, 1568, 1446, 1329, 1309, 1218, 993, 970, 804 cm^{-1}; ^1H NMR δ: 7.04 (broad s, 1H), 7.06 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.10 (d, *J* = 8.2 Hz, 1H), 7.38-7.42 (m, 2H), 8.23 (d, *J* = 7.5 Hz, 1H), 8.57 (d, *J* = 8.2 Hz, 1H), 10.86 (s, 1H), 11.76 (s, 1H), 13.62 (s, 1H); ^{13}C NMR δ: 98.2, 112.0, 112.3, 116.9, 118.3, 122.3, 122.5, 123.2, 124.7, 128.4, 132.6, 140.1, 145.2, 146.5, 150.1, 171.2. Its NMR spectra were in good accordance with those reported previously.[10,13] HRMS (ESI) calcd for $C_{16}H_{10}BrN_3NaO_2$ [$M+Na^+$] 377.9854, found 377.9833.

ACKNOWLEDGEMENTS

This work was supported by Japan society for Promotion of Science KAKENHI (19K05849) for T. S.

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