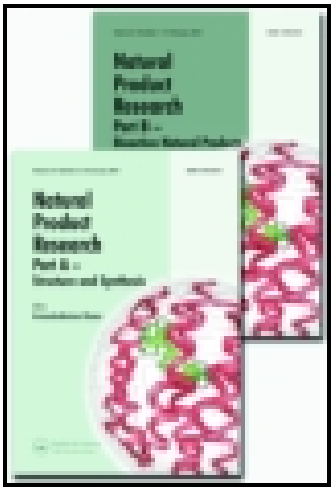

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Studies on the acetylation and NMR reassignment of indirubin derivatives

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Studies on the acetylation and NMR reassignment of indirubin derivatives

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The analysis of 1D- and 2D-NMR spectroscopic data confirmed that the amino N-1′ protons of indirubin and indirubin-3′-oxime resonate at a higher frequency than N-1 protons. The amino N-1′ protons in both indirubin and indirubin-3′-oxime are not favourable for acetylated reaction due to their intramolecular hydrogen bonding with the amide carbonyl group. The new N-1-acetylindirubin-3′-acetoxime has been synthesised using acetic anhydride. The reassignment of the NMR data of indirubin, indirubin-3′-oxime and N-1-acetylindirubin was confirmed with the aid of DEPT, HSQC, HMBC and NOESY methods.

Keywords: indirubin; indirubin-3′-oxime; acetylation; spectral reassignment; intramolecular hydrogen bond

1. Introduction

Indirubin (**1**) is a dark-red isomer of the blue indigo. Both are natural dyes which are found in plants such as *Strobilanthes cusia*, *Polygonum tinctorium*, *Indigofera tinctoria*, *Indigofera suffrutticosa*, *Isatis indigotica*, and is also found in the urine of various mammals, including man (Tai & Cuong, 2008). Indirubin has been reported as the active ingredient of a traditional Chinese medicinal recipe 'Danggui Longghui Wan', a mixture of 11 herbal medicines that is used to treat chronic diseases such as chronic myelocytic leukaemia in traditional Chinese medicine (Meijer, Shearer, Bettayeb, & Ferandin, 2006). In recent years, studies on indirubins have been stimulated due to the first report on the inhibitory activity of indirubin-3′-oxime (**2**) against cyclin-dependent kinases (CDKs) (Hoessel et al., 1999).

The first chemical synthesis of indirubin was accomplished in 1881 by Baeyer using a condensable reaction between indoxyl and isatin (Merz & Eisenbrand, 2006). The method was considerably improved and is now being used as the main method for the synthesis of ≈200 derivatives of indirubin, so far.

Many reports showed that indirubin is a powerful inhibitor of CDKs at different stages of the cell cycle (Lee et al., 2005), and glycogen synthase kinase-3β (GSK-3β), which is involved in neurodegenerative disorders (Schnitzer, Schmid, Zhou, Eisenbrand, & Brüne, 2005). In addition, indirubin-3′-oxime is also a compound effective as a potent inhibitor of c-Jun NH2-terminal kinase (JNK), an important regulator of neuronal apoptosis (Xie et al., 2004).

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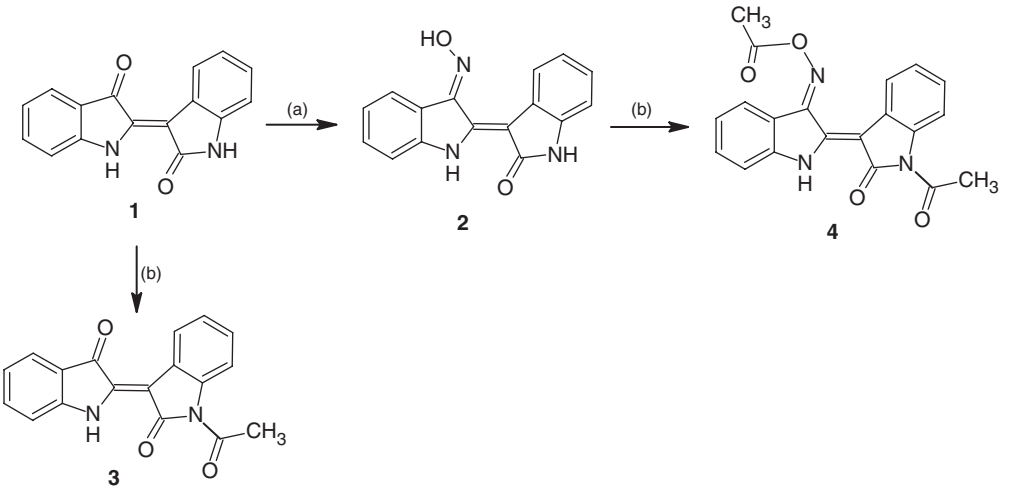


(a) $NH_2OH{\cdot}HCl$, pyridine, 120°C. (b) Ac_2O, reflux.

Scheme 1. Synthesis of acetyl derivatives of indirubin and indirubin-3′-oxime.

According to our research, the acetylation of indirubin-3′-oxime has not been reported so far. In this article, we report on the synthesis of N-1-acetylindirubin (**3**) and the new N-1-acetylindirubin-3′-acetoxime (**4**). The ^{1}H- and ^{13}C-NMR reassignment of indirubin (**1**), indirubin-3′-oxime (**2**), and the spectral assignment of the acetylated compounds **3** and **4** were carefully obtained by using 1D- and 2D-NMR and IR methods.

2. Results

The general synthetic approach for the acetylated derivatives of indirubin and indirubin-3′-oxime is shown in Scheme 1. Indirubin **1** (≈80%) was obtained from indigo powder, prepared from the leaves of *S. cusia*, by purification using column chromatography on silica gel (Cuong, Thuy, Ha, & Tai, 2007). Indirubin-3′-oxime (**2**) was synthesised from the reaction of **1** with hydroxylamine in pyridine. N-1-acetylated indirubin (**3**) was easily formed by the reaction of **1** with Ac_2O at reflux for 5 h. Compound **4** has been formed by the reaction of **2** using Ac_2O at reflux for 5 h. Compounds **3** and **4** were purified by column chromatography on silica gel with dichloromethane as fluent.

3. Discussion

3.1. *Reassignment of ^{1}H- and ^{13}C-NMR parameters of indirubin and indirubin-3′-oxime*

The ^{1}H- and ^{13}C-NMR spectral data of indirubin (**1**) and indirubin-3′-oxime (**2**) were assigned firstly by Hoessel et al. (1999). However, there was incorrect assignment of the amino protons at N–1H and N–1′H, as well as at some carbon atoms, as shown in Table 1. We have found that in DMSO-d_6 solvent, the resonances of N–1′H protons of indirubin (**1**) and indirubin-3′-oxime (**2**) are changed for N–1H protons, e.g. they resonate at a higher frequency than N–1H, and were confirmed by data from HMQC and HMBC experiments. The ketone carbon signal of **1** at δ188.58 (C-3′) had the clear ^{13}C–^{1}H

Table 1. The ^{1}H- and ^{13}C-NMR spectral data of indirubin (**1**), and indirubin-3′-oxime (**2**) (DMSO-d_6).

	^{1}H-NMR				^{13}C-NMR				HMBC
Pos.	**1**	**1** (Hoessel et al., 1999)	**2**	**2** (Hoessel et al., 1999)	**1**	**1** (Hoessel et al., 1999)	**2**	**2** (Hoessel et al., 1999)	**2**
1	10.87 (s)	11.01 (s)	10.70 (s)	11.73 (s)					C-2, C-3, C-3a, C-7a
2					170.90	171.11	170.91	170.95	
3					106.54	106.97	98.88	98.88	
3a					121.42	121.58	122.63	122.66	
4	8.77 (d, 7.5)	8.77 (d, 7.7)	8.65 (d, 7.5)	8.65 (d, 7.2)	124.63	129.15	122.94	125.92	C-3, C-6, C-7a
5	7.02 (t, 7.5)	7.02 (m)	6.95 (dt, 7.5, 1)	6.95 (m)	121.21	121.21	120.31	121.49	C-3a, C-7
6	7.25 (t, 7.5)	7.26 (m)	7.13 (dt, 7.5, 1)	7.13 (m)	129.22	124.62	125.86	123.09	C-4, C-7a
7	6.90 (d, 7.5)	6.91 (d, 8.1)	6.90 (d, 8.0)	6.90 (d, 7.6)	109.53	109.57	108.78	108.91	C-3a, C-5
7a					140.87	138.48	138.29	138.34	
1′	11.01 (s)	10.88	11.73 (s)	10.72 (s)					C-2′, C-3′, C-3′a, C-7′a
2′					138.30	152.35	145.22	145.32	
3′					188.58	188,27	151.28	151.22	
3′a					118.99	119.37	116.48	116.54	
4′	7.65 (d, 7.5)	7,66 (d, 7.4)	8.24 (d, 8.0)	8.24 (d, 7.6)	124.29	124.20	127.92	127.95	C-6′, C-7′a
5′	7.02 (t, 7.5)	7.02 (m)	7.03 (m)	7.03 (m)	121.21	121.19	121.39	120.43	C-7′
6′	7.57 (t, 7.5)	7.58 (m)	7.39 (m)	7.41 (m)	137.04	136.85	131.97	132.02	C-4′, C-7′a
7′	7.42 (d, 8.0)	7.42 (d, 8.1)	7.39 (m)	7.41 (m)	113.39	113.15	111.45	111.52	C-3′a, C-5′
7′a					152.46	141.07	144.82	144.83	
OH	–	–		13.48 (s)					

correlation in the HMBC spectrum to the downfield amino proton signal at δ 11.01 (N–1′H) and the aromatic proton signal at δ 7.65 (H-4′), whereas in **2** the oxime carbon signal at δ 151.28 (C-3′) correlated to the proton of the hydroxyl group N–OH (δ 13.48) and the amino proton N–1′H (δ 11.73). The upfield amino proton singlets at δ 10.87 in **1** and at δ 10.70 in **2** had the ^{1}H–^{13}C long-range correlations to all four carbon signals of C-2 (amide), C-3, C-3a and C-7a, confirming the assignment of these singlets to the amino N–1H protons. Similarly, the downfield amino proton signals at δ 11.01 in **1** and at δ 11.73 were assigned to the amino N-1′ proton due to the HMBC correlations between those signals and the carbon signals of C-2′, C-3′, C-3′a and C-7′a.

The reassignment of the amino proton signals in **1** and **2** was further confirmed by two pairs of the correlations in the NOESY spectrum between the N–1H proton and the aromatic proton H-7, as well as between the N–1′H proton and the aromatic proton H-7′. With the aid of the HSQC, HMBC and NOESY methods, the correct spectroscopic parameters of indirubin (**1**) and indirubin-3′-oxime (**2**) were elucidated and described in Table 1. Table 1 shows the reassignment of protons N–1H, N–1′H, and carbons C-4, C-6, C-7a, C-2′ and C-7′a for indirubin, and carbons C-4, C-5, C-6 and C-5′ for indirubin-3′-oxime, in comparison with the literature (Hoessel et al., 1999).

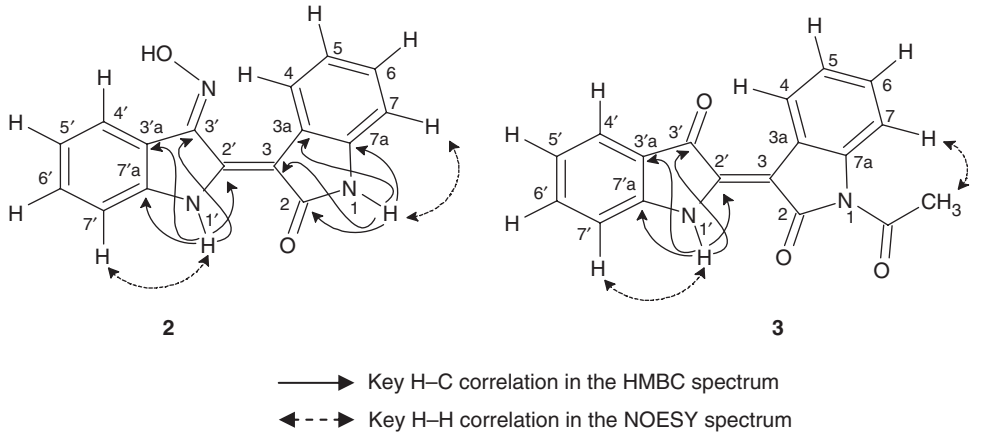
The fact that the chemical shift of the amino N-1′ proton in **1** and **2** moved downfield in comparison with those of the amide N-1 proton is due to the intramolecular hydrogen bond between the amino proton and the $C{=}O_{amide}$ group. The IR spectrum of **1** in KBr at different concentrations confirms such an intramolecular hydrogen bond. The IR spectra of both low and high indirubin-concentrated pellets showed the sharp singlet band at 3347 cm^{-1}, indicating the stretching frequency for the intramolecular hydrogen bonded N-1′ amino group. The broad bands at 3434 and 3191 cm^{-1} in the low-concentrated pellet are due to the stretching frequency of the amide group. At a higher concentration of indirubin, the vibration of the amide group lies at one broad band at 3182 cm^{-1}, suggesting the amide group is intermolecular hydrogen bonded with the C=O group.

3.2. *Acetylation of indirubin and indirubin-3′-oxime*

Some Chinese scientists reported the synthesis of N-1-acetyl indirubin (Ji & Zhang, 1985). Recently, the N-1′-acetyl indirubin was synthesised (Kim et al., 2005; Moon et al., 2006), in which the reported ^{1}H-NNR data were not assigned. We found that the spectroscopic characteristics of the amino N-1′ protons in N-1-acetyl compounds **3** and **4** are similar to those of **1** and **2** in the HMBC spectra, i.e. having correlation to all four carbon atoms C-2′, C-3′, C-3′a and C-7′a. The NOESY spectra of compound **3** showed two important ^{1}H–^{1}H correlations between the amino proton N–1′H at δ 11.33 and the aromatic proton H-7′ at δ 7.45 and between the methyl group at δ 2.71 and the aromatic proton H-7 at δ 8.22 (Figure 1). The close resonance between C-6′/C-7a, C-5′/C-3a, and C-4′/C-5 and also other signals was determined by the combination of HSQC, HMBC and DEPT spectral evidence.

The reported N-1′-acetyl indirubin (Kim et al., 2005; Moon et al., 2006) had only ^{1}H-NMR data, and some of them were different to those of N-1-acetyl indirubin (**3**). We report here the synthesis and full spectral assignment of N-1-acetyl indirubin (**3**).

The ^{1}H-NMR of the diacetylated derivative of indirubin-3′-oxime (**4**) has two methyl groups at 2.74 and 2.50. The ESI-MS data of **4** showed a positive ion at m/z 362 $[M+H]^{+}$ and a negative ion at m/z 360 $[M-H]^{-}$, corresponding to the molecular formula



2 **3**

Figure 1. Some of the main spectral correlations of compounds **2** and **3**.

$C_{20}H_{15}N_3O_4$. The structure of N-1-acetylindirubin-3′-acetoxime (**4**) was also elucidated by 1D- and 2D-NMR methods, in which it should be noted that the resonance of the protons H-4, H-4′, and H-7 were shifted further downfield due to the deshielded effect of the methoxy group.

Recently, Beauchard et al. (2006) and Ferandin et al. (2006) reported that the N-1′-protons of synthesised derivatives of indirubin and indirubin-3′-oxime resonated at a higher frequency than N-1-protons without explanation. Ferandin et al. (2006) reported the synthesis of 3′-acetoxime derivatives with acetic anhydride for normally 30 min at 0°C, whereas alkaloid **4** was refluxed for 5 h. It is suggested that the high temperature and excess of acetic anhydride are reasons for the formation of diacetylated indirubin-3′-oxime **4**.

4. Conclusion

The analysis of 1D- and 2D-NMR spectroscopic data confirms that the amino N-1′-protons of indirubin and indirubin-3′-oxime resonate at a higher frequency than N-1-protons. The intramolecular hydrogen bond between the proton of the amino group and the amide group is the reason for the poor activity of the amino group in the acetylated reaction, when compared with the amide group. The ^{1}H- and ^{13}C-NMR reassignments of indirubin-3′-oxime at N–1H and N–1′H and at carbons C-4, C-5, C-6, and C-5′ were confirmed by combination of NMR and IR methods. N-1-acetylindirubin (**3**) and the new N-1-acetylindirubin-3′-acetoxime (**4**) were synthesised, and their structures were determined by spectral methods.

5. Experimental

5.1. *Apparatus*

^{1}H- and ^{13}C-NMR, NOESY, HSQC, HMBC spectra was recorded by an AVANCE 500 MHz (Bruker, Germany) spectrometer. The solvent used was DMSO-d_6 and chemical shifts are shown in δ (ppm) with TMS as an internal reference. Sample concentrations were

typically in the range of 5–15 mg per 0.5 mL. Mass spectroscopy ESI-MS was measured by an Agilent 1100 Series LC/MSD Trap SL (Institute of Chemistry, Vietnam Academy of Science and Technology).

5.2. *Preparation of indirubin (1)*

Indirubin was isolated from indigo powder, a dark blue powder produced from the leaves of *S. cusia*, as described in (Cuong et al., 2007).

5.3. *Synthesis of indirubin-3′-oxime (2)*

Indirubin (**1**) (262 mg, 1 mmol) was dissolved in 15 mL of pyridine. Then hydroxylamine hydrochloride (181 mg, 2.6 mmol) was added and the solution was refluxed at 120°C. After 5 h the solvent was removed at low pressure and poured into 1 N hydrochloric acid (100 mL). The precipitate was filtered off and washed several times with water. The crude product was purified by redissolving in 1N sodium hydroxide (50 mL) and reprecipitated in 1N hydrochloric acid to give a red solid, yield 263.5 mg (93%).

Indirubin (**1**): ESI-MS *m/z*: 263 $[M+H]^+$, 261 $[M-H]^-$. ^{1}H- and ^{13}C-NMR (DMSO, 500 MHz, see Table 1).

Indirubin-3′-oxime (**2**): ESI-MS *m/z*: 278 $[M+H]^+$, 276 $[M-H]^-$. ^{1}H- and ^{13}C-NMR (DMSO, 500 MHz, see Table 1).

5.4. *Synthesis of N-1-acetyl indirubin (3) and N-1-acetyl indirubin-3′-acetoxime (4)*

The mixture of 10 mL anhydride acetic acid and 262 mg (1 mmol) of **1** or 277 mg (1 mmol) of **2** was placed into a 50 mL round-bottomed flask. The reaction mixture was mixed well with a magnetic stirrer and refluxed for 5 h by oil bath. When the reaction terminated, the solvent was removed at low pressure. Residues were washed several times with water, and then dried to obtain crude products. Purer products were obtained by column chromatography with dichloromethane as the eluted solution.

N-1-acetylindirubin (**3**), $C_{18}H_{12}N_2O_3$; red powder, ESI-MS *m/z*: positive mode: 305 $[M+H]^+$, 275 $[M+H–CH_2{=}O]^+$, 263 (100%) $[M+H–CH_2{=}CO]^+$, negative mode: 303 $[M-H]^-$, 261 $[M-H–CH_2{=}CO]^-$. ^{1}H-NMR (DMSO, 500 MHz), δ (ppm): 11.33 (1H, s, H-1′); 8.98 (1H, dd, $J=8.0$; 1 Hz, H-4); 8.22 (1H, d, $J=8.0$ Hz, H-7); 7.67 (1H, d, $J=7.5$ Hz, H-4′); 7.61 (1H, dt, $J=7.5$, 1.5 Hz, H-6′); 7.45 (1H, d, $J=8.0$ Hz, H-7′); 7.38 (1H, dt, $J=8$, 1.5 Hz, H-6); 7.27 (1H, dt, $J=8$, 1.0 Hz, H-5); 7.07 (1H, dt, $J=7.5$, 0.5 Hz, H-5′); 2.71 (3H, s, $COCH_3$); ^{13}C-NMR (DMSO, 125 MHz) δ (ppm): 188.42 (C-3′); 170.33 ($COCH_3$) 169.68 (C-2); 152.16 (C-7′a); 139.81 (C-2′); 137.68 (C-7a); 137.41 (C-6′); 128.96 (C-6); 124.67 (C-4′); 124.48 (C-5); 123.85 (C-4); 122.20 (C-3a); 122.11 (C-5′); 118.98 (C-3′a); 115.22 (C-7); 113.76 (C-7′); 104.25 (C-3); 26.71 ($COCH_3$).

N-1-acetylindirubin-3′-acetoxime (**4**), $C_{20}H_{15}N_3O_4$; red powder, ESI-MS *m/z*: positive mode: 384 $[M+Na]^+$, 362 $[M+H]^+$; negative mode: 360 $[M-H]^-$. ^{1}H-NMR (DMSO, 500 MHz), δ (ppm): 11.69 (1H, s, H-1′); 9.28 (1H, d, $J=8.0$ Hz, H-4); 8.26 (1H, d, $J=7.5$ Hz, H-4′), 8.24 (1H, d, $J=8$ Hz, H-7); 7.55 (1H, dt, $J=7.5$; 1 Hz, H-6′); 7.52 (1H, d, $J=8.0$ Hz, H-7′); 7.32 (1H, dt, $J=7.5$, 1 Hz, H-6); 7.21 (1H, t, $J=7.5$ Hz, H-5); 7.14 (1H, dt, $J=7.5$, 1.0 Hz, H-5′); 2.74 (3H, s, $COCH_3$), 2.50 (3H, s, $OCOCH_3$).

^{13}C-NMR (DMSO, 125 MHz) δ (ppm): 170.08 ($COCH_3$); 169.08 (C-2); 166.80 ($OCOCH_3$) 156.04 (C-3′); 146.31 (C-7′a); 143.84 (C-2′); 136.14 (C-7a); 134.26 (C-6′); 129.23 (C-4′); 127.06 (C-6); 123.88 (C-5); 123.84 (C-4); 122.15 (C-3, C-5′); 115.05 (C-3′a); 114.34 (C-7); 112.51 (C-7′); 100.99 (C-3); 26.36 ($COCH_3$); 18.69 ($COCH_3$).

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