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Isolation, synthesis, and cytotoxicity evaluation of two impurities in nomegestrol acetate

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Abstract

Nomegestrol acetate (NOMAc) is a synthetic progesterone analog and classified as a fourth-generation progestin. It has been approved in many countries for oral contraception, hormonal replacement therapy (HRT), and treatment of various gynecological disorders. There are several synthetic routes reported for the synthesis of NOMAc and they all share the very similar last three to five steps toward the conversion of 6-methylene to 6-methyl-6,7-unsaturated structure. Therefore the final product from different processing routes may have similar impurity profiles. In the analysis of NOMAc, we identified two impurities, impurity **A** (listed in EP 8.0) and impurity **B** (not specified in EP 8.0). Both impurities were further confirmed by synthesis. In addition, both impurities and NOMAc were evaluated for their *in vitro* cytotoxicities against L02 liver cells, mesenchymal stem cells, MCF-7 breast cancer cells, and C33A cervical cancer cells. These three analogs are not cytotoxic to the four cell lines at low concentrations (<20 μM). NOMAc and impurity **A** showed cytotoxicity to L02, MCF-7, and C33A cells at high concentrations, while impurity **B** did not show significant cytotoxicity to any of the cell lines tested.

KEYWORDS
cytotoxicity, impurity, nomegestrol acetate, progestin, steroid, synthesis

1 | INTRODUCTION

Nomegestrol acetate (NOMAc), or 6-methyl-3,20-dioxo-19-norpregna-4,6-dien-17-yl acetate, is a fourth generation synthetic analog of progesterone. It has high affinity and selectivity for human progesterone receptor (PR) and is devoid of any estrogenic, androgenic, glucocorticoid or mineralocorticoid activity. In addition, it has excellent anti-gonadotropic activity, good progesterone receptor-mediated anti-estrogenic activity, and moderate anti-androgenic activity.[1–6] NOMAc has been approved and marked by commercial names such as Zoley®, Lutenyl®, Uniplant®, Naemis®, etc. in many countries as oral contraceptives (OCs) and hormone replacement therapeutics.[7–11] Unlike earlier generations of progestins that were derived from the structure of testosterone (estranes and gonanes), NOMAc and other fourth generation of progestins, including dienogest (DNG), trimegestone (TMG), and nesterone (NES), were derived from the structure of pregnane.[12–14] These newly developed progestins are also called pure progestins due to their high affinity for PR and lack of affinities for other steroidal receptors.[15] Progestins inhibit ovulation by suppressing the natural production of menstrual-cycle hormones,[16] and are often used in combination with an estrogen for oral contraception,[17–19] HRT,[20,21] endometrosis,[22–24] and gynecological

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disorders, such as menstrual disturbances, dysmenorrhoea, and premenstrual syndrome.[25,26] Studies indicated that the use of earlier generations of progestins may have some minor side effects,[27,28] but their long-term use have been credited with some highly beneficial effects in prevention of osteoporosis, breast, ovarian, and endometrial cancers.[29–32] NOMAc and other fourth generation progestins are expected to have fewer or no undesired side effects and are expected to offer even more health benefits to long-term users.

Three synthetic methods for NOMAc (**6**) share the last five steps (Scheme 1), the differences among them are the methods of obtaining the precursor **1**.[33–35] Another synthetic method for NOMAc also has similar steps in the early stage and is followed by the modification at the 17 carbon position.[36] Therefore, all four reported synthetic procedures may display some common impurities resulted from the modification at the 6 and 7 positions of the steroidal structure as shown in Scheme 1. Pharmaceutical impurities have attracted more and more attention from not only the scientific community but also the regulatory agencies due to their potential harmful effects.[37] Studies are encouraged to identify more unknown impurities and assess their safety in normal treatment application.

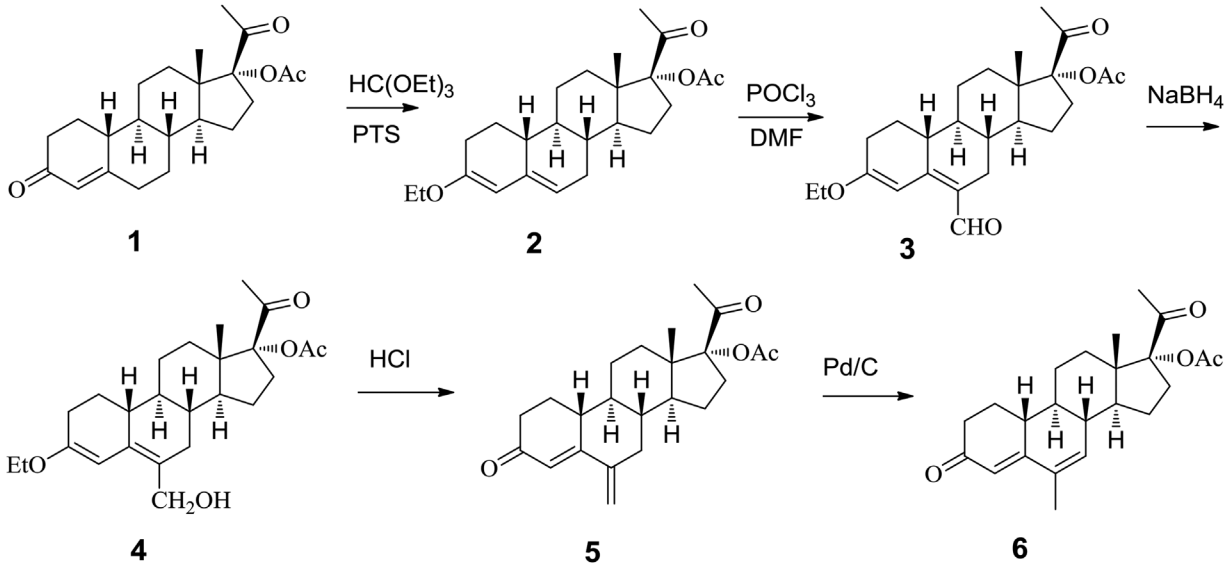
For the product of NOMAc, a known impurity (impurity **A)** with the structure of 6-α-methyl-3,20-dioxo-19-norpregna-4-en-17-yl acetate has been specified in EP 8.0 with a limit of <0.2%. There are other impurities not specifically identified in EP 8.0, but there is a combined limit of <0.3%. In our recent analysis of NOMAc, we identified impurity **A** and another impurity (**B**), a hydroxyl substitution at 6-methyl position of NOMAc, or 6-hydroxymethyl-3,20-dioxo-19-norpregna-4,6-dien-17-yl acetate (Figure 1). In addition, we developed processes to synthesize both impurity **A** and impurity **B** and obtained both in multi-gram quantities (Schemes 2 and 3).

A preliminary cytotoxicity screening assay was performed for impurity **A**, impurity **B**, and NOMAc against the survival of L02 cells and mesenchymal stem cells (MSCs). L02 is a human normal liver cell line that is a frequent choice to study hepatocellular toxicity of test drugs.[38–40] MSCs play important roles for tissue repair and regeneration and can differentiate into a variety of cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts, and neurons, thus are often used as cell substrates for cytotoxicity assays.[41–44] Breast cancer and cervical cancer are highly associated with estradiol.[45] NOMAc has demonstrated to reduce the production of estradiol and thus has inhibitory activity against the growth of cancer cells.[46] Therefore, MCF-7 (breast cancer cells) and C-33A (cervical cancer cells) were also used to assess the anticancer activity of impurity **A**, impurity **B**, and NOMAc.

2 | RESULTS AND DISCUSSION

There are at least four reported methods for the synthesis of NOMAc; three of them share the last five steps and the other one also has similar chemistry in its early stages. Impurity **A** has been specified as a common and hard to be removed substance in the final product. Impurity **B** has not been identified as an impurity of NOMAc in EP 8.0. One earlier patent reported its preparation and implicated its pharmaceutical application.[47] Another publication implicated that this impurity **B** could be a possible metabolite (metabolite #3) of NOMAc,[48] but there has been no confirmation of impurity **B** being the metabolite #3 up to today even though crystal structure of impurity **B** has been published.[49] However, during our analysis of NOMAc, we identified impurity **B** as an impurity in the final product at below 0.1% level. Therefore, it is not likely a metabolite of NOMAc.

The introduction of impurity **A** is certainly due to the reduction of the double bond of the 6-methylene of intermediate **5** or the 6,7-double bond of NOMAc (Figure 1) during the catalytic double bond translocation reaction. Whereas, the introduction of impurity **B** is likely due to the incomplete reduction of intermediate **3** that would become



SCHEME 1 Common procedures for the synthesis of NOMAc

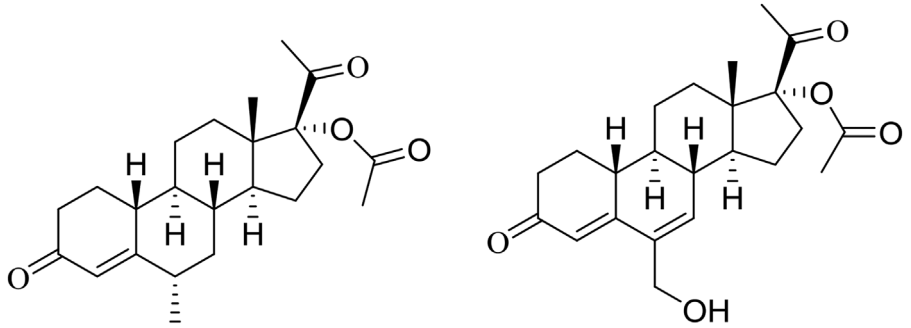
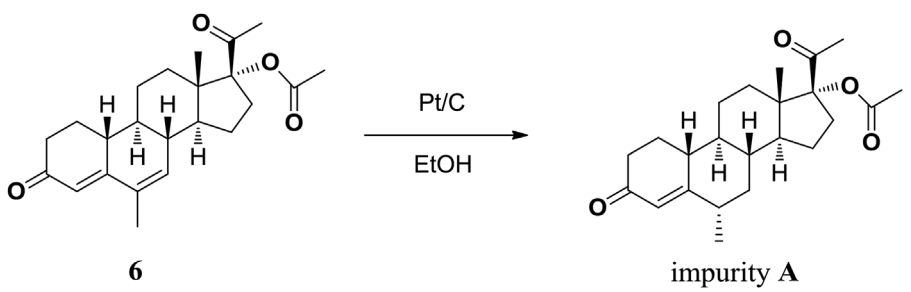


FIGURE 1 Chemical structures of impurity **A** and impurity **B**

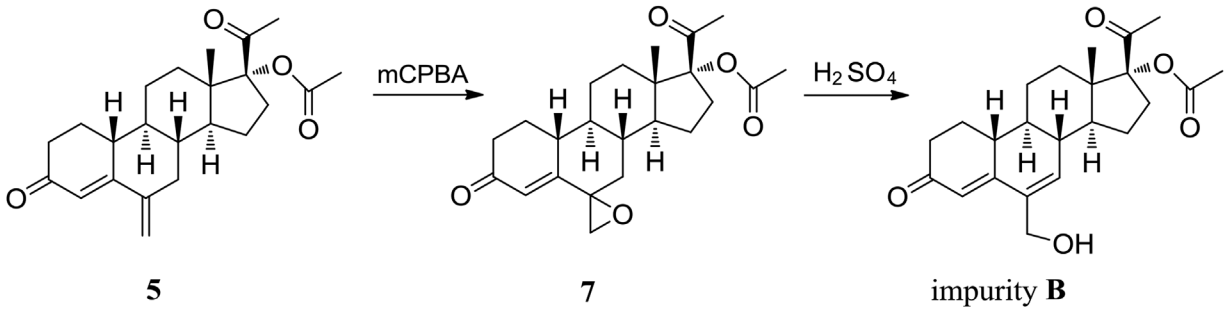
intermediate **8** as a result of the subsequent acidification (Scheme 4). Intermediate **8** is then subjected to the catalytic double bond translocation reaction to afford impurity **B**. Another possible (but unlikely) route of introducing impurity **B** is that intermediate **4** does not dehydrate during the acidification process and form intermediate **9** which would go through the catalytic double bond translocation reaction and to give impurity **B**.

In order to confirm the proposed chemistry for introduction of impurity **A**, we carried out a reaction using intermediate **5** to undergo catalytic double bond translocation reaction with extended reaction time.[35] The reaction mixture did have impurity **A**, NOMAc, and other by-products (data not shown). When the NOMAc was used to further undergo catalytic double bond translocation reaction (palladium on active carbon with added cyclohexene) at extended reaction time (Scheme 2), the impurity **A** was produced in much higher percentage (~89%) in the reaction mixture and thus much easier to be purified. After multi-rounds of re-crystallization, impurity **A** was obtained with multi-gram quantity and in high purity. To confirm the formation of impurity **B** via intermediate **8**, intermediate **3** was acidified to give **8** that then underwent catalytic double bond translocation reaction (palladium on active carbon with added cyclohexene), the reaction mixture did show an impurity **B** absorption (data not shown), but also contained lots of other by-products and was hard to be purified. Efforts to obtain intermediate **9** via acidification of intermediate **4** were not successful (always resulted in intermediate **5**). In order to obtain impurity **B** more efficiently, intermediate **5** was subjected to undergo epoxidation at the 6-methylene position with mCPBA to afford intermediate **7** that was then treated with acid to form impurity **B** with high purity after silica gel column chromatography (Scheme 3).

Impurities **A**, **B**, and intermediate **7** were characterized by both ^{1}H NMR and ^{13}C NMR. Though impurity **A** is a confirmed impurity in NOMAc, no NMR data for impurity **A** is available in the literature. It is very difficult to assign ^{1}H NMR resonance peaks to the corresponding protons due to overlapped signals between 1.0 and 2.5 ppm. But several resonance signals are clearly identifiable in the ^{1}H NMR spectra for both the isolated impurity **A** and the synthesized impurity **A**. Notably, ~5.87 ppm for C4-**H**, ~2.10 ppm for C-CO-C**H**$_3$ at #21 carbon, ~2.05 ppm for C**H**$_3$-CO-O- at #23 carbon, ~1.10–1.12 ppm for 6-α-C**H**$_3$ or on #24 carbon, ~0.70 ppm for −C**H**$_3$ on the #18 carbon. Similar resonance peaks are also identifiable in the ^{1}H NMR spectra for isolated impurity **B** and the synthesized impurity **B**, such as, ~6.36 ppm for C4-**H**, ~6.01 ppm for C7-**H**, ~4.32 ppm for −C**H**$_2$-OH on #24 carbon, ~2.10 ppm for C-CO-C**H**$_3$ at #21 carbon, ~2.06 ppm for C**H**$_3$-CO-O- at #23 carbon, ~0.72 ppm for -C**H**$_3$ on the #18 carbon. For intermediate **7**, a signal at ~5.92 ppm is assigned for C4-**H**; a signal at ~3.25 ppm is assigned for 1 proton on the epoxide moiety and ~2.87 ppm for another proton on the epoxide ring; a signal at ~2.13 ppm is assigned for C-CO-C**H**$_3$ at #21 carbon; a signal at ~2.08 ppm is assigned for C**H**$_3$-CO-O- at #23 carbon. One typical resonance at ~2.93–2.98 ppm (td) is observable on all three analogs' ^{1}H NMR spectra, it likely belongs to one of the two protons on #16 carbon.

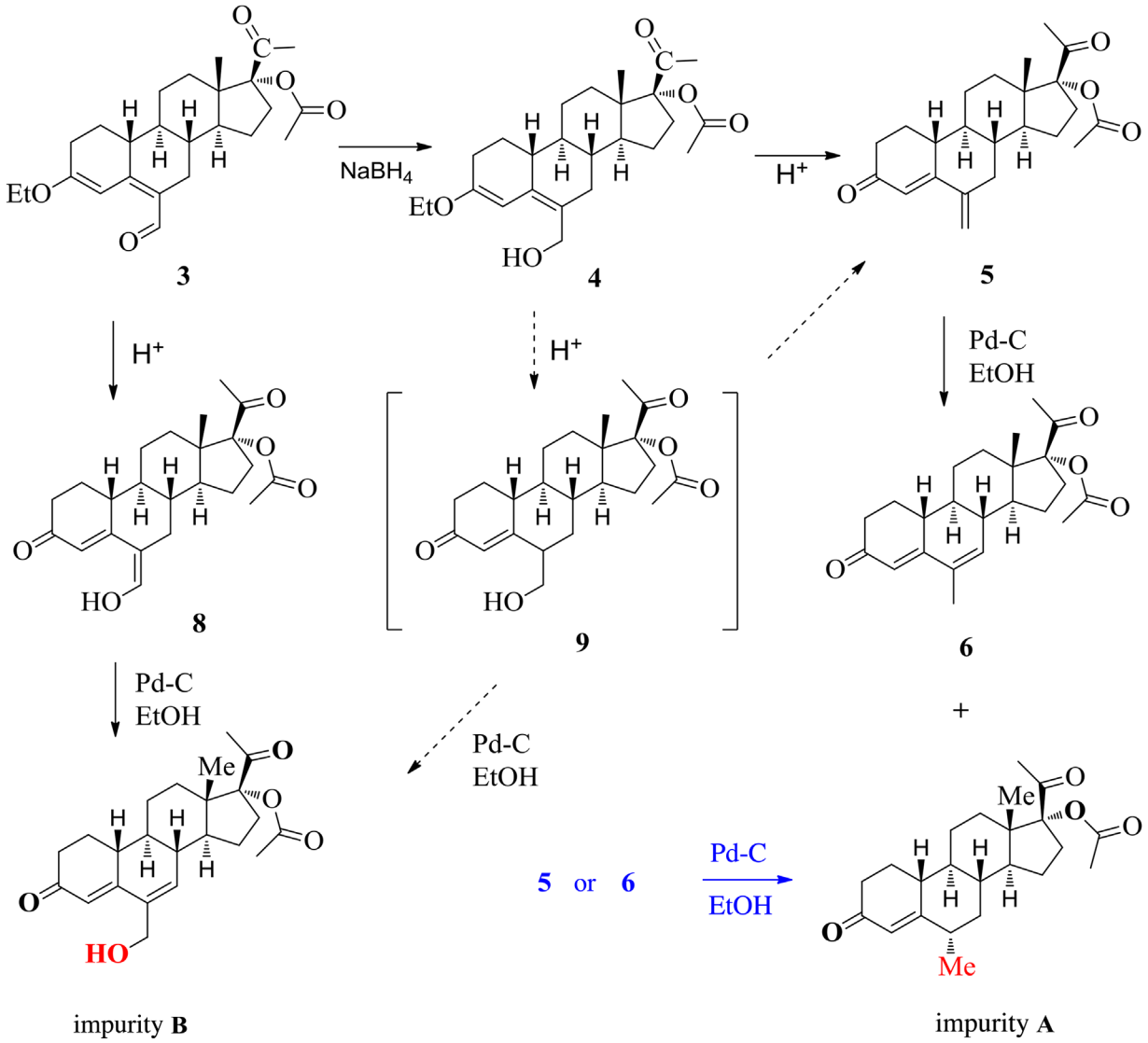


SCHEME 2 Preparation of impurity **A**



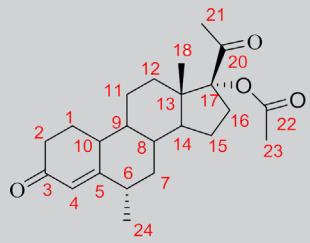
SCHEME 3 Preparation of impurity **B**

^{13}C NMR and ^{13}C NMR-DEPT135 spectra nicely displayed each individual carbon (^{13}C NMR) or proton-attached (^{13}C NMR-DEPT135) signal. Based on the resonance signal information from both ^{13}C NMR spectra, each signal was assigned to the corresponding carbon (Table 1). For impurity **A**, the ~121.57 ppm certainly belongs to **C**-4 carbon (based on ^{13}C NMR-DEPT135); the signal at ~96.71 ppm certainly belongs to **C**-17 carbon; the signal at ~204.12 ppm likely belongs to **C**-20 carbon; the signal at ~200.07 ppm likely belongs to

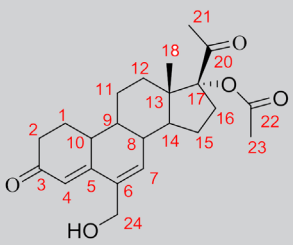


SCHEME 4 Proposed mechanism of introduction of impurities **A** and **B**

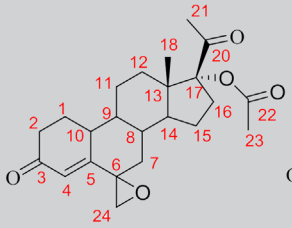
TABLE 1 Assignment of ^{13}C NMR spectra of impurities **A**, **B**, intermediate **7**, and NOMAc



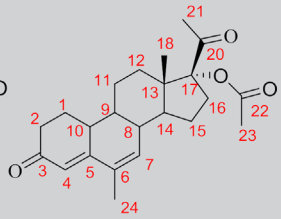
Impurity **A**



Impurity **B**



Intermediate **7**



NOMAc

	A	B	7[a]	NOMAc[a,b]
C-1	35.55	37.04	36.36	37.51
C-2	26.12	26.85	26.76	26.97
C-3	200.07	200.17		
C-4	121.58	121.47	124.37	121.77
C-5	170.09	156.57		
C-6	50.24	135.32		
C-7	40.72	139.84	38.31	139.56
C-8	38.01	41.00	41.42	41.00
C-9	42.78	45.32	50.35	45.56
C-10	40.39	40.90	37.31	41.06
C-11	25.78	24.93	25.71	24.92
C-12	30.31	30.31	30.31	30.26
C-13	47.01	47.52		
C-14	48.90	48.53	48.32	48.66
C-15	23.64	23.19	23.47	23.16
C-16	31.04	30.99	30.91	30.98
C-17	96.71	96.45		
C-18	14.47	14.20	14.47	14.19
C-19				
C-20	204.12	203.94		
C-21	26.37	26.46	26.49	26.40
C-22	170.74	170.69		
C-23	21.27	21.20	21.26	21.20
C-24	17.76	62.78	52.34	19.32

[a]Data were from DEPT-^{13}C NMR only.
[b]Data were extracted from Ref. [35] for comparison purpose.

C-3 carbon; the signal at ~170.74 ppm likely belongs to **C**-5 carbon; the signal at ~170.09 ppm likely belongs to **C**-22 carbon. Signals at ~50.24, 48.90, 42.78, 40.39, 38.01 ppm belong to one of the 5 single proton attached carbon units, **C**-6, **C**-8, **C**-9, **C**-10 and **C**-14. Signals at ~40.72, 35.55, 31.04, 30.31, 26.12, 25.78 and 23.64 ppm belong to one of the seven double proton attached carbon units, **C**-1, **C**-2, **C**-7, **C**-11, **C**-12, **C**-15, and **C**-16. Finally, signals at ~26.37, 21.27, 17.76, and 14.47 ppm belong to **C**-21, **C**-23, **C**-24, and **C**-18, respectively. Based on the same criteria, we assigned each of the corresponding C-13 NMR spectra for impurity **B** and intermediate **7**, as summarized in Table 1.

The human liver cell line L02 and mesenchymal stem cells were used to investigate the cytotoxicities of impurity **A**, impurity **B**, and NOMAc. The results demonstrated that NOMAc inhibited the viability of L02 cells line at ≥20 μM in both 24 and 48 h incubations, suggesting that it is probably cytotoxic to liver at high doses (Figure 2A). It did not inhibit the viability of human MSCs (Figure 2B). Breast and cervical cancer are associated with sex hormones.[50,51] NOMAc is a selective

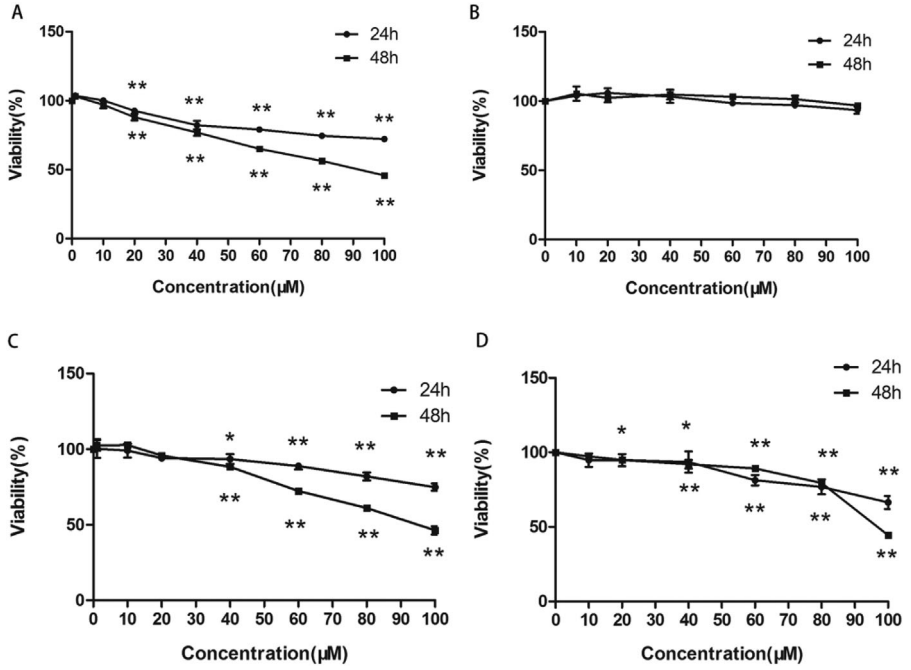


FIGURE 2 Viability of cells treated with NOMAc at different concentrations. The L02 (A), MSCs (B), MCF-7 (C), or C33A (D) cells were incubated with different concentrations of nomegestrol acetate for 24 and 48 h. The cell viability was determined by CKK-8 assay. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control

progestogen and structurally similar to progesterone with anti-estrogenic activity by inhibiting the production of estradiol. NOMAc has been reported to have anti-proliferative effect on human breast and endometrial cancer cells.[32] Our results also indicated that NOMAc inhibited the viability of MCF-7 breast cancer cells and C-33A cervical cancer cells at ≥40 and ≥20 μM, respectively, in both 24 and 48 h incubations (Figure 2C and D). In the 24 h incubation assay, NOMAc has IC_{50} values greater than 100 μM against all four cell lines, whereas in the 48 h incubation assay, it has IC_{50} values greater than 80 μM against the same cell lines.

Impurity **A** demonstrated similar pattern of cytotoxicities to those of NOMAc against these four cell lines with slightly less cytotoxicity. Impurity **A** inhibited the viability of L02 cells line at ≥20 μM, inhibited the viability of MCF-7 at ≥40 μM and C33A at ≥60 μM (Figure 3A, C, D). It is interesting to note that impurity **A** increased the viability of human MSCs at 20 μM (Figure 3B). Impurity **B** did not show significant cytotoxicity up to 60 μM against the viability of L02, MCF-7, and C33A cell lines (Figure 4A, C, D). It also increased the viability of human MSCs at ≥10 μM (Figure 4B). Impurity **A** showed IC_{50} values greater than 100 μM against all four cell lines in the 24 h incubation and greater than 100 μM against L02, MSCs, and MCF-7, and greater than 80 μM against C33A cells in the 48 h incubation. Whereas impurity **B** had IC_{50} values greater than 100 μM against all four cell lines in both the 24 and 48 h incubations.

NOMAc is currently marketed with doses of 2.5, 3.75, and 5.0 mg orally with plasma concentrations ranged from 2.5 to 5 nM, which are far less than the concentrations that showed cytotoxictity in this study. Impurities **A** and **B** have less than 0.2% in the API and thus have plasma concentrations in the range of 5–10 pM. Therefore, all these analogs will not have any measurable cytotoxicity against the four cell lines tested under normal therapeutic dosages.

3 | CONCLUSIONS

Two impurities have been identified during our quality analysis of NOMAc. Impurity **A** is a known impurity for NOMAc and is listed in EP 8.0 as a "must be measured" substance that has been known to be introduced during the 6-methylene double bond catalytic translocation. Impurity **B** has not been identified to be an impurity in NOMAc before and for the first time was identified and confirmed as a process impurity due to the incomplete reduction of 6-formyl group, which was subsequently transformed to the 6-hydroxymethyl group during the 6-methylene double bond translocation process. Analytical methods for both impurities were developed and could be used for NOMAc quality control. We also developed synthetic procedures to prepare both impurities **A** and **B** and were able to obtain both compounds in multiple grams with high purity. At high concentrations, both NOMAc and impurity **A** showed very milder cytotoxicities to L02 liver cells, MCF-7 breast cancer cells, and C-33A cervical cancer cells. Impurity **B** did not show significant cytotoxicity to L02 liver cells, MCF-7 breast cancer cells, and C-33A cervical cancer cells. All three analogs did not show cytotoxicities to mesenchymal stem cells, instead impurities **A** and **B** showed some stimulation of growth to mesenchymal stem cells. Overall, the dosages used for medical treatment contain far less *in vivo* concentrations of these analogs that would trigger any cytotoxicity against the cell lines tested.

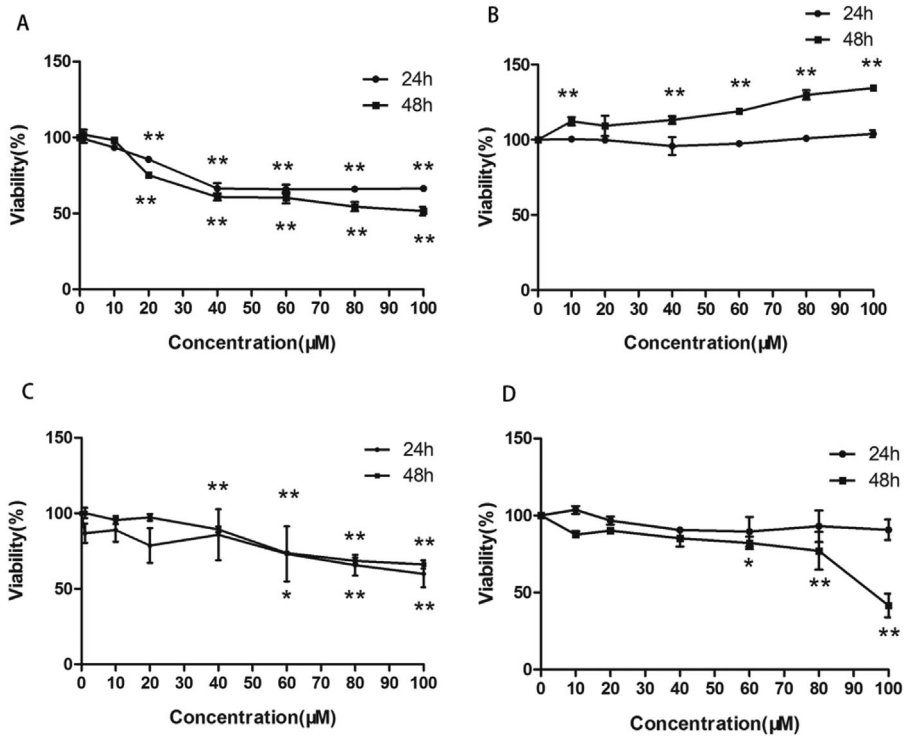


FIGURE 3 Viability of cells treated with impurity **A** at different concentrations. The L02 (A), MSCs (B), MCF-7 (C), or C33A (D) cells were incubated with different concentrations of nomegestrol acetate for 24 and 48 h. The cell viability was determined by CKK-8 assay. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control

4 | EXPERIMENTAL

4.1 | General

Intermediate **5** and NOMAc were obtained from Lijiang Yinghua BioPharmaceutical Company (Lijiang, Yunnan, China). Reference impurity **A** was purchased from Guangzhou Daersike Biotech Co. (Panyu, Guangzhou). All other chemicals were purchased commercially as reagent grade and used directly without further purification. The reactions were monitored by analytical thin-layer chromatography (TLC) on silica gel F254 glass plates and visualized under UV light (254 and 365 nm). Flash column chromatography was performed on silica gel (200–300 mesh). ^{1}H NMR spectra were recorded with a Bruker Avance III 400 MHz NMR spectrometer at room temperature. Chemical shifts (in ppm) were recorded as parts per million (ppm) downfield to tetramethylsilane (TMS). The following abbreviations are used for multiplicity of NMR signals: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; td, triplet of doublets; dt, doublet of triplets; dq, doublet of quartets; b, broad; ^{13}C NMR or DEPT-^{13}C (distortionless enhancement by polarization transfer ^{13}C NMR) spectra were recorded with Bruker Avance III 400 MHz NMR spectrometer (100 MHz) and calibrated with $CDCl_3$ (δ = 77.23 ppm). LC-mass spectrum was recorded with a Bruker amaZon SL instrument. High-resolution mass spectra were recorded with a LTQOrbitrap XL (ThermoFisher) mass spectrometer.

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

4.2 | Analysis of NOMAc

4.2.1 | Analysis of the purified product NOMAc

The NOMAc was synthesized according to procedure of Lu et al.[35] and obtained in 99.67% purity at 245 nm and 99.97% purity at 290 nm (Supporting Information Figures S1 and S2). It should be noticed that both NOMAc and impurity **B** do not have good UV absorption at 245 nm, whereas impurity **A** does not have good absorption at 290 nm. Thus, they have to be analyzed at different wavelengths of UV light in order to be measured, that is, impurity **A** is analyzed at 245 nm, impurity **B** is analyzed at 290 nm.

4.2.2 | Purification of impurity A from the mother liquid of NOMAc

A 5.0 kg of mother liquid in methanol solution from the crystallization of NOMAc (supplied by Lijiang Yinghua BioPharmaceutical Company) was repeatedly concentrated to get the majority of NOMAc out to finally obtain 300 mL enriched solution that was then chromatographed on silica gel column with petroleum ether/ethyl acetate (10:1 to 4:1) gradient elution to obtain the crude impurity **A** (with NOMAc) and impurity **B** crudes, respectively. The crude impurity **A** was subjected to numerous chromatography on silica gel column (for a total of six rounds) with petroleum ether/ethyl acetate (50:1 to 5:1) gradient elution to finally give 87 mg of impurity **A**. ^{1}H NMR ($CDCl_3$, ppm): 5.86 (s, 1H), 2.92–2.97 (td, 1H), 2.37–2.41 (m, 1H), 2.21–2.30 (m, 3H), 2.10

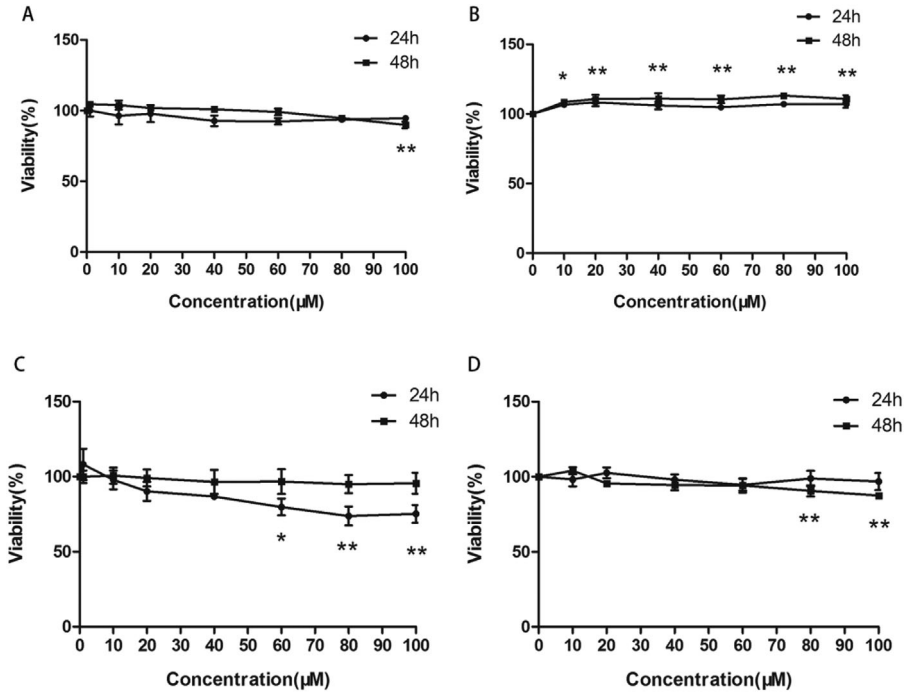


FIGURE 4 Viability of cells treated with impurity **B** at different concentrations. The L02 (A), MSCs (B), MCF-7 (C), or C33A (D) cells were incubated with different concentrations of nomegestrol acetate for 24 and 48 h. The cell viability was determined by CKK-8 assay. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control

(s, 3H), 2.05 (s, 3H), 1.87–2.00 (m, 3H), 1.70–1.82 (m, 5H), 1.54–1.59 (m, 2H), 1.30–1.33 (m, 2H), 1.10–1.12 (d, 3H), 0.84–1.05 (m, 2H), 0.69 (s, 3H); ^{13}C NMR (DEPT, $CDCl_3$, ppm): δ 121.57, 50.24, 48.90, 42.78, 40.72, 40.39, 38.01, 35.55, 31.04, 30.31, 26.37, 26.12, 25.78, 23.64, 21.27, 17.76, 14.47. Note: Both the ^{1}H NMR and the ^{13}C NMR DEPT-135 spectra indicated the presence of small amounts of impurities in the isolated impurity **A**.

4.3 | Synthesis and analysis of impurity A

4.3.1 | Synthesis of impurity A

To a 500 mL round bottle was added ethanol (300 mL), 5% Pd/C (5 g), cyclohexene (5 mL). The solution was heated to reflux and was added NOMAc (15 g, 40.5 mmol) and continued to stir under reflux for 2 h. The reaction was cooled to rt and filtered off the solid, concentrated to dryness. The crude was recrystallized from acetone three times and then from ethanol twice to afford impurity **A** (5.2 g, yield 34%, HPLC 99.58% at 245 nm). Mp 173–174°C; ^{1}H NMR ($CDCl_3$, ppm): 5.88 (s, 1H), 2.92–2.99 (td, 1H), 2.37–2.44 (m, 1H), 2.20–2.33 (m, 3H), 2.11 (s, 3H), 2.06 (s, 3H), 1.87–2.01 (m, 3H), 1.67–1.82 (m, 5H), 1.48–1.59 (m, 2H), 1.26–1.37 (m, 2H), 1.11–1.13 (d, 3H), 0.91–1.08 (m, 2H), 0.70 (s, 3H); ^{13}C NMR ($CDCl_3$, ppm): δ 204.12, 200.07, 170.74, 170.09, 121.57, 96.71, 50.25, 48.92, 47.01, 42.80, 40.74, 40.41, 38.02, 35.55, 31.05, 30.33, 26.37, 26.13, 25.79, 23.64, 21.26, 17.76, 14.48; ESI-MS: *m/z* 373.2349 $[M+1]^+$; calcd. MW for $C_{23}H_{32}O_4$ 372.2301. Anal. calcd. (%) for $C_{23}H_{32}O_4$: C 74.16, H 8.66. Found: C 74.05, H 8.52.

4.3.2 | Analysis of impurity A

The synthesized impurity **A** was analyzed at both 245 and 290 nm and showed it has a very weak absorption at 290 nm (Supporting Information Figure S3). It showed a purity of 99.58% at 245 nm with a retention time of 19.367 min. The reference impurity **A** was analyzed at the same condition and showed a purity of 99.13% at 245 nm with a retention time of 19.414 min (Supporting Information Figure S4). When mixed of the synthesized impurity **A** and the reference impurity **A** at 1:1 ratio, the mixture showed a purity of 99.42% at 245 nm with a retention time of 19.442 min (Supporting Information Figure S5).

4.4 | Synthesis and analysis of impurity B

4.4.1 | Purification of impurity B from the mother liquid of NOMAc

The crude impurity **B** (from above 4.1.2.) was repeatedly chromatographed on silica gel column (for a total of four rounds) with petroleum ether/ethyl acetate (20:1 to 4:1) gradient elution to finally give 44 mg impurity **B**. ^{1}H NMR ($CDCl_3$, ppm): 6.36 (s, 1H), 6.01 (s, 1H), 4.32–4.33 (d, 2H), 2.96–3.03 (td, 1H), 2.54–2.58 (d, 1H), 2.30–2.35 (t, 3H), 2.15–2.20 (t, 1H), 2.10 (s, 3H), 2.06 (s, 3H), 1.93–1.99 (m, 4H), 1.57–1.61 (m, 4H), 1.25–1.50 (m, 2H), 1.24–1.27 (m, 2H), 0.72 (s, 3H); Ref. [47] ^{1}H NMR: 6.37 (s, 1H), 6.01 (s, 1H), 4.34 (s, 2H), 2.10 (s, 3H), 2.07 (s, 3H), 1.63 (s, 1H), 0.73 (s, 3H); ^{13}C NMR (DEPT, $CDCl_3$, ppm): δ 139.79, 121.43, 62.71, 48.54, 45.33, 41.01, 40.89, 37.39, 30.99, 30.30, 26.85,

26.44, 24.93, 23.18, 21.18, 14.19; ESI-MS: *m/z* 387.3 $[M+1]^+$; calcd. MW for $C_{23}H_{30}O_5$ 386.21.

4.4.2 | Synthesis of impurity B

To a 200 mL round bottle was added intermediate **5** (5.0 g, 13.5 mmol), dichloromethane (50 mL), and 3-chloroperbenzoic acid (5.0 g, 29.0 mmol) in dichloromethane (20 mL). The solution was stirred at refluxing for 4 h, cooled to rt and poured onto ice-water, extracted with dichloromethane, washed with brine, dried over sodium sulfate. The solvent was removed *in vacuo* and the residue was purified by a silica gel column to give **7** (3.5 g, yield 67%, HPLC 98.8% at 254 nm). ^{1}H NMR ($CDCl_3$, ppm): 5.92–5.93 (d, 1H), 3.25–3.26 (d, 1H), 2.93–2.99 (td, 1H), 2.87–2.88 (d, 1H), 2.36–2.46 (m, 3H), 2.13 (s, 3H), 2.08 (s, 3H), 1.97–2.04 (m, 2H), 1.87–1.92 (m, 1H), 1.78–1.82 (m, 2H), 1.62–1.65 (m, 5H), 1.49–1.50 (dd, 1H), 1.35–1.40 (m, 2H), 1.07–1.13 (m, 1H), 0.74 (s, 3H); ^{13}C NMR (DEPT, $CDCl_3$, ppm): δ 124.37, 52.34, 50.35, 48.32, 41.42, 38.31, 37.31, 36.36, 30.91, 30.31, 26.76, 25.71, 23.47, 21.26, 14.47. Anal. calcd. (%) for $C_{23}H_{30}O_5$: C 71.48, H 7.82. Found: C 71.44, H 7.71.

To a round bottle was added the above intermediate **7** (3.0 g, 7.7 mmol), THF (30 mL), and stirred until all solid dissolved. The solution was cooled to 0°C and added 1 N H_2SO_4 (5 mL) and then stirred at rt overnight. The solution was poured into water, extracted with dichloromethane, washed with brine, and dried over sodium sulfate. The solvent was removed *in vacuo* and the residue was purified by a silica gel column to give impurity **B** (1.2 g, yield 40%, HPLC 99.73 at 290 nm). Mp 105–107°C; ^{1}H NMR ($CDCl_3$, ppm): 6.38 (s, 1H), 6.03 (s, 1H), 4.35 (s, 2H), 2.98–3.06 (td, 1H), 2.57–2.62 (dt, 1H), 2.31–2.38 (m, 3H), 2.17–2.23 (t, 1H), 2.09–2.12 (2s, 6H), 1.91–2.02 (m, 3H), 1.81–1.89 (m, 1H), 1.60–1.67 (m, 4H), 1.43–1.52 (m, 1H), 1.31–1.42 (qd, 1H), 1.23–1.29 (m, 2H), 0.74 (s, 3H); ^{13}C NMR ($CDCl_3$, ppm): δ 203.94, 200.17, 170.69, 156.57, 139.84, 135.32, 121.47, 96.45, 62.78, 48.53, 47.52, 45.32, 41.00, 40.09, 37.40, 30.98, 30.31, 26.85, 26.45, 24.93, 23.19, 21.20, 14.20; ESI-MS: *m/z* 387.2146 $[M+1]^+$; calcd. MW for $C_{23}H_{30}O_5$ 386.2093. Anal. calcd. (%) for $C_{23}H_{30}O_5$: C 71.48, H 7.82. Found: C 71.39, H 7.68.

4.4.3 | Analysis of impurity B

NOMAc was analyzed on a Shimadzu LC-15C liquid chromatograph with impurity **B** showing a retention time around 3.755 min and NOMAc showing a retention time around 11.044 min (Supporting Information Figure S6). The synthesized impurity B along was analyzed in the same condition and showed a retention time around 3.722 min (Supporting Information Figure S7). When the synthesized impurity **B** was added to the NOMAc, the HPLC results indicated an increased absorption at 3.751 min (Supporting Information Figure S8).

4.5 | Evaluation of cytotoxicity *in vitro*

The cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8, Sigma). Briefly, the cells were harvested and seeded at a density of approximately 5×10^3 cells per well in 96-well plates. NOMAc, impurity **A** and impurity **B** were dissolved in DMSO. The solutions were then added to the culture medium at the concentrations indicated with final concentration of DMSO at less than 0.1% for 24 and 48 h. The cytotoxicity was assessed by the CCK-8. The absorbance at 450 nm was measured by a microplate reader.

4.6 | Statistical analysis

All data were presented as mean ± SD and statistical analysis was performed using one-way analysis of variance (one-way ANOVA) among multiple groups. A *p*-value less than 0.05 was considered significant for all analysis.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.
HPLC analysis and spectroscopic data (^{1}H and ^{13}C NMR) for impurities **A** and **B** as well as intermediate **7** are available.

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