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Semi-synthetic modification of nimbolide to 6-homodesacetylnimbin and 6-desacetylnimbin and their cytotoxic studies

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Semi-synthetic modification of nimbolide to 6-homodesacetylnimbin and 6-desacetylnimbin and their cytotoxic studies

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A new C-seco tetranortriterpenoid named as 6-homodesacetylnimbin **2**, has been synthesised for the first time through semi-synthetic modification of nimbolide **1**, a potent molecule with anticancer activity. Attempts were made to transesterify the –COOMe moiety in nimbolide using titanium (IV) isopropoxide and ethanol so as to obtain a molecule with –COOEt moiety. However, a novel product was envisaged during the course of the reaction, which was identified as 6-homodesacetylnimbin, a higher homologue of 6-desacetylnimbin **3** through spectroscopic and crystallographic methods. Also, 6-desacetylnimbin has been synthesised through acid hydrolysis of nimbolide. The compounds were screened for their cytotoxic properties through brine shrimp lethality bioassay method using *Artemia salina*.

Keywords: 6-Homodesacetylnimbin; Nimbolide; 6-Desacetylnimbin; $Ti(^{i}OPr)_4$; Brine shrimp

1. Introduction

One of the most exciting applications of limonoids is their utility in treatment of cancer [1] apart from antifeedant and insect-growth regulatory activity [2]. Neem preparations contain not only azadirachtin [3] as the active insect antifeedant or growth regulator but also a variety of other limonoids, some of which are cytotoxic to N1E-115 neuroblastoma (mouse), 143B.TK-osteosarcoma (human) and Sf9 (insect) cultured cell lines [4]. The most potent of these limonoids is nimbolide [5], with an IC_{50} ranging from 4 to 10 μM, and averaging 6 μM. Other limonoids of decreasing potency and their average IC_{50} values (μM) are epoxyazadiradione 27 μM, salannin 112 μM, and nimbin, 6-deacetylnimbin and azadirachtin each >200 μM (practically non-toxic).

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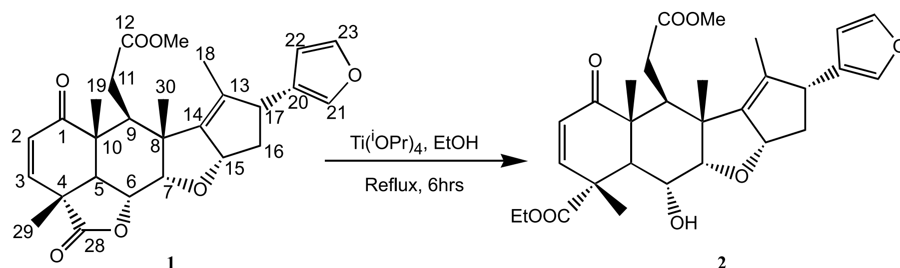
Accordingly, nimbolide **1** has been isolated from the neem leaves and semi-synthetically modified with a view to enhance the bioefficacy of the native compound. In the course of our study, 6-homodesacetylnimbin **2**, a higher homologue of 6-desacetyl nimbin **3** has been envisaged through titanium (IV) isopropoxide catalysed reaction of nimbolide in the presence of ethanol. Also, 6-desacetylnimbin was synthesised through acid hydrolysis of nimbolide. The cytotoxic property of 6-homodesacetylnimbin was determined and compared with that of nimbolide, 6-desacetylnimbin and nimbin **4** using a brine shrimp lethality bioassay method using *Artemia salina* [6]. We report the results of our findings.

2. Results and discussion

The C-seco limonoids are characterised by the presence of –COOMe moiety at the C-12 position. Generally, presence of bulky substituents drastically alters the bioefficacy of a molecule. We attempted to induce steric hindrance through transesterification of the alkyl group in the –COOMe moiety. However, transesterification [7] of nimbolide using $Ti(^{i}OPr)_4$ and ethanol yielded a novel product **2** whose structure was ascertained using NMR (1D and 2D), IR, mass spectra and HPLC analyses. The NMR of the new compound showed a shift in frequency from 4.65 ppm and 73.41 ppm in nimbolide to 3.80 ppm and 66.10 ppm for the H-6 and C-6, respectively. However, no significant change was observed for the carbon signal of C-28. The proton signals corresponding to a quartet at 4.1 ppm and a triplet at 1.2 ppm and carbon signals at 61.2 ppm and 17.4 ppm confirm the presence of $-COOCH_2CH_3$ moiety. Nimbolide eluted at a retention time of 20.76 min while the product eluted at 26.31 min on a reverse-phase HPLC. IR band at 1778 cm^{-1} corresponding to the lactone carbonyl in nimbolide was shifted to 1712 cm^{-1} in the product representing a new ester carbonyl. The M^+ peak is observed at 513 Da.

These results suggest the following structure for **2** (scheme 1), called 6-homodesacetylnimbin. The plausible mechanism of the reaction is depicted in scheme 2. Under the reaction conditions THE –COOMe group at C-12 has remained intact and the lactone ring has opened to yield the product. $Ti(^{i}OPr)_4$ has selectively coordinated with the oxygen of the lactone carbonyl rather than the ester carbonyl, facilitating the attack of the alcohol on C-28. Absence of any reaction with nimbin, viz. a limonoid possessing an ester and sans lactone, confirms that the lactone moiety is the reaction site.

Finally, the structure of **2** has also been confirmed by X-ray diffraction analysis. Rod-shaped crystals of the 6-homodesacetylnimbin was grown from a solution of chloroform/hexane (4:1). It crystallised in $P2_12_12_1$ space group with cell parameters, viz. *a* 6.711 (2), *b* 13.897 (2), *c* 29.0760 (10). The structure was solved using the programme



Scheme 1. Conversion of nimbolide (**1**) to 6-homodesacetylnimbin (**2**).

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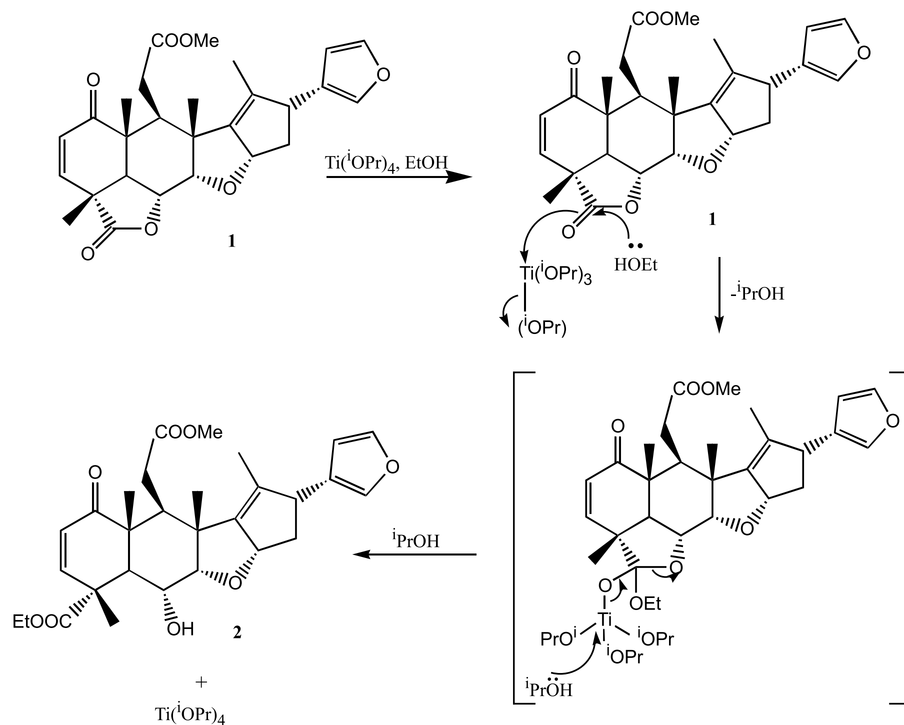

Scheme 2. Conversion of nimbolide (**1**) to 6-homodesacetylnimbin (**2**): reaction mechanism.

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SHELXS97 and was refined using SHELXL97 to an *R* factor of 0.092 [8]. The ortep diagram of the molecule is given in figure 1.

Nimbolide on acid hydrolysis using $MeOH/H_2SO_4$ produced 6-desacetylnimbin (**3**) whose structure was ascertained by comparing with literature data [9]. The plausible mechanism of the reaction is depicted in scheme 3. The mechanism involves protonation of the lactone

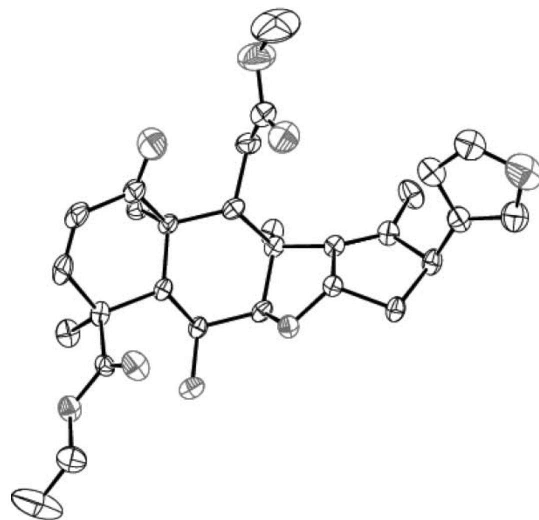


Figure 1. The X-ray structure of compound **2** drawn by ORTEP at 30% probability level (CCDC No. 267936).

carbonyl followed by attack of the alcohol on C-28 resulting in a tetrahedral intermediate yielding the desired product. Further bond breaking and bond making yields the product.

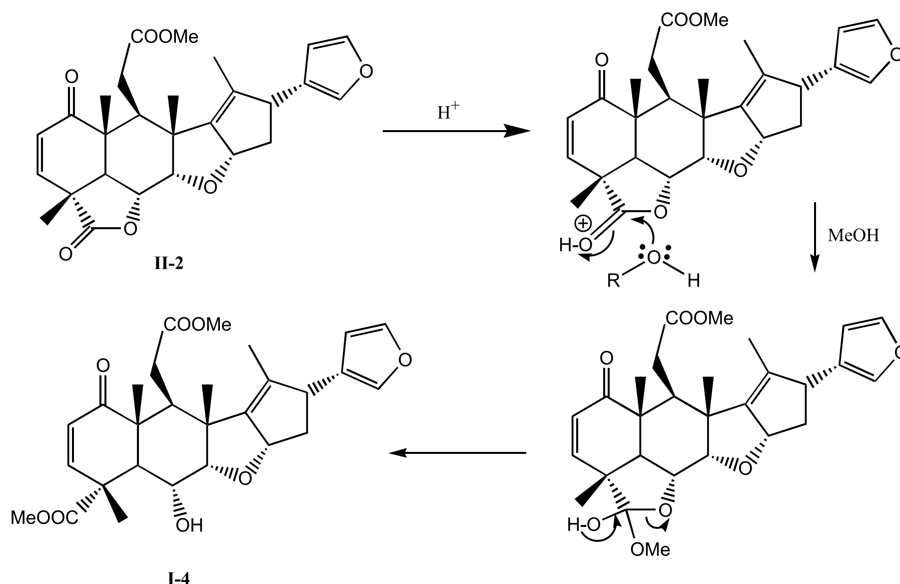
From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumour compounds in terrestrial plant extracts [10]. Hence, the cytotoxicity of nimbolide, 6-homodesacetylnimbin, 6-desacetylnimbin and nimbin were determined using brine shrimp lethality bioassay method using *Artemia salina*. This involves exposing groups of 10 *Artemia* aged 12 h to various concentrations of the test compound and the activity determined after 24 h by counting the number of dead organisms in the II/III instar. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. To determine whether the organisms died of starvation, a control was maintained. However, a hatched brine shrimp nauplii can survive up to 48 h without food. Eight different concentrations of the test compounds were taken in DMSO and their effect on the brine shrimp lethality is determined using Finney Probit Analysis software [11]. The ED_{50} values are given in table 1.

Absence of any activity and very little activity exhibited by nimbin and 6-desacetylnimbin respectively depicts that the lactone ring in nimbolide is very essential for anticancer activity. However, ED_{50} value for 6-homodesacetylnimbin illustrates moderate activity. Hence, steric hindrance through the presence of a bulky group or a lactone ring over the C-28 position may be critical for imparting cytotoxic activity. In conclusion, we have synthesised a novel molecule, viz. 6-homodesacetylnimbin, that appears to be a potent molecule for anticancer activity.

3. Experimental

3.1 General experimental procedures

Melting points were determined using a Raaga industries melting point apparatus and are uncorrected. NMR spectra were recorded on a Bruker 300 MHz instrument using TMS as an



Scheme 3. Conversion of nimbolide (**1**) to 6-desacetylnimbin (**3**): reaction mechanism.

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Table 1. ED_{50} values: brine shrimp lethality bioassay method.

S. no.	*Compound*	*ED_{50} (ppm)*	*Dose for cytotoxic activity (ppm)*
1	Nimbolide	540.85	54.08
2	Homo desacetyl nimbin	1941.97	194.19
3	Desacetyl nimbin	13850.96	1385.09
4	Nimbin	–	–

[a]Dose for cytotoxic activity is 1/10th the dose of ED_{50}.

internal standard and $CDCl_3$ as the solvent. Mass spectrometry was performed on a Shimadzu QP 1000A and QP 5000 mass spectrometer. High-performance liquid chromatography was performed on a Shimadzu instrument with LC-10ATVP high-pressure pump and a C18 Luna 5μ column (250 × 4.60 mm) and the peaks detected at 215 nm (SPD-10 AVP UV–Vis Detector), the mobile phase being acetonitrile/water system (60:40) at a flow rate of 0.5 ml/min.

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3.2 Isolation of nimbolide

Neem leaves were collected, shade dried and extracted with acetone. Column chromatography over 60–120 mesh silica gel using hexane/ethyl acetate eluent (1:1) yielded pure nimbolide (**1**). The structure of nimbolide was confirmed by correlating the spectroscopic data with literature.

3.3 Conversion of nimbolide to 6-homodesacetylnimbin

Three hundred milligrams (0.63 mmol) of nimbolide was dissolved in 75 ml alcohol. To this solution was added 0.5 ml titanium (IV) isopropoxide and the mixture was refluxed for 6 h. The completion of the reaction was monitored using TLC and the reaction mixture was concentrated under reduced pressure. Fifty millilitres of water was added to the reaction mixture and extracted using ethyl acetate. The organic layer was dried over sodium sulphate and concentrated under reduced pressure. Flash column chromatography of the crude product over silica gel (70–325 mesh) using hexane/ethyl acetate (86:14) furnished the pure product **2** (174.6 mg, 54.37%). A complete assignment of proton and carbon values for 6-homodesacetylnimbin is given below.

3.3.1 Structure determination. $C_{29}H_{36}O_8$ (512); crystalline, mp 192–195°C, UV (MeOH) λ_{max}: 220, 224, 227 nm.

^{1}H NMR (300 MHz, $CDCl_3$): δ 5.84 (1H, d, $J = 10.17$, 2-H); 6.42 (1H, d, $J = 10.17$, 3-H); 3.41 (1H, d, $J = 11.20$, 5-H); 3.92 (1H, ddd, 3.02, 11.27, 14.57, 6-H); 4.02 (1H, d, $J = 3.30$, 7-H); 2.76 (1H, t, $J = 3.85$, 9-H); 2.90 (1H, dd, $J = 5.77$, 16.23, 11a-H); 2.2 (1H, q, $J = 4.12$, 7.15, 11.82, 18.43, 11b-H); 5.55 (1H, t, $J = 6.60$, 15-H); 2.18 (2H, q, 4.12, 7.15, 16-H); 3.68 (1H, s, 17-H); 1.58 (3H, d, 3.57, 18-H); 1.21 (3H, s, 19-H); 7.24 (1H, s, 21-H); 6.33 (1H, s, 22-H); 7.32 (1H, s, 23-H); 1.68 (3H, s, 29-H); 1.29 (3H, s, 30-H); 3.65 (3H, s, –OMe), 4.15 (2H, q, $J = 6.80$, 14.30, $COCH_2CH_3$); 1.25 (3H, t, $J = 7.1$, $COCH_2CH_3$).

^{13}C NMR (300 MHz, $CDCl_3$): δ 202.22 (C-1); 126.75 (C-2); 148.25 (C-3); 41.38 (C-4); 47.36 (C-5); 66.16 (C-6); 86.86 (C-7); 49.53 (C-8); 39.03 (C-9); 47.68 (C-10); 34.29 (C-11);

173.63 (C-12); 134.76 (C-13); 146.81 (C-14); 87.44 (C-15); 43.34 (C-16); 47.23 (C-17); 12.78 (C-18); 13.99 (C-19); 126.30 (C-20); 138.93 (C-21); 110.38 (C-22); 142.97 (C-23); 174.90 (C-28); 16.31 (C-29); 17.08 (C-30); 51.57 (C-OMe); 61.70 ($COCH_2CH_3$); 17.46 ($COCH_2CH_3$).

3.4 Conversion of nimbolide to 6-desacetylnimbin

Two hundred milligrams (0.42 mmol) of nimbolide was dissolved in 95 ml methanol at 0°C. To this solution 6 ml of concentrated H_2SO_4 in 5 ml methanol was added dropwise using a pressure equalizer. The reaction was stirred for 2 h at room temperature. The completion of the reaction was monitored using TLC and the reaction was quenched with 20 g of solid sodium bicarbonate. The reaction mixture was filtered and concentrated under reduced pressure. Fifty millilitres of water was added to the reaction mixture and extracted using ethyl acetate. The organic layer was dried over sodium sulphate and concentrated under reduced pressure. Flash column chromatography of the crude product over silica gel (70–325 mesh) using hexane/ethyl acetate (75:25) furnished the pure product **3** (yield: 142 mg, 68.93%). A complete assignment of proton and carbon values for 6-desacetyl nimbin is given below.

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3.4.1 Structure determination. $C_{28}H_{34}O_8$ (498); crystalline, mp 190–195°C, UV (MeOH) λ_{max}: 221, 224 nm.

^{1}H NMR (300 MHz, $CDCl_3$): δ 5.85 (1H, d, $J = 9.90$, 2-H); 6.41 (1H, d, $J = 10.17$, 3-H); 3.39 (1H, d, $J = 11.82$, 5-H); 3.92 (1H, ddd, $J = 3.30$, 11.27, 14.57, 6-H); 4.02 (1H, d, $J = 3.30$, 7-H); 2.76 (1H, t, $J = 3.57$, 9-H); 2.90 (1H, dd, $J = 5.7$, 16.50, 11a-H); 2.25 (1H, d, $J = 3.55$, 5.50, 11b-H); 5.45 (1H, ddd, $J = 1.92$, 6.60, 12.65, 15-H); 2.03 (2H, m, 16-H); 3.68 (1H, s, 17-H); 1.68 (3H, d, $J = 1.37$, 18-H); 1.21 (3H, s, 19-H); 7.32 (1H, d, 1.37, 21-H); 6.33 (1H, s, 22-H); 7.24 (1H, s, 23-H); 1.59 (3H, s, 29-H); 1.290 (3H, s, 30-H); 3.70 (3H, s, -OMe), 3.65 (3H, s, –OMe).

^{13}C NMR (300 MHz, $CDCl_3$): δ 202.12 (C-1); 126.75 (C-2); 148.02 (C-3); 47.70 (C-4); 43.59 (C-5); 66.10 (C-6); 86.86 (C-7); 47.26 (C-8); 38.99 (C-9); 47.41 (C-10); 34.29 (C-11); 173.60 (C-12); 134.83 (C-13); 146.75 (C-14); 87.38 (C-15); 41.37 (C-16); 49.55 (C-17); 12.78 (C-18); 16.33 (C-19); 126.75 (C-20); 138.93 (C-21); 110.37 (C-22); 142.98 (C-23); 175.74 (C-28); 16.33 (C-29); 17.08 (C-30); 51.5 (COO*Me*); 52.93 ($COOCH_3$).

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