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Synthesis and evaluation of cytotoxic effects of hanultarin and its derivatives

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ABSTRACT

One of the known cytotoxic lignans is (−)-1-*O*-feruloyl-secoisolariciresinol designated as hanultarin, which was isolated from the seeds of *Trichosanthes kirilowii*. In this Letter, we described the first synthesis of 1-*O*-feruloyl-secoisolariciresinol, 1,4-*O*-diferuloyl-secoisolariceresinol and their analogues. The cytotoxicities of these compounds were evaluated against several cancer cell lines. Interestingly, we found that the feruloyl diester derivative of secoisolariciresinol was the most active cytotoxic compound against all the cancer cells tested in this experiment. The IC_{50} values of the1,4-*O*-diferuloyl-secoisolariceresinol were in the range of 7.1–9.8 μM except one cell line. In considering that both ferulic acid and secoisolariciresinol are commonly found in many plants and have no cytotoxicity, this finding is remarkable in that simple covalent bonds between the ferulic acid and secoisolariciresinol cause a cytotoxic effect.

Lignans are polyphenols found in plants and their precursors are found in a wide variety of plant-based foods, including seeds, whole grains, legumes, fruits, and vegetables.[1,2] Lignans have attracted much interest over the years on account of their broad range of biological activities such as antifungal,[3] antioxidant activity,[4] and cytotoxicity.[5] Secoisolariciresinol is one of the common lignans contained in food plants.[6,7] A racemic mixture of secoisolariciresinol is biosynthesized in some plants.[8] It has been reported that secoisolariciresinol was converted to enterodiol and enterolactone, which are compounds that have been postulated to have anti-carcinogenic properties.[9,10] The Yamauchi group reported first stereo-selective synthesis of *meso*-secoisolariciresinol and its biological activity was compared with (+) and (−)-secoisolariciresinol.[11] The optically active compounds, (+) and (−)-secoisolariciresinol, stimulated the IgM production of HB4C5 cells. However, *meso*-secoisolariciresinol had no effect of IgM production-stimulating activity toward the same cells.

In contrast to the well known biological activity of the secoisolariciresinol, its simple derivatives such as an ester were not well studied. Recently, a novel cytotoxic lignan was isolated from the seeds of *Trichosanthes kirilowii* and fully characterized as shown in Figure 1.[12] The isolated lignan was identified as mono ferulic acid ester of (−)-(2*R*,3*R*)-secoisolariciresinol and designated as hanultarin. The known 1,4-*O*-diferuloyl-secoisolariciresionol was also isolated from the seeds and fully characterized. Both compounds showed a comparable cytotoxic effect to the cisplatin against several cancer cell lines.

Interestingly, hanultarin has an inhibitory effect on actin polymerization, which was confirmed by an immune-fluorescence analysis.[12]

In this letter, we report first synthesis of hanultarin and its analogues and evaluate the cytotoxic activity of the obtained derivatives against several cancer cell lines. Further, we discussed the origin of cytotoxic effects of hanultarin and its analogues based on the structural characteristics of these molecules.

The secoisolariciresinol compound **7** is a key intermediate for the synthesis of hanultarin. One classical synthetic route toward the lignin structure utilizes the direct oxidative coupling reaction with structurally simple precursors.[13,14] However, the coupling reaction of phenol derivatives generates complex coupling products due to several radical intermediates.[15] Stereo-selective synthesis of (+) and (−)-secoisolariciresinol was achieved by multi-step synthesis using a chiral erythro-aldol product.[4,11,16]

Although the first synthesis of 2,3-dibenzylidenesuccinates via a double Stobbe condensation was achieved with the vanillin and an unprotected phenolic group by the Brown group,[17] other groups failed to synthesize 2,3-dibenzylidenesuccinates via Stobbe condensation without protection of the phenol hydroxyl group.[15,18] In our study, a large scale synthesis of the secoisolariciresinol was carried out by a double Stobbe condensation, starting from a succinate ester and the vanillin without protection of the phenolic hydroxyl groups as shown in Scheme 1.[19]

Briefly, the first Stobbe reaction with the vanillin **1** and the dimethyl succinate in the presence of lithium produced the condensation product **2**. Compound **2** was esterified with anhydrous MeOH in the presence of catalytic H_2SO_4 prior to the next Stobbe condensation. Then, the second Stobbe condensation of **3** was carried out with additional vanillin and followed esterification. Yields

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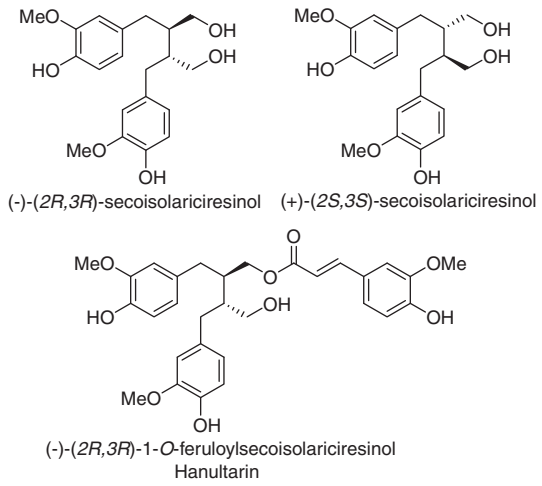
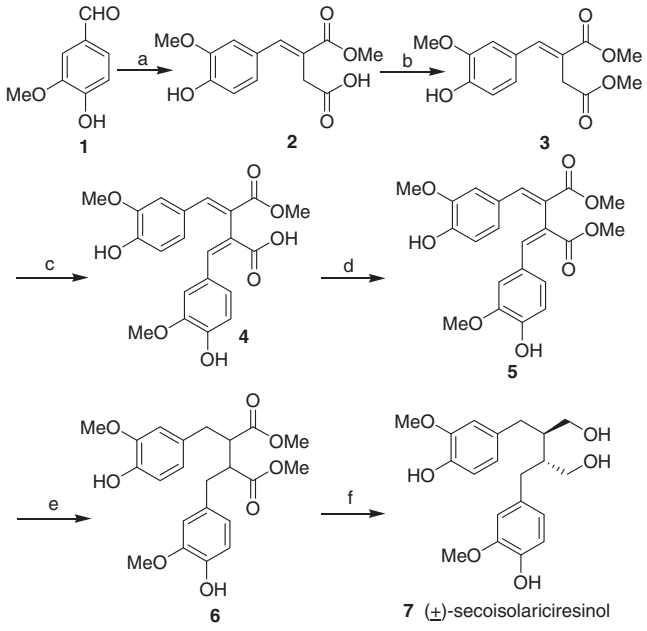


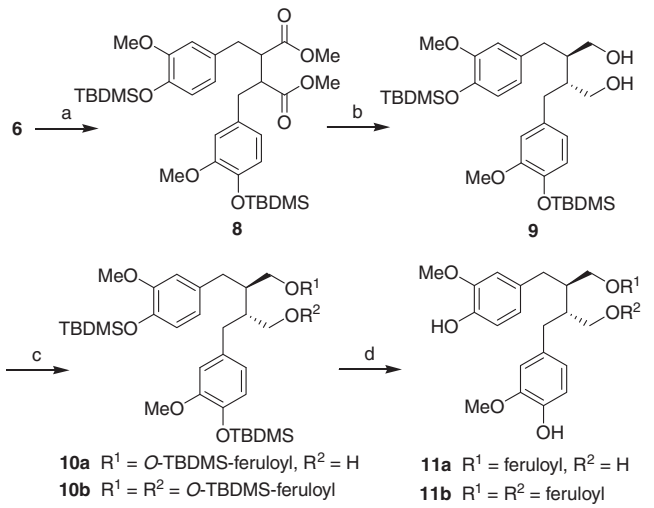
Figure 1. Structures of (±)-secoisolariciresinol and hanultarin.



Scheme 1. Synthesis of (±)-secoisolariciresinol. Reagents and conditions: (a) dimethyl succinate, lithium, dry MeOH, reflux, 48 h; (b) H_2SO_4, dry MeOH, reflux, 24 h; (c) vanillin, lithium, dry MeOH, reflux, 48 h; (d) H_2SO_4, dry MeOH, reflux, 24 h; (e) 10% Pd/C (5:1 substrate/catalyst), dry MeOH, H_2 gas, 24 h; (f) $LiAlH_4$, dry THF, −15 °C, 1 h.

of the sequential Stobbe condensation were 94% and 50%, respectively. Compound **5** was subjected to the catalytic hydrogenation with 10% Pd/C and produced the hydrogenated compound **6**. In this hydrogenation step, theoretically, three stereo isomers can be produced such as *meso* and one pair of enantiomer. In a similar reaction carried out by the Wang group[15], the hydrogenation of a similar structure containing the *t*-butyl group on phenyl rings generated 40% of *meso* derivative and 59% of the racemic mixture. However, the hydrogenation of **5** yielded only the racemic mixture of **6**, which was confirmed by the analysis of the ^1H and ^{13}C NMR.[19] The final (±)-secoisolariciresinol was produced by the reduction of compound **6** with $LiAlH_4$ and the formation of racemate was also confirmed by specific rotation, which was almost zero.[19]

For the synthesis of hanultarin, the phenolic alcohols of compound **6** should be protected prior to the reduction to avoid generation of the side products in coupling reaction with the



Scheme 2. Synthesis of hanultarin and its derivatives. Reagents and conditions: (a) TBDMS-Cl, imidazole, DMF, 3 h; (b) $LiAlH_4$, dry THF, −15 °C, 1 h; (c) DCC, DMAP, *O*-TBDMS-ferulic acid, DCM, 4 h; (d) TBAF, dry THF, 2 h.

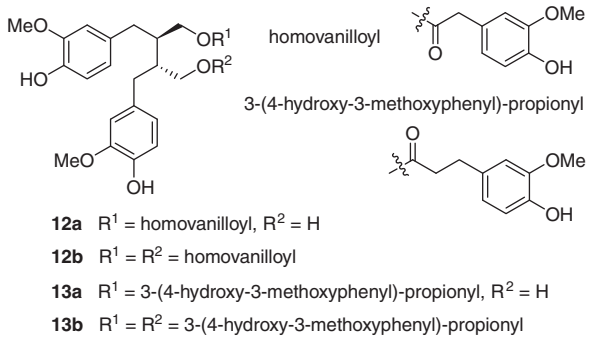


Figure 2. Structures of mono and diester analogues of the (±)-secoisolariciresinol.

protected ferulic acid. As shown in Scheme 2, *t*-butyldimethyl-silyl group was selected for the protection of phenolic alcohols and conversion to the compound **8** was almost quantitative. Then, the compound **9** was synthesized by the reduction of **8** with $LiAlH_4$. The DCC coupling reaction of **9** with the TBDMS-protected ferulic acid (1.5 equiv) yielded the mixture of mono and diester derivatives. The isolated yields of mono and diester derivatives were 23% and 50%, respectively. Finally, TBAF treatment of **10a** produced the target hanultarin **11a**. Both hanultarin (**11a**) and 1,4-*O*-diferuloyl-secoisolariciresionol (**11b**) were fully characterized by ^1H NMR, ^{13}C NMR, FT-IR and HR-Mass spectra[20] and perfectly matched to the isolated structures.[12] However, the specific optical rotation of both compounds (**11a** and **11b**) were determined to be almost zero, which means the formation of the racemic mixture of hanultarin and 1,4-*O*-diferuloyl-secoisolaricire sionol.

The feruloyl moiety of the hanultarin plays a key role in the cytotoxic effect against the cancer cell lines because of the relative low cytotoxicity of the secoisolariciresinol.[12] Therefore, further modification of the hanultarin focused on the feruloyl moiety as shown in Figure 2. First, the feruloyl group was replaced by one carbon short homovanilloyl moiety, which has a carboxy methyl group bound to the phenyl ring (**12a**, **12b**). Second modified compounds were more flexible derivatives without a double bond in the feruloyl group (**13a**, **13b**). Mono and diester derivatives of these analogues were prepared by the same method of the hanultarin.

Table 1
Cytotoxicity assay of hanultarin and its analogues

Compounds	Cell line (IC_{50}, μM)				
	A549[a]	B16F10[b]	HCT15[c]	SKOV3[d]	SKMel2[e]
7	>110.4	>110.4	>110.4	>110.4	>110.4
11a	29.5(5.6)[f]	21.4(24.1)	29.3	63.9	17.3(5.6)
11b	7.1(16.8)[g]	9.8(16.8)	8.3	21.7	8.3(14.0)
12a	65.3	71.8	52.2	>76.0	>76.0
12b	17.5	13.3	15.9	30.8	14.2
13a	65.7	68.4	49.6	>74.0	64.0
13b	12.1	12.8	10.0	35.9	10.3
Cisplatin	37.3(33.9)	31.3(16.8)	41.3	27.3	28.0(23.5)

[a] Human small lung cancer cell.
[b] Mouse melanoma cell.
[c] Human colon cell.
[d] Human ovary cell.
[e] Human skin cell.
[f] In parenthesis, IC_{50} of (−)-**11a**.[12]
[g] In parenthesis, IC_{50} of (−)-**11b**.[12]

The cytotoxicities of secoisolariciresinol, hanultarin and its analogues were evaluated against human lung cancer (A549), mouse melanoma cell (B16F10), human colon cell (HCT-15), human ovary cell (SKOV3) and human skin cell (SKMel2) lines using a sulforhodamine B (SRB) assay method.[21] Triplicate experiments were carried out against all the cell lines at a dose range of 1.56–50 μg/mL.[19] The IC_{50} values of each compound are summarized in Table 1.

The (±)-secoisolariciresinol **7** was inactive against all the cell lines at the given ranges of dose concentrations.[12] The IC_{50} values of the (±)-hanultarin **11a** were less than that of the control cisplatin against all the cells except the SKOV3 cell. In comparison to the isolated (−)-hanultarin, the cytotoxicities of (±)-**11a** were five times less toxic against the A549, three times less against the SKMel2 and comparable to the (−)-hanultarin against the B16F10 cell line. This means that (+)-derivative of the (±)-hanultarin is less active against all the cell lines. However, the (±)-1, 4-*O*-diferuloyl-secoisolariciresinol **11b** was two times more active than the isolated (−)-1,4-*O*-diferuloyl-secoisolariciresinol. This indicates that (+)-**11b** is more cytotoxic than the (−)-**11b** derivative. Interestingly, the diester derivative **11b** was two to four times more active than the mono ester **11a** against all the cell lines used in this study.

As shown in Table 1, the homovanilloyl mono ester **12a** and the 3-(4-hydroxy-3-methoxyphenyl)-propionyl mono ester **13a** showed two to four times less toxicity than the feruloyl mono ester **11a**. However, diester derivatives **12b** and **13b** were more active than (±)-hanultarin **11a** and their mono ester derivatives **12a** and **13a**. The diester **12b** and **13b** were a little less active than the ferulic acid diester **11b**. This suggests that the cytotoxicity of these compounds originates from the diester structure of the secoisolariciresinol. The diferuloyl ester derivative **11b** was the most active compound against all the cell lines. It is not common that the derivatives originating from dietary plants show the μ molar concentration of the IC_{50} values against the cancer cell lines.

The diferuloyl ester derivative **11b** was the most active compound against all the cell lines. It is not common that the derivatives originating from dietary plants show the μ molar concentration of the IC_{50} values against the cancer cell lines.

An immunocytochemical analysis of A549 lung cancer cells was carried out to determine the influence of the cellular structure on exposure to these derivatives. After treatment of each compound against the A549 cell lines at the dose range of the IC_{50} value, the cells were sequentially exposed to microtubule, actin and nuclei-specific antibodies.[19] As shown in Figure 3, all the actin and microtubule fibers were disrupted to form aggregate-like structures at a dose of near the IC_{50} value of each compound, except the nucleus, which were stable in these treatments. An inhibitory effect of the hanultarin on actin polymerization was reported via an immune-fluorescence analysis using normal epidermal keratinocytes.[12] However, it was difficult to discriminate the inhibitory effect between antin and microtubule fibers against the A549 cells exposed to the synthetic (±)-hanultarin.

All the diester derivatives including the diferuloyl ester of secoisolariciresinol showed a similar pattern to the hanultarin. At this stage, we cannot confirm whether these cell shrinkages originated from the process of the normal cell death or the inhibitory effect of the polymerization of actin or microtubule fibers. It might be proved in a specific actin or microtubule depolymerization assay in vitro.

In conclusion, we described the first synthesis of the feruloyl mono ester and the diferuloyl ester of secoisolariciresinol. These structures were fully characterized and well matched to the known spectral data except for the formation of a racemic mixture.

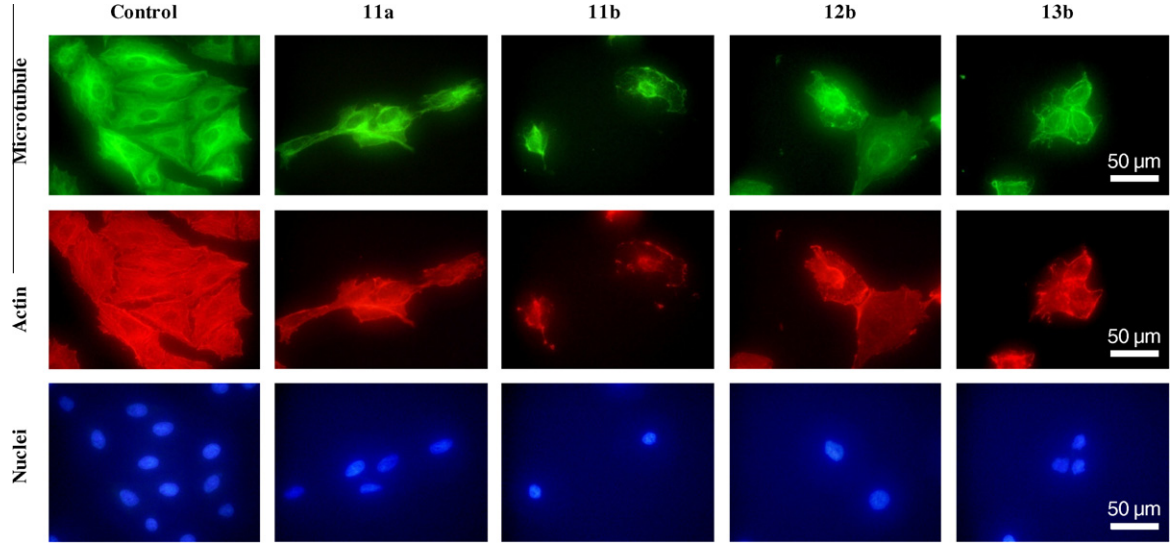


Figure 3. Immunocytochemical analysis of A549 lung cell lines after treatments of hanultarin and its analogues. Microtubules and actins were labeled with anti-β tubulin primary antibodies and rhodamine-conjugated phalloidin respectively. The nuclei were labeled with biotin-conjugated antibodies, and detected by FITC-conjugated streptavidin. The human small lung cancer cell lines were treated with each compound at about IC_{50} (12.5 μg/mL).

Additionally, the analogues of the hanultarin were also prepared to determine the structural effect on the cell toxicity. The cytotoxicity experiments revealed that the diester derivatives were more active than the mono ester derivatives against various cancer cell lines. The IC_{50} values of the (±)-1,4-*O*-diferuloyl-secoisolariciresinol were in the range of the several μ molar concentration, which is remarkable in that simple covalent bonds between the ferulic acid and secoisolariciresinol cause a cytotoxic effect. The immunocytochemical analysis of the A549 cells after treatment of the mono or diester derivatives showed the cell shrinkages, which might be originated from the inhibitory effect of the polymerization of actin or microtubule fibers.

Now further modification of the hanultarin derivative and a detailed mechanistic analysis of the cytotoxicity are in progress.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.014.

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19. See the Supplementary data for the detailed experimental.
20. Full characterization data:
*(±)-hanultarin (**11a**): IR ν_{max}:* 3419, 2917, 2850, 1697, 1602, 1515, 1269, 1156 *cm^{-1}:* ^{1}H NMR (400 MHz, CD_3OD) δ 1.92 (1H, m), 2.19 (1H, m), 2.56 (2H, d, *J* = 7.6 Hz), 2.65 (2H, dd *J* = 7.2, 13.6 Hz), 3.49 (1H, dd, *J* = 6.0, 14.4 Hz), 3.62 (1H, dd, *J* =), 3.66 (3H, s), 3.67 (3H, s), 3.82 (3H, s), 4.04 (1H, dd, *J* = 6.4, 11.2 Hz), 4.28 (1H, dd, *J* = 6.0, 11.2 Hz), 6.30 (1H, d, *J* = 16 Hz), 6.51 (3H, m), 6.57 (1H, d, *J* = 2 Hz), 6.62 (2H, dd, *J* = 2, 8 Hz), 6.75 (1H, d, *J* = 8 Hz), 7.00 (1H, dd, *J* = 1.6, 8.0 Hz), 7.11 (1H, d, *J* = 2.0 Hz), 7.51 (1H, d, *J* = 16.0 Hz). ^{13}C NMR (100 MHz, CD_3OD) 35.53, 36.01, 40.78, 56.33, 56.61, 62.87, 66.13, 111.80, 113.40, 113.53, 115.70, 116.00, 116.05, 116.63, 122.81, 122.85, 127.83, 133.39, 133.87, 145.70, 145.79, 147.00, 148.99, 149.02, 149.54, 150.80, 169.45; ESI-MS: Calcd 538.2203. Found: 537.2130 [M−H]; observed $[\alpha]_D^{20}$ −0.15 (c 0.1, MeOH).
*(±)-1,4-O-di-feruloyl-secoisolariciresionol (**11b**):* IR ν_{max}: 3418, 2926, 1698, 1514, 1267, 1157 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.21 (2H, m), 2.72 (4H, m), 3.77 (6H, s), 3.91 (6H, s), 4.21 (2H, dd, *J* = 5.2, 11.2 Hz), 4.39 (2H, dd, *J* = 5.6, 11.2 Hz), 5.89 (2H, s), 6.28 (2H, d, *J* = 16.0 Hz), 6.52 (2H, d, *J* = 2.0 Hz), 6.61 (2H, dd, *J* = 2.4, 8.0 Hz), 6.80 (2H, d, *J* = 8.0 Hz), 6.90 (2H, d, *J* = 8.0 Hz), 7.01 (2H, d, *J* = 2.0 Hz), 7.06 (2H, dd, *J* = 1.6, 8.0 Hz), 7.58 (2H, d, *J* = 16.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 35.26, 40.12, 55.74, 55.96, 64.44, 109.44, 111.24, 114.11, 114.71, 115.18, 121.71, 123.07, 126.82, 131.69, 143.90, 145.13, 146.43, 146.76, 148.05, 167.23; ESI-MS: Calcd 714.2676. Found: 713.2604 [M−H]; observed $[\alpha]_D^{20}$ −0.01 (c 0.1, MeOH).
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