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Synthesis and anticancer evaluation of complex unsaturated isosteviol-derived triazole conjugates

Background: For the last two decades, diterpenoid isosteviol and its derivatives have gained significant attention for novel chemical transformation in the drug discovery field. **Results:** An efficient way towards the synthesis of structurally diverse isosteviol derivatives was described here employing unsaturated functionalities as attractive templates for further transformation such as epoxidation. These structurally diverse compounds exhibited promising cytotoxic activities on different types of cancer cell lines, leading to drug discovery derived from natural products for the treatment of cancer. **Conclusion:** In this work, novel isosteviol derivatives with Michael acceptors were synthesized to expand the diversity and complexity of a class of isosteviol-derived triazole conjugates to facilitate the development of potential antitumor agents.

The **diterpenoids**, readily available from abundant *Stevia rebaudiana* plant, represent attractive platforms for the multifaceted medicinal and structural chemistry discoveries. In particular, since the end of the 1990s, *ent*-beyrane diterpenoid isosteviol has attracted a great deal of attention from medicinal chemists as a native scaffold for organic chemistry and drug discovery [1–3]. With its unique rigid skeletal structure with six stereo centers, isosteviol derivatives have been shown to have numerous biological activities [4–9], particularly in the anticancer area [10–15].

These characteristics led to the efforts of employing both isosteviol and its derivatives to the synthesis of different templates for diverse small-molecule libraries, including stereochemical approaches to connect heterocyclic ring systems to the isosteviol core. As a result, series of pyrazoline [15], pyrazole [15] and isoxazolidine [16] derivatives (**Figure 1**) have been recently synthesized.

Generally, the major approaches for modifying the isosteviol core involved chemical transformation of its D-ring, particularly the ketone group. For example, the Beckmann rearrangement and Beckmann fragmentation reactions have been applied by Georg *et al.* [17]

in an elegant, diversity-oriented approach for the synthesis of stereochemically complex isomeric lactam templates for small-molecule libraries (**Figure 1**). In addition, complex enantiopure fused derivatives have been investigated by Dehaen's group [18] to employ pericyclic reactions.

In our previous study, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction [19,20] as a highly efficient and rapid technique for the synthesis of large varieties of complex compounds has been successfully employed to the assembly of isosteviol core into diverse triazole conjugates types **1** and **2** (**Figure 2**) [13].

This strategy, involving the modification of both ketone and carboxylic groups, allowed us to further enrich the diversity of synthesized triazole small-molecule conjugates. With this strategy, we identified a panel of isosteviol-derived triazole conjugates possessing promising anticancer activities [13]. In the current study, we described the novel electrophilic isosteviol conjugates **3a–f** bearing triazole ring with conjugated and unsaturated functions, and further explored their modifications to discover novel and efficient templates for generating stereochemically diverse and complex small-molecules.

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Key terms

Diterpenoids: The largest class of plant derived metabolites with diverse and complex twenty-carbon backbone possess many biological functions.

Michael acceptors: Electrophilic α,β -unsaturated compounds with electron-withdrawing substituents such as ketone, ester or amide units.

MMP: Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and are capable of degrading extracellular matrix proteins.

HPSE2: Heparanase (HPSE) degrades heparan sulfate (HS), while heparanase 2 (HPSE2), a homolog of HPSE, lacks HS-degrading activity and acts as a competitive binder with HPSE for HS.

RT-qPCR: Reverse transcription quantitative polymerase chain reaction.

Results & discussion

In order to expand the scope to generate stereochemically complex small-molecules, novel isosteviol-derived triazole conjugates bearing diverse functionalities were designed and further explored. The key triazole conjugates type **1** and **2**, including four new derivatives **1e–f**, **2e–f** with aliphatic alanine and leucine residues, were synthesized by following the general procedures previously described [13]. The ketone group and C-15 position of conjugates **1a–f** opened the door to a wide variety of further modifications of these small molecules while type **2** conjugates have tertiary hydroxyl group, which possibly could be involved in further transformations. Although the extremely hindered tertiary hydroxyl group of derivatives **2** did not allow us to involve it in an acylation reaction to further modify these compounds, it can be easily eliminated to afford a next generation of unsaturated triazole conjugates **3a–f** (Figure 3).

In order to overcome the obstacles of side products formation from standard TFA, SOCl_2 and DCC-mediated conditions [21–24] for the dehydration reaction of derivatives **2a–f** and their poor solubility in organic solvents such as CH_3CN , THF and toluene, we explored mild heating of **2a–f** in concentrated formic acid in absence of any additives that provided derivatives **3a–f** with good yields (Table 1). No significant side products were observed under this optimized condition.

Since the triazole ring is considered to be a mimic of the amide unit [22,24], the novel derivatives **3a–f** can be categorized as Michael-type acceptors. The **Michael acceptors**, also known as electrophilic compounds, are characterized as α,β -unsaturated compounds with electron-withdrawing substituents such as ketone, ester or amide units [23], which has been proven to attribute to their anticancer activities. Moreover, numerous biologically active natural compounds belong to these large group of electrophilic natural products [25].

The anticancer study of newly synthesized α,β -unsaturated derivatives **3a–f** revealed moderate improvements compared with conjugates **2a–f** (Table 2). ABAE (adult bovine aortic endothelial) cells were used as normal control cells here and no significant effects were observed in general.

Next, we recognized that dehydroisosteviol conjugates **3a–f** are attractive templates for further derivatization to achieve skeletal diversity and stereochemical complexity. It has been shown that chemical transformations of the isosteviol core can be frequently performed in a stereoselective manner in the absence of any enantioselective catalysts or chiral auxiliaries [16,21]. The high stereoselectivity of those reactions can be attributed to the unique space structure of the rigid isosteviol skeleton with several chiral centers.

Figure 1. Isosteviol and selected known heterocyclic derivatives as diverse templates suitable for small-molecule library discovery.

Figure 2. Synthetic routes towards diverse isosteviol triazole conjugates.

Therefore, we decided to involve the unsaturated derivative **3a** in an epoxidation reaction with *m*-CPBA to demonstrate the potency of conjugates **3a–f** to access stereochemical diversity. Treating the compound **3a** with a slight excess of *m*-CPBA under standard conditions [26] afforded epoxidation product **4a** within several hours. The epoxide **4a** was isolated after column chromatography as a colorless caramel in good yield. Employment of the nuclear Overhauser effect spectroscopy (NOESY) established the configuration of the oxirane ring in the conjugate **4a** (Figure 4).

In the NOESY experiment, selective irradiation of H-C15 (3.72 ppm) demonstrated its strong correlation with 20-CH₃ group (0.74 ppm), which unambiguously indicated the β -orientation of the oxirane ring in compound **4a** (Figure 3). The same correlation was clearly supported by 2D NOESY experiment (see supporting materials). The stereoselective formation of the epoxide **4a** can be rationalized by attacking the double

bond of **3a** from the less sterically hindered side. The novel type of the epoxide **4a** with unique stereochemical structure opens numerous possibilities for further derivatizations employing versatile epoxide chemistry.

Recent studies have revealed several potential mechanisms of action of isosteviol derivatives for anticancer activity [11,27]. From our gene microarray data from breast cancer patient tissues, *MMP11* was found to be upregulated while *HPSE2* was downregulated in breast cancer compared with normal control [28,29]. As shown in Figure 5, the expression of *MMP11* in isosteviol compounds **1a** and **3a** treated MDA231 breast cancer cells were downregulated compared with the expression from the no drug control. On the other hand, the levels of *HPSE2* in all drug treated MDA231 breast cancer cells were elevated compared with that in no drug control. The RT-qPCR results for these samples elucidated the mechanism of these isosteviol compounds to prevent cancer cell proliferation.

Figure 3. Unsaturated isosteviol-derived triazole conjugates.

Table 1. Optimization of the dehydration reaction.

Conjugate 2	Conditions	Yield of 3 (%)
R = -C ₃ H ₆ Ph (2a)	TFA/THF/60°C/24 h	42% [†]
(2a)	TFA/THF/60°C/48 h	60% [‡]
(2a)	HCOOH/60°C/8 h	78% [‡]
R = -CH ₂ C(O)-Leu (2f)	TFA/(THF or CH ₃ CN)/60°C/48h	10% [†]
(2f)	HCOOH/60°C/8 h	65% [‡]

[†]Estimated by ¹H NMR of crude reaction mixture.[‡]Isolated yield after flash column chromatography.

Experimental

¹H and ¹³C NMR spectra were recorded on an Oxford Activated Shield NMR instrument (Varian, Inc.) operating at 400 MHz for ¹H, and 100 MHz for ¹³C using CD₃OD, DMSO-d₆ or CDCl₃ as the solvents. Melting points were determined on a Fisher-Johns melting point apparatus. All chemicals were purchased from Sigma Aldrich or Acros and were used as received, unless stated otherwise. High resolution mass spectra was acquired on a Thermo Scientific Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer with electro spray ionization (ESI) mode. Reactions were carried out in oven-dried glassware under nitrogen atmosphere, unless otherwise noted. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and visualized by UV and phosphomolybdic acid (PMA) staining. Flash column chromatography was performed on E. Merck silica gel 60 (40–63 mm).

Yields refer to chromatographically and spectroscopically pure compounds. Compounds **1a–d** and **2a–d** were reported in our previous work [13].

The following solvent systems were used for preparative column chromatography: **1e** and **1f** MeOH (from 10 to 20%) in DCM; **2e** and **2f** MeOH (from 10 to 20%) in DCM; **3a** Hexane:EtOAc = 3:1; **3b** Hexane:EtOAc = 3:1; **3c**–Hexane:EtOAc = 3:1; **3d**–MeOH 5% in DCM; **3e–f** MeOH (from 5 to 10%) in DCM; **4a** Hexane:EtOAc = 3:1.

General procedure for the dehydration reaction

The corresponding hydroxyl derivative **2a–f** (0.04 mmol) was dissolved in 1 ml of concentrated formic acid (98%) and stirred at 60°C for 16 h. Reaction mixture was cooled down to room temperature, diluted with methanol and all volatiles were rotaevaporated.

Table 2. Cytotoxic activities of isosteviol derivatives against human cancer cell lines.

Compound	IC ₅₀ (μM)	
	MDA-231 [†] (breast)	HL-60 [‡] (leukemia)
2a	39.2 ± 5.23	28.8 ± 0.63
2b	38.1 ± 1.82	35.7 ± 1.08
2c	50.1 ± 3.08	46.8 ± 1.47
2d	>100	>100
2e	>100	>100
2f	>100	>100
3a	41.9 ± 0.69	25.6 ± 0.51
3b	35.3 ± 2.29	29.0 ± 0.58
3c	47.4 ± 0.78	31.5 ± 0.60
3d	52.3 ± 2.18	51.1 ± 0.68
3e	>100	>100
3f	52.9 ± 1.06	51.3 ± 0.98

± values are standard deviation.

[†]Cell viability was analyzed by the MTT assay. All measurements were performed in triplicate. Data were represented as mean ± standard deviation.[‡]Cell viability was analyzed by the CellTiter-Glo® Luminescent Cell Viability Assay. All measurements were performed in triplicate. Data were represented as mean.

Figure 4. Synthesis of enantiopure epoxide **4a** and its NOESY correlations. (A) ^1H NMR spectra of compound **4a** (400 MHz, CDCl_3 , 20°C). (B) 1D NOESY spectra; irradiated proton is indicated with arrow.

The pure product **3a–f** was obtained after silica gel flash column chromatography.

(2S)-2-(2-(4-(((4R,6aR,9S,11bS)-4,9,11b-Trimethyl-8-oxotetradecahydro-6a,9-methanocyclohepta[a]naphthalene-4-carbonyl)oxy)methyl)-1H-1,2,3-triazol-1-yl)acetamido)propanoic acid (1e)

White solid (84%). ^1H NMR (CDCl_3 , 300 MHz, ppm): 7.87 (s, 1H), 6.93 (d, $J = 6.9$ Hz, 1H), 5.19 (dd, $J = 45.0$, 12.6 Hz, 2H), 5.09 (dd, $J = 84.1$, 16.2 Hz, 2H), 4.52 (p, $J = 7.2$ Hz, 1H), 2.74 (dd, $J = 19.1$, 3.6 Hz, 1H), 2.25–2.17 (m, 1H), 1.92–1.50 (m, 10H), 1.46 (d, $J = 7.3$ Hz, 3H), 1.43–1.23 (m, 3H), 1.20 (s, 3H), 1.17–0.98 (m, 4H), 0.97 (s, 3H), 0.84 (td, $J = 13.2$, 4.2 Hz, 1H), 0.34 (s, 3H). ^{13}C NMR (CDCl_3 , 75 MHz, ppm): δ 195.4, 176.9, 164.6, 142.9, 126.0, 57.3, 56.8, 54.4, 54.4, 54.0, 52.7, 49.1, 49.1, 48.5, 48.1, 43.9, 41.2, 39.7, 39.7, 39.6, 37.9, 37.8, 37.6, 30.9, 29.2, 21.5, 20.1, 19.7, 19.0, 17.6, 13.1; HRMS (ESI $^+$) calcd for $\text{C}_{28}\text{H}_{40}\text{N}_4\text{NaO}_6$ $[\text{M}+\text{Na}]^+$ 551.2846, found: 551.2841. MP 117–119°C.

(2S)-4-Methyl-2-(2-(4-(((4R,6aR,9S,11bS)-4,9,11b-trimethyl-8-oxotetradecahydro-6a,9-methanocyclohepta[a]naphthalene-4-

carbonyl)oxy)methyl)-1H-1,2,3-triazol-1-yl)acetamido)pentanoic acid (1f)

White solid (86%). ^1H NMR (CDCl_3 , 300 MHz, ppm): 7.90 (s, 1H), 7.02–6.92 (m, 1H), 5.19 (dd, $J = 42.7$, 12.6 Hz, 2H), 5.12 (dd, $J = 87.9$, 16.2 Hz, 2H), 4.62–4.47 (m, 1H), 2.68 (dd, $J = 19.0$, 3.5 Hz, 1H), 2.18 (d, $J = 13.6$ Hz, 1H), 1.96–1.24 (m, 19H), 1.19 (s, 3H), 1.16–0.97 (m, 4H), 0.97 (s, 3H), 0.96 (m, 6H), 0.83 (td, $J = 13.2$, 4.3 Hz, 1H), 0.34 (s, 3H). ^{13}C NMR (CDCl_3 , 75 MHz, ppm): 227.1, 176.8, 174.1, 164.8, 142.8, 126.0, 57.3, 56.8, 54.4, 54.0, 52.6, 51.3, 49.0, 48.0, 43.8, 41.2, 40.8, 39.7, 39.6, 37.9, 37.7, 37.5, 29.2, 24.9, 22.8, 21.8, 21.4, 20.1, 19.7, 19.0, 13.0. HRMS (ESI $^+$) calcd for $\text{C}_{31}\text{H}_{47}\text{N}_4\text{O}_6$ $[\text{M}+\text{H}]^+$ 571.3490, found: 571.3505. MP 123–125°C.

(2S)-2-(2-(4-(((4R,6aR,8R,9S,11bS)-8-Hydroxy-4-(methoxycarbonyl)-4,9,11b-trimethyltetradecahydro-6a,9-methanocyclohepta[a]naphthalen-8-yl)-1H-1,2,3-triazol-1-yl)acetamido)propanoic acid (2e)

White solid (75%). ^1H NMR ($\text{DMSO}-d_6$, 300 MHz, ppm): 12.68 (s, 1H), 8.66 (d, $J = 7.3$ Hz, 1H), 7.71 (s, 1H), 5.09 (s, 2H), 5.03 (s, 1H), 4.24 (p, $J = 7.2$ Hz, 1H), 3.56 (s, 3H), 2.29–2.09 (m, 2H), 2.06 (d,

Figure 5. Validation of expression of HPSE2 and MMP11 using RT-qPCR. MDA231 cancer cells were treated with **1a** and **3a** for 48 h at the concentration of 40 μ M. Results were shown as mean $\pm$ SEM from triplicates ($n = 3$).

$J = 13.0$ Hz, 1H), 1.97–1.67 (m, 7H), 1.59–1.48 (m, 2H), 1.41–1.33 (m, 2H), 1.31 (d, $J = 7.3$ Hz, 1H), 1.23–1.16 (m, 1H), 1.13 (s, 3H), 1.12–0.98 (m, 3H), 0.98–0.80 (m, 2H), 0.69 (s, 3H), 0.34 (s, 3H). ^{13}C NMR (DMSO- d_6 , 75 MHz, ppm): 177.6, 174.1, 165.7, 156.8, 123.8, 80.8, 56.7, 55.8, 54.5, 51.7, 51.5, 49.8, 48.2, 43.8, 45.7, 43.6, 41.5, 41.3, 38.0, 37.9, 36.6, 28.9, 22.9, 22.0, 20.8, 19.1, 17.8, 13.4. HRMS (ESI $^+$) calcd for $\text{C}_{28}\text{H}_{42}\text{N}_4\text{O}_6$ [M+H] $^+$ 531.3177, found: 531.3168. MP 256–258°C.

(2*S*)-2-(2-(4-((4*R*,6*aR*,8*R*,9*S*,11*bS*)-8-Hydroxy-4-(methoxycarbonyl)-4,9,11*b*-trimethyltetradecahydro-6*a*,9-methanocyclohepta[*a*]naphthalen-8-yl)-1*H*-1,2,3-triazol-1-yl)acetamido)-4-methylpentanoic acid (**2f**)

White solid (85%). ^1H NMR (CD_3OD , 300 MHz, ppm): 7.74 (s, 1H), 5.23–5.09 (m, 2H), 4.51–4.38 (m, 1H), 3.61 (s, 3H), 2.34 (dd, $J = 14.3, 2.4$ Hz, 1H), 2.26–2.17 (m, 1H), 2.15 (d, $J = 13.4$ Hz, 1H), 2.06–1.52 (m, 15H), 1.51–1.20 (m, 4H), 1.17 (s, 3H), 1.16–0.99 (m, 5H), 0.97 (d, $J = 5.9$ Hz, 3H), 0.92 (d, $J = 5.9$ Hz, 3H), 0.78 (s, 3H), 0.44 (s, 3H). ^{13}C NMR (CD_3OD , 75 MHz, ppm): 178.3, 174.1, 166.6, 156.9, 123.3, 81.0, 57.0, 55.9, 54.5, 51.4, 50.9, 50.2, 49.5, 45.4, 43.6, 41.4, 40.7, 40.3, 39.7, 37.8, 37.7, 36.2, 27.8, 24.6, 21.9, 21.6, 20.5, 20.4, 18.6, 12.4. HRMS (ESI $^+$) calcd for $\text{C}_{31}\text{H}_{49}\text{N}_4\text{O}_6$ [M+H] $^+$ 573.3647, found: 573.3663. MP 228–229°C.

(4*R*,6*aR*,9*S*,11*bS*)-Methyl-4,9,11*b*-trimethyl-8-(1-(3-phenylpropyl)-1*H*-1,2,3-triazol-4-yl)-1,2,3,4,4*a*,5,6,9,10,11,11*a*,11*b*-dodecahydro-

6a,9-methanocyclohepta[a]naphthalene-4-carboxylate (3a)

Colorless caramel (78%). $^1\text{H-NMR}$ (CDCl_3 , 400 MHz, ppm): δ 7.34 (s, 1H), 7.33–7.15 (m, 5H), 6.32 (s, 1H), 4.32 (t, J = 7.1 Hz, 2H), 3.63 (s, 3H), 2.67 (t, J = 7.5 Hz, 2H), 2.25 (p, J = 7.2 Hz, 2H), 2.16 (d, J = 13.2 Hz, 1H), 1.92–1.55 (isosteviol skeleton, 10H), 1.44–0.77 (isosteviol skeleton, 9H), 1.25 (s, 3H), 1.18 (s, 3H), 0.58 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz, ppm): δ 178.3, 143.9, 140.3, 136.1, 133.7, 128.7, 128.6, 126.5, 119.3, 61.4, 57.1, 52.6, 51.3, 49.5, 47.3, 45.3, 44.0, 39.7, 38.3, 37.8, 37.4, 32.9, 32.7, 32.1, 31.8, 29.8, 29.0, 24.3, 21.8, 20.7, 19.4, 14.0. HRMS (ESI^+) calcd. for $\text{C}_{32}\text{H}_{44}\text{N}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 502.3434, found 502.3422.

(4R,6aR,9S,11bS)-Methyl-4,9,11b-trimethyl-8-(1-phenyl-1H-1,2,3-triazol-4-yl)-1,2,3,4,4a,5,6,9,10,11,11a,11b-dodecahydro-6a,9-methanocyclohepta[a]naphthalene-4-carboxylate (3b)

Colorless caramel (85%). $^1\text{H-NMR}$ (CDCl_3 , 400 MHz, ppm): δ 7.82 (s, 1H), 7.73 (d, J = 7.7 Hz, 2H), 7.52 (t, J = 7.8 Hz, 2H), 7.42 (t, J = 7.4 Hz, 1H), 6.42 (s, 1H), 3.64 (s, 3H), 2.21–2.12 (m, 1H), 1.95–1.50 (m, 11H), 1.45–1.25 (m, 4H), 1.25 (s, 3H), 1.19 (s, 3H), 1.16–0.79 (m, 5H), 0.60 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz, ppm): δ 178.1, 144.5, 137.1, 135.7, 134.4, 129.7, 128.5, 120.4, 117.1, 110.0, 61.2, 56.9, 52.5, 51.2, 47.3, 45.2, 43.9, 39.5, 38.2, 37.7, 37.2, 32.7, 28.9, 24.1, 21.6, 20.6, 19.2, 13.9. HRMS (ESI^+) calcd. for $\text{C}_{29}\text{H}_{38}\text{N}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 460.2964, found 460.2956.

(4R,6aR,9S,11bS)-Methyl-8-(1-((R)-1-methoxy-1-oxo-3-phenylpropan-2-yl)-1H-1,2,3-triazol-4-yl)-4,9,11b-trimethyl-1,2,3,4,4a,5,6,9,10,11,11a,11b-dodecahydro-6a,9-methanocyclohepta[a]naphthalene-4-carboxylate (3c)

Colorless caramel (86%). $^1\text{H-NMR}$ (CDCl_3 , 400 MHz, ppm): δ 7.38 (s, 1H), 7.29–7.17 (m, 3H), 7.06–6.97 (m, 2H), 6.29 (s, 1H), 5.54 (t, J = 7.4 Hz, 1H), 3.75 (s, 3H), 3.64 (s, 3H), 3.54–3.43 (m, 2H), 2.16 (dd, J = 13.1, 3.3 Hz, 1H), 1.94–1.20 (m, 15H), 1.18 (s, 3H), 1.09 (s, 3H), 1.06–0.77 (m, 4H), 0.57 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz, ppm): δ 178.1, 168.7, 143.6, 135.8, 134.8, 133.9, 129.0, 128.7, 127.5, 119.3, 63.8, 61.1, 56.9, 53.0, 52.5, 51.2, 47.2, 45.1, 43.9, 39.5, 39.1, 38.2, 37.7, 37.2, 32.7, 29.7, 28.8, 24.0, 21.6, 20.5, 19.2, 13.9. HRMS (ESI^+) calcd. for $\text{C}_{33}\text{H}_{44}\text{N}_3\text{O}_4$ $[\text{M}+\text{H}]^+$ 546.3332, found 546.3320.

(2R)-2-(2-(4-((4R,6aR,9S,11bS)-4-(Methoxycarbonyl)-4,9,11b-trimethyl-1,2,3,4,4a,5,6,9,10,11,11a,11b-dodecahydro-6a,9-methanocyclohepta[a]naphthalen-**8-yl)-1H-1,2,3-triazol-1-yl)acetamido)-3-phenylpropanoic acid (3d)**

Off-white solid (67%). $^1\text{H-NMR}$ (CD_3OD , 400 MHz, ppm): δ 7.80 (s, 1H), 7.35–7.23 (m, 2H), 7.22–7.17 (m, 3H), 6.27 (s, 1H), 5.25–5.00 (m, 2H), 4.70 (dd, J = 8.5, 5.0 Hz, 1H), 3.63 (s, 3H), 3.24 (dd, J = 13.9, 4.9 Hz, 1H), 2.99 (dd, J = 13.9, 8.7 Hz, 1H), 2.18–2.08 (m, 1H), 1.94–1.55 (m, 8H), 1.46–1.25 (m, 7H), 1.18 (s, 3H), 1.15 (s, 3H), 1.13–0.85 (m, 5H), 0.60 (s, 3H). $^{13}\text{C-NMR}$ (CD_3OD , 100 MHz, ppm): δ 178.2, 166.2, 143.2, 136.7, 135.8, 133.6, 131.0, 128.9, 128.4, 128.1, 126.5, 121.9, 67.7, 61.1, 56.8, 52.4, 51.4, 50.3, 44.9, 43.6, 39.3, 38.8, 37.7, 37.4, 37.1, 36.9, 32.2, 30.2, 28.7, 27.9, 23.5, 23.0, 22.6, 21.4, 20.3, 18.9, 13.0, 13.0, 10.0. HRMS (ESI^+) calcd. for $\text{C}_{34}\text{H}_{45}\text{N}_4\text{O}_5$ $[\text{M}+\text{H}]^+$ 589.3390, found 589.3401. MP 115–120°C.

(2R)-2-(2-(4-((4R,6aR,9S,11bS)-4-(Methoxycarbonyl)-4,9,11b-trimethyl-1,2,3,4,4a,5,6,9,10,11,11a,11b-dodecahydro-6a,9-methanocyclohepta[a]naphthalen-8-yl)-1H-1,2,3-triazol-1-yl)acetamido)propanoic acid (3e)

Off-white solid (73%). $^1\text{H-NMR}$ (CD_3OD , 400 MHz, ppm): δ 7.94 (s, 1H), 6.29 (s, 1H), 5.19–5.15 (m, 2H), 4.41 (q, J = 7.1 Hz, 2H), 3.63 (s, 3H), 2.17–2.09 (m, 1H), 1.97–1.54 (m, 9H), 1.43 (d, J = 7.2 Hz, 3H), 1.40–1.20 (m, 7H), 1.18 (s, 3H), 1.17 (s, 3H), 1.14–0.83 (m, 4H), 0.60 (s, 3H). $^{13}\text{C-NMR}$ (CD_3OD , 100 MHz, ppm): δ 179.6, 167.6, 144.6, 137.2, 135.0, 123.5, 69.1, 62.5, 58.2, 53.8, 52.8, 51.7, 49.0, 46.3, 45.0, 40.7, 39.1, 38.8, 38.5, 33.6, 30.1, 29.3, 24.5, 22.8, 21.7, 20.3, 17.7, 14.4. HRMS (ESI^+) calcd. for $\text{C}_{28}\text{H}_{41}\text{N}_4\text{O}_5$ $[\text{M}+\text{H}]^+$ 513.3077, found 513.3071. MP 189–190°C.

(2R)-2-(2-(4-((4R,6aR,9S,11bS)-4-(Methoxycarbonyl)-4,9,11b-trimethyl-1,2,3,4,4a,5,6,9,10,11,11a,11b-dodecahydro-6a,9-methanocyclohepta[a]naphthalen-8-yl)-1H-1,2,3-triazol-1-yl)acetamido)-4-methylpentanoic acid (3f)

White needles (65%). $^1\text{H-NMR}$ (CD_3OD , 400 MHz, ppm): δ 7.93 (s, 1H), 6.29 (s, 1H), 5.37–5.03 (m, 2H), 4.45 (dd, J = 8.7, 5.8 Hz, 1H), 3.63 (s, 3H), 2.16–2.07 (m, 1H), 1.96–1.50 (m, 13H), 1.18 (s, 3H), 1.17 (s, 3H), 1.13–1.01 (m, 3H), 0.95 (dd, J = 17.7, 6.1 Hz, 6H), 0.60 (s, 3H). $^{13}\text{C-NMR}$ (CD_3OD , 100 MHz, ppm): δ 179.6, 175.6, 167.9, 144.6, 137.2, 135.0, 123.5, 62.5, 58.2, 53.8, 52.8, 52.4, 51.7, 46.3, 45.0, 41.6, 40.7, 39.1, 38.8, 38.5, 33.6, 29.3, 26.1, 24.4, 23.4, 22.8, 21.8, 21.7, 20.3, 14.4. HRMS (ESI^+) calcd. for $\text{C}_{31}\text{H}_{47}\text{N}_4\text{O}_5$ $[\text{M}+\text{H}]^+$ 555.3546, found 555.3560. MP 127–130°C.

48 h and then incubated with 100 μ l of 0.5 mg/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution for 4 h. The supernatant was discarded and DMSO was added to each well. Absorbance at 570 nm was measured using a SpectraMax M2 reader (Molecular Devices, Sunnyvale, USA). The number of viable cells in the control group was assigned a relative value of 100%.

Cell titer Glo assay

The leukemia cell line HL-60 was cultured in DMEM medium, 10% FBS was supplied to the culture system. For the growth assays, HL-60 was plated in 96-well plates at 10,000 cells/well. Cells were treated with isosteviol triazole compounds at various concentrations. On Day 2, cells were lysed with CellTiter-Glo[®] Luminescent Cell Viability Assay reagent (Promega) following manufacture's instruction and luminescence was read using the PerkinElmer Victor³ V plate reader (PerkinElmer, MA, USA). Percent cell growth was calculated relative to control cells.

Real-time RT-qPCR validation

cDNA was generated using SuperScript[®] VILO[™] MasterMix (Invitrogen). All primers required were designed using Primer Premiere 6 software, and purchased from Integrated DNA Technologies (IDT). The real-time RT-qPCR reactions were prepared using SYBR[®] Select Master Mix (Life Technologies), and performed using BioRad CFX96 Real-Time PCR Detection System. The following conditions were used: 95°C for 2 min, 40 cycles of 95°C for 10 s and 60°C for 1 min. Fold change of gene expression was calculated with the $2^{-\Delta\Delta C_T}$ method, using β -actin as the house keeping gene.

Conclusion

In conclusion, we designed an efficient synthetic methodology for generating novel structurally complex isosteviol-derived triazole conjugates. The series of α,β -unsaturated derivatives have been shown to display promising anticancer activities against different types of cancer cell lines. We recognized that they are attractive templates for further derivatization to achieve skeletal diversity and stereochemical complex-

ity. The potential of these compounds as templates for further stereoselective modification was demonstrated here using epoxidation reaction as an example. Taken together, this study explored isosteviol-derived triazole conjugates to expand the structural diversity of biologically active compounds, thereby facilitating the development of drug leads derived from natural products for the treatment of cancer.

Future perspective

In view of the dehydroisosteviol as an attractive template for further derivatization to achieve skeletal diversity and stereochemical complexity, our future studies will be focused on investigating the possible transformation from these dehydroisosteviol compounds (some of the examples such as olefin metathesis are depicted in Figure 6). Moreover, animal studies have already been initiated to evaluate their anticancer effects. In addition, it is necessary to address the advantages of these natural product derived drugs over traditional therapeutics by comparing their physicochemical and physiological properties, such as protease stability, cell membrane permeability and immunogenicity. The prospective small molecules bearing α,β -unsaturated isosteviol skeleton not only have promising biological activity as many other isosteviol derivatives, but also represent ideal structural templates for future medicinal chemistry application due to abundant functional groups stereospecifically linked at different position of carbocyclic diterpenoid platform.

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Executive summary

- An efficient synthetic approach towards α,β -unsaturated isosteviol conjugates has been developed. The method involves versatile 'click' chemistry technique and optimized mild dehydration procedure.
- The designed novel isosteviol conjugates were evaluated on anticancer activities.
- Potency of these α,β -unsaturated isosteviol compounds as reactive electrophiles was examined through epoxidative reaction. It has also been shown that further transformation of the double bond of these unsaturated conjugates could proceed in stereoselective fashion.

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