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LAT Transport Inhibitors from *Pittosporum venulosum* Identified by NMR Fingerprint Analysis

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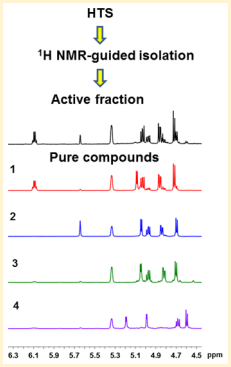
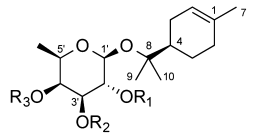
S *Supporting Information*

ABSTRACT: ^{1}H NMR fingerprints were used as the guiding principle for the isolation of minor compounds related to the L-type amino acid transporter inhibitors venulosides A (**1**) and B (**2**). Two new monoterpene glycosides, namely, venulosides C (**3**) and D (**4**), were isolated from a Queensland collection of the plant *Pittosporum venulosum*. Compounds **3** and **4** were found to inhibit L-leucine transport in LNCaP cells with IC_{50} values of 11.47 and 39.73 μM, respectively. The venulosides are the first reported natural product inhibitors of leucine transport in prostate cancer cells, and the isolation of the minor compounds provides some early SAR information.



L-Type amino acid transporters (LATs) mediate the transport of large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, and tryptophan across cell membranes. As essential nutrient transporters, the LAT family of membrane-spanning proteins are expressed in both normal and tumor cells, and up-regulation of LAT expression has been found in a number of human tumors,[1−4] including prostate cancer.[5,6] Moreover, it has been demonstrated that inhibition of the LAT transporters suppresses the activity of mammalian target of rapamycin complex 1 (mTORC1) and M-phase cell cycle in castration-resistant prostate cancer, therefore providing a novel therapeutic target in the pathways responsible for tumor cellular growth and proliferation.[7] With the aim of finding new natural product inhibitors of LAT1 (SLC7A5) and LAT3 (SLC43A1), we have recently undertaken a high-throughput screen (HTS) designed to identify LAT-specific small-molecule inhibitors, resulting in the disclosure of the structures and LAT-inhibitory activity of venulosides A (ESK246) (**1**) and B (ESK242) (**2**) sourced from a Queensland collection of the plant *Pittosporum venulosum*.[8] This paper reports on the structures and activity of two minor metabolites, namely, venulosides C (**3**) and D (**4**), sourced from the same plant collection. We demonstrate the use of a ^{1}H NMR fingerprint to comprehensively detect metabolites in the fraction and to guide isolation of the minor compounds. Combination of spectra of the individual compounds shows that the NMR fingerprint is a powerful approach for metabolomics identification.

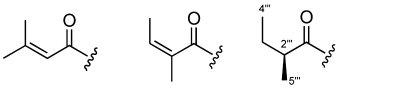


1 R_1 = Ac, R_2 = senecioyl, R_3 = H

2 R_1 = Ac, R_2 = angeloyl, R_3 = H

3 R_1 = Ac, R_2 = 2‴-methybutyryl, R_3 = H

4 R_1 = Ac, R_2 = H, R_3 = 2‴-methylbutyryl



senecioyl angeloyl (2‴*S*)-methylbutyryl

RESULTS AND DISCUSSION

The HTS was designed to monitor leucine uptake using an androgen-responsive prostate cancer cell line, LNCaP, and a subset of the Nature Bank fraction library containing 4488 plant-sourced fractions was screened for activity. This fraction library was prepared by injecting plant extract equivalent to 50 mg of freeze-dried biota onto the analytical HPLC and collecting 11 lead-like enhanced (LLE) fractions.[9] In the LAT

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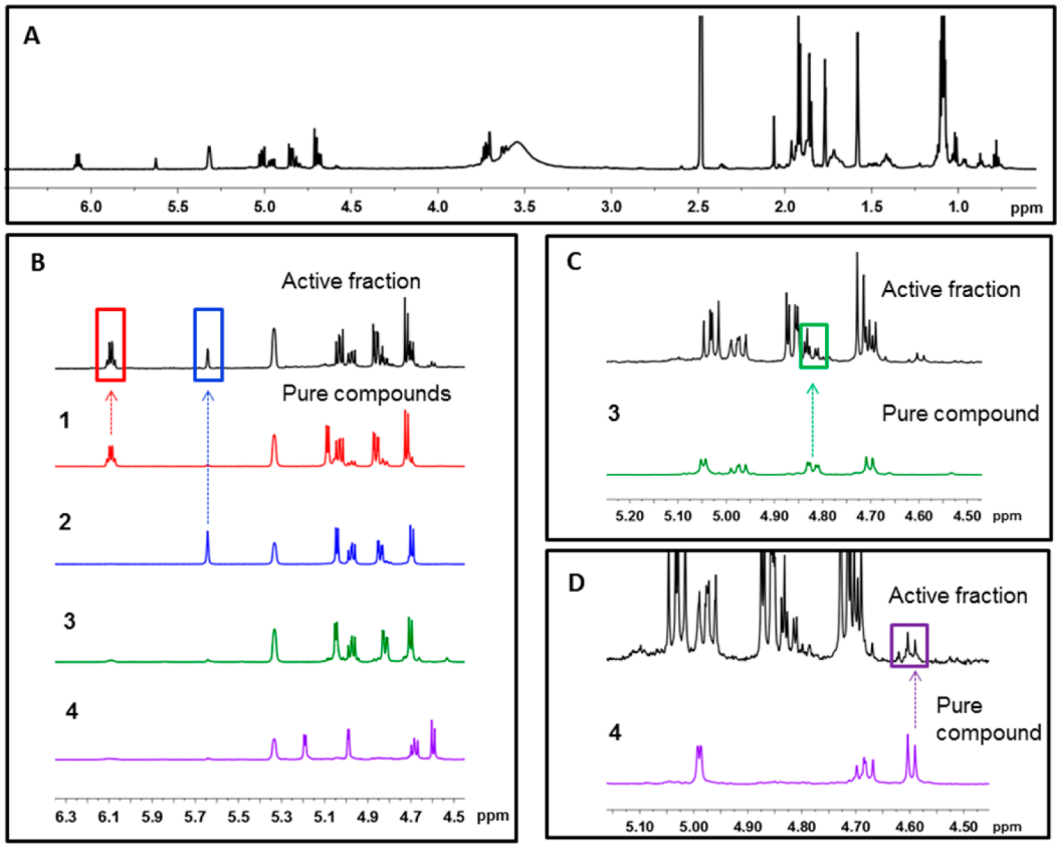

Figure 1. ^{1}H NMR fingerprint analysis. (A) Complete NMR fingerprint of the HTS active fraction. (B) Comparison of the active fraction NMR fingerprint with those of the spectra acquired for the pure compounds **1**–**4**. The spectra are expanded in the region δ_H 6.35 to 4.35, and the resonances used to exemplify the presence of venuloside A (**1**) are highlighted with a red rectangle and that of venuloside B (**2**) with a blue rectangle. (C) Comparison of the active fraction NMR fingerprint with that of the spectrum acquired for venuloside C (**3**). The spectra are expanded in the region δ_H 5.25 to 4.45, and the resonance used to exemplify the presence of **3** in the active fraction is highlighted with a green rectangle. (D) Comparison of the active fraction NMR fingerprint with that of the spectrum acquired for venuloside D (**4**). The spectra are expanded in the region δ_H 5.15 to 4.45, and the resonance used to exemplify the presence of **4** in the active fraction is highlighted with a purple rectangle. All depicted spectra were acquired in DMSO-d_6 at 600 MHz.

screen, LLE fraction 11 sourced from the mixed collection of the plant *P. venulosum* showed potent inhibition of leucine uptake and cell growth, while having no significant effect on the cell morphology. In order to accelerate the bioactive compound isolation process, the fractionation procedure was repeated and small-scale analytical UV-LC-MS and ^{1}H NMR fingerprints were obtained on the active fraction (for a complete metabolic fingerprint, see Supporting Information (SI) Figure S1). The LC-UV-MS information revealed that the constituents had limited UV absorbance and that the ESI mass spectrum contained many fragments (SI, Figure S2). The ^{1}H NMR spectrum, on the other hand, was well resolved and showed the presence of multiple compounds, providing a comprehensive fingerprint of all of the small molecules contained in this fraction (Figure 1A). Recently we reported the use of ^{1}H NMR metabolic fingerprints of a natural product fraction library and its effectiveness in the identification and isolation of new and novel natural products.[10] Herein, we showcase this methodology in the isolation of the minor active constituents of the active HTS fraction.

The comparison of the active fraction fingerprint with that of the pure compounds identified via ^{1}H NMR-guided isolation is shown in Figure 1B, C, and D. In Figure 1B, the two main natural products **1** and **2** are clearly visible in the active fraction NMR spectrum, exemplified by the presence of multiplets at δ_H 6.08 and 5.63 as well as other glycosidic oxymethine resonances. In addition to the two major compounds, ^{1}H NMR data indicated the presence of two other minor metabolites, demonstrated by resonances at δ_H 4.81 (dd, J = 7.1, 3.6 Hz) (Figure 1C) and 4.58 (d, J = 8.1 Hz) (Figure 1D). Minor constituents were also obvious in the aliphatic region of the spectrum with resonances at δ_H 1.03 (d, J = 6.5 Hz), 0.98 (apparent doublet), 0.89 (apparent triplet), and 0.80 (d, J = 7.6 Hz) (SI Figure S3). NMR-guided large-scale isolation on 10 g of dried plant material, targeting the NMR signals for the two minor metabolites, resulted in the purification and identification of two new natural products, namely, venulosides C (**3**) and D (**4**).

Venuloside C (**3**) was isolated as an optically active oil ($[\alpha]^{25}_D$ = +11 *c* 0.1, MeOH) with a molecular formula of $C_{23}H_{38}O_7$ established by HRESIMS and ^{13}C NMR measurements. The ^{1}H NMR data in C_6D_6 (chosen as it provided the best dispersion of the glycosidic oxymethine and the aliphatic signals) showed structural similarities to those of venulosides A (**1**) and B (**2**), with the exception of vinyl ester resonances, which were replaced by resonances for four sp^3-hybridized carbons, comprising two methyls (δ_H 1.09, d, J = 7.0 Hz, δ_C 16.6 and δ_H 0.87, d, J = 7.2 Hz, δ_C 11.5), one diastereotopic methylene (δ_H 1.70, m, δ_H 1.40, m, δ_C 26.94), and one methine (δ_H 2.34 br q, δ_C 41.2) resonance. ^{1}H–^{1}H COSY correlations

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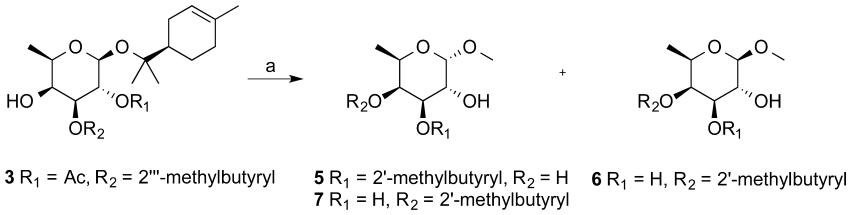
Scheme 1. Hydrolysis of 3[a]

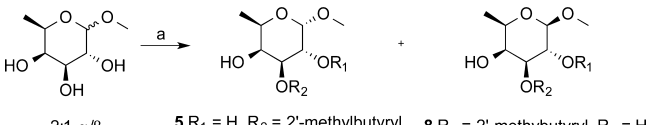


3 R_1 = Ac, R_2 = 2‴-methylbutyryl **5** R_1 = 2′-methylbutyryl, R_2 = H **6** R_1 = H, R_2 = 2′-methylbutyryl
7 R_1 = H, R_2 = 2′-methylbutyryl

[a]Reagents and conditions: (a) 5% HCl, MeOH, 60 °C, 16 h.

placed the five ester-group resonances in a contiguous spin system, and $^1H-^{13}C$ HMBC correlations established the presence of a 2-methylbutyryl group. Substitution of the unsaturated angeloyl and senecioyl groups found in **1** and **2** with the saturated 2-methylbutyryl moiety in **3** was supported by the molecular formula, showing two additional hydrogen atoms and lacking one index of hydrogen deficiency. The remaining NMR data were consistent with the presence of a β-fucopyranoside moiety, an α-terpineol substituent at C-1′, and an acetyl group at C-2′ identical to compounds **1** and **2**, revealing the planar structure of **3** to be α-terpineol 8-*O*-β-(2′-*O*-acetyl-3′-*O*-2‴-methylbutyryl)fucopyranoside. The absolute configuration of venuloside A (**1**) was established via acid hydrolysis and synthesis.[8] On the basis of the co-occurrence of **1** and **3** in the identical plant sample, the absolute configurations of the fucose and the α-terpineol moieties were concluded to be D and 4*R*, respectively. The absolute configuration of the methylbutyryl ester unit at C-2‴ in **3** was determined via an NMR titration experiment, comparing hydrolyzed natural product to synthetic material of known configuration. Hydrolysis of **3** in 5% HCl in MeOH afforded a mixture of methyl 3-*O*-2′-methylbutyryl-α-fucopyranoside (**5**), methyl 3-*O*-2′-methylbutyryl-β-fucopyranoside (**6**), and methyl 4-*O*-2′-methylbutyryl-α-fucopyranoside (**7**) (Scheme 1). The semisynthetic compound **5** was compared to the synthetic material of known methylbutyryl configuration. Treatment of methyl D-fucopyranoside (2:1 α/β) with (2*S*)-methylbutyryl chloride under basic conditions afforded methyl 3-*O*-(2′*S*)-methylbutyryl-α-fucopyranoside (**5**) as well as methyl 2-*O*-(2′*S*)-methylbutyryl-β-fucopyranoside (**8**) and methyl 2-*O*-(2′*S*)-methylbutyryl-α-fucopyranoside (**9**) as the major isomers (Scheme 2). The reaction was repeated with (2*R*)-methylbutyryl chloride to afford the corresponding (2*R*)-C-2′ epimers of compounds **5**, **8**, and **9**.

Scheme 2. Synthesis of Compounds 5, 8, and 9[a]



2:1 α/β **5** R_1 = H, R_2 = 2′-methylbutyryl **9** R_1 = 2′-methybutyryl, R_2 = H **8** R_1 = 2′-methybutyryl, R_2 = H

[a]Reagents and conditions: (a) (2*S*)- or (2*R*)-methylbutyryl chloride, pyridine/CH_2Cl_2, 16 h.

In a ^1H NMR-based titration experiment, a C-2′ epimeric mixture of methyl 3-*O*-2′-methylbutyryl-α-fucopyranoside (**5**) was formed by adding molar equivalents of the (2′*S*)-**5** epimer to (2′*R*)-**5** (Figure 2A). The ^1H NMR spectrum of the epimeric mixture showed a Δ_δ = 0.015 ppm (9.0 Hz) difference between the resonances associated with OH-4 as well as a Δ_δ = 0.005 ppm (3.0 Hz) difference between the resonances associated with H-4′, providing a point of differentiation between the two C-2′ epimers of compound **5**. The addition of the hydrolysis-sourced naturally occurring **5** to the epimeric mixture (Figure 2B) resulted in the enhancement of the resonances associated with the (2′*S*)-**5** epimer, securing the absolute configuration of the C-2′ stereogenic center as *S*. The structure of **3** was therefore concluded to be (4*R*)-α-terpineol 8-*O*-β-D-[2′-*O*-acetyl-3′-*O*-(2‴*S*)-methylbutyryl]fucopyranoside.

Venuloside D (**4**) was isolated as an optically active oil ($[\alpha]^{25}_D$ = +26 *c* 0.1, MeOH) and had a molecular formula identical to that of compound **3**. Comparison of the 1D and 2D NMR data in C_6D_6 revealed that the significant differences between the structures of **3** and **4** were in the substitution of the fucopyranoside ring and, in particular, a shift in the resonances of one of the esterified centers. In the 1D NMR spectra of compound **3** the C-2′ (δ_H 5.57, dd, *J* = 10.5, 8.0, δ_C 69.7) and C-3′ (5.02, dd, *J* = 10.5, 3.3, δ_C 74.2) glycosidic positions that bear the acetyl and methylbutyryl substituents, respectively, were found to have significantly deshielded ^1H NMR shifts compared to the hydroxylated H-4′ (3.63, br s, δ_C 70.3) resonance. In contrast, the NMR spectrum of compound **4** suggested that the methylbutyryl substituent had migrated to the C-4′ position (δ_H 5.13, dd, *J* = 3.6, 1.0, δ_C 72.9), with the C-3′ now bearing the free hydroxy substituent. $^1H-^1H$ COSY and $^1H-^{13}C$ HMBC data confirmed the structural assignment for venuloside D to be α-terpineol 8-*O*-β-(2′-*O*-acetyl-4′-*O*-2‴-methylbutyryl)fucopyranoside. On the basis of the structural homology with venuloside A (**1**) and the close correspondence of chemical shifts of the terpineol, fucose, and methylbutyryl residues with those reported for **3**, the structure of **4** is proposed to be (4*R*)-α-terpineol 8-*O*-β-D-[2′-*O*-acetyl-4′-*O*-(2‴*S*)-methylbutyryl]fucopyranoside.

The venulosides were found to inhibit leucine transport in LNCaP cells with IC_{50} values ranging from 8.12 to 39.73 μM (Table 1). Compounds **1**–**3**, with esterification at the glycosidic C-3′ position, were more potent than the C-4′-esterified **4**. The unsaturated angelic acid ester **2** was less active than the saturated methylbutyryl-containing analogue **3**, indicating that a Michael acceptor is not a requirement for the activity of the series. Venulosides A–D are the first reported natural product inhibitors of leucine transport in prostate cancer cells.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a Jasco P-1020 polarimeter using a 1 dm cell in the solvent indicated. IR spectra were recorded on KBr plates using a Bruker Tensor 27 FTIR instrument. UV spectra were recorded as MeOH solutions on a Jasco V650 UV/vis spectrophotometer. NMR spectra were recorded at 30 °C on a 600 MHz Varian Unity INOVA spectrometer, equipped with either a triple resonance cold probe or a

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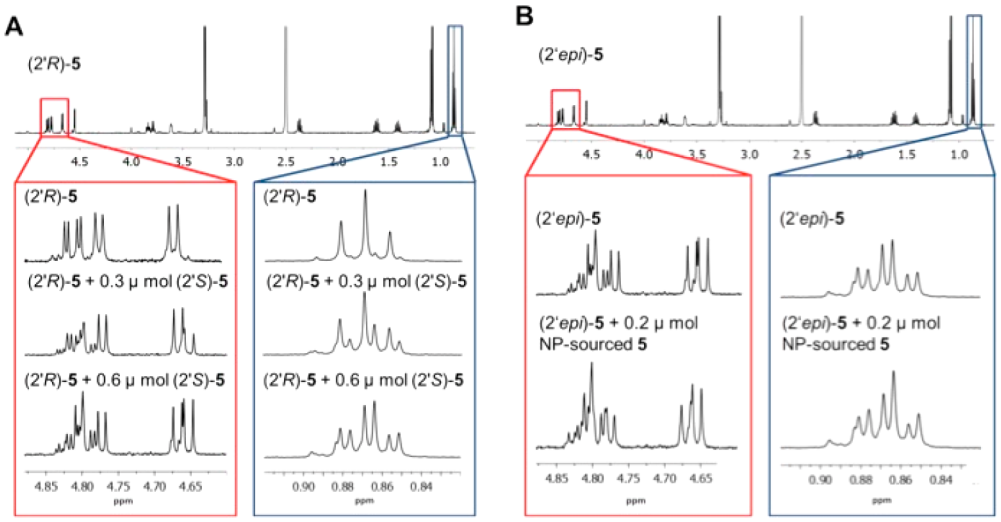

Figure 2. ^{1}H NMR-based titration. (A) Addition of the (2′*S*)-**5** epimer to (2′*R*)-**5**. (B) Addition of the hydrolysis-sourced naturally occurring **5** to the synthetically prepared C-2′ epimeric mixture of **5**. Spectra were acquired in DMSO-$_{d6}$ at 600 MHz.

Table 1. Leucine Transport Inhibitory Activity of Venulosides A–D (1–4)

compound	IC_{50} (μM)[a]
1	8.12 ± 1.2
2	29.17 ± 1.2
3	11.47 ± 1.2
4	39.73 ± 1.5
BCH[b]	4060 ± 1.1

[a]IC_{50} values represent concentration (μM) of compounds that inhibits 50% of L-leucine transport in LNCaP cells. Data show mean ± SEM (n = 3). [b]Positive control, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH).

triple resonance room-temperature probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks for C_6D_6 at δ_H 7.20 and δ_C 128.0 and DMSO-d_6 at δ_H 2.50 and δ_C 39.45. LRESIMS were recorded on a Waters ZQ single-quadrupole ESI spectrometer. HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer. A Waters Oasis HLB (400 mg) extraction cartridge was used to purify the small-scale extracts. Prefractionation was performed on a Phenomenex Onyx Monolithic C_{18} column [100 × 4.6 mm]. A BIOLINE orbital shaker was used for the large-scale extraction of the plant material. Normal-phase flash chromatography was carried out with Merck silica gel 60 (0.040–0.063 mm). A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A Phenomenex Onyx Monolithic C_{18} column [100 × 10 mm] was used for semipreparative HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H_2O was Millipore Milli-Q PF filtered.

Plant Collection, Extraction, and Isolation. The plant *Pittosporum venulosum* (F. Muell) (Nature Bank code 11711.8) was collected in July 1995 from State Forest 144, Mt. Windsor Tableland, Queensland, Australia. The plant was identified by P. I. Forster and S. J. Figg. A voucher specimen (PIF17245) has been lodged with the Queensland Herbarium. A slightly modified isolation procedure from that previously reported[8] was used. *P. venulosum* (10 g) was dried, ground, and sequentially extracted in *n*-hexane (250 mL) to give 480 mg of crude *n*-hexane extract, CH_2Cl_2 (250 mL) to give 470 mg of crude DCM extract, and MeOH (2 × 250 mL) to give 360 mg of crude MeOH extract. The combined DCM and MeOH extracts were subjected to a solvent–solvent partition with the compounds of interest concentrated in the *n*-hexane fraction (117 mg). The combined *n*-hexane extracts (597 mg) was subjected to flash silica oxide chromatography (10 cm × 4 cm) eluting with a gradient from 100% *n*-hexane to 100% EtOAc. The fraction eluting with 8:2 (*n*-hexane/EtOAc) yielded venuloside A (**1**) (135 mg, 1.35% dry weight), while the fraction eluting with 7:3 (*n*-hexane/EtOAc) yielded venuloside B (**2**) (67 mg, 0.67% dry weight). Fractions eluting with 7:3 and 6:4 *n*-hexane/EtOAc containing venulosides C and D (**3** and **4**) were recombined and further purified on a silica oxide flash column (15 cm × 4 cm). Fractions eluting with 85:15 *n*-hexane/EtOAc yielded venuloside C (**3**) (19 mg, 0.19% dry weight), while fractions eluting with 80:20 *n*-hexane/EtOAc yielded venuloside D (**4**) (15 mg, 0.15% dry weight).

Small-Scale NMR Fingerprint Analysis. A single replicate of the LLE fraction 11 was analyzed by ^{1}H NMR spectroscopy as previously described.[10] The sample was dissolved in 200 μL of DMSO-d_6 and run in a 3 mm NMR tube. The standard VnmrJ 3.2 Proton pulse sequence was run with the following parameters: pw = 45°, p1 = 0 μs, d2 = 0 s, d1 = 1 s, at = 1.7 s, sw = 9615 Hz, nt = 128 scans.

*Venuloside C (**3**):* clear oil; $[\alpha]^{25}_D$ = +11 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.59), 234 (3.56) nm; IR (KBr film) 3428, 2933, 1737, 1461, 1373, 1237, 1132, 1060 cm^{-1}; ^{1}H NMR (600 MHz, C_6D_6) δ 5.57 (1H, dd, J = 10.5, 8.0 Hz, H-2′), 5.47 (1H, br s, H-2), 5.02 (1H, dd, J = 10.5, 3.3 Hz, H-3′), 4.43 (1H, d, J = 8.0 Hz, H-1′), 3.63 (1H, br s, H-4′), 3.00 (1H, br q, J = 7.5 Hz, H-5′), 2.34 (1H, br q, J = 7.0 Hz, H-2‴), 2.14 (1H, m, H-3A), 1.98 (1H, m, H-6A), 1.95 (1H, n, H-5A), 1.92 (1H, m, H-3B), 1.89 (1H, m, H-6B), 1.88 (3H, s, H-2″), 1.70 (1H, m, H-3‴A), 1.62 (1H, m, H-4), 1.67 (3H, br s, H-7), 1.40 (1H, m, H-3‴B), 1.33 (1H, m, H-5B), 1.19 (3H, s, H-9), 1.11 (3H, s, H-10), 1.09 (3H, d, J = 7.0 Hz, H-5‴), 0.87 (3H, t, J = 7.2 Hz, H-4‴), 0.86 (3H, d, J = 7.5 Hz, H-6′); ^{13}C NMR (150 MHz, C_6D_6) δ 175.4 (C-1‴), 168.4 (C-1″), 133.6 (C-1), 121.0 (C-2), 95.8 (C-1′), 79.5 (C-8), 74.2 (C-3′), 70.3 (C-4′), 70.1 (C-5′), 69.7 (C-2′), 44.4 (C-4), 41.2 (C-2‴), 31.3 (C-6), 27.03 (C-3), 26.94 (C-3‴), 24.1 (C-5), 23.84 (C-7), 23.56 (C-10), 23.51 (C-9), 20.6 (C-2″), 16.6 (C-5‴), 16.4 (C-6′), 11.5 (C-4‴); HRESIMS m/z [M + Na]$^+$ 449.25216 (calcd for $C_{23}H_{38}O_7Na$, 449.25098).

*Venuloside D (**4**):* clear oil; $[\alpha]^{25}_D$ = +26 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.53), 234 (3.48) nm; IR (KBr film) 3427, 2937, 2973, 2936, 1738, 1370, 1234, 1130, 1069 cm^{-1}; ^{1}H NMR (600 MHz, C_6D_6) δ 5.44 (1H, m, H-2), 5.33 (1H, dd, J = 10.5, 7.8 Hz, H-2′), 5.13 (1H, dd, J = 3.6, 1.0 Hz, H-4′), 4.42 (1H, d, J = 7.8 Hz, H-1′), 3.61 (1H, dd, J = 10.0, 2.8 Hz, H-3′), 3.07 (1H, br q, H-5′), 2.25 (1H, m, H-2‴), 2.11 (1H, m, H-3A), 1.94 (2H, br m, H-6A/H-5A), 1.87 (1H, m, H-3B), 1.86 (1H, m, H-6B), 1.84 (3H, s, H-2″), 1.68 (1H, m, H-3‴A), 1.65 (3H, br s, H-7), 1.61 (1H, m, H-4), 1.34 (1H, m, H-3‴B), 1.29 (1H, m, H-5B), 1.19 (3H, s, H-9), 1.13 (3H, d, J = 6.5 Hz, H-6′), 1.12 (3H, s, H-10), 1.06 (3H, d, J = 7.0 Hz, H-5‴), 0.84 (3H, t,

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J = 7.5 Hz, H-4‴); ^{13}C NMR (150 MHz, C_6D_6) δ ^{13}C NMR (150 MHz, C_6D_6) δ 176.6 (C-1‴), 170.6 (C-1″), 133.6 (C-1), 121.0 (C-2), 95.3 (C-1′), 79.4 (C-8), 73.6 (C-2′), 72.9 (C-4′), 72.4 (C-3′), 69.0 (C-5′), 44.3 (C-4), 41.2 (C-2‴), 31.3 (C-6), 27.1 (C-3), 27.0 (C-3‴), 24.0 (C-5), 23.8 (C-7), 23.7 (C-10), 23.5 (C-9), 20.6 (C-2″), 16.9 (C-5‴), 16.8 (C-6′), 11.6 (C-4‴); HRESIMS m/z [M + Na]$^+$ 449.2514 (calcd for $C_{23}H_{38}O_7Na$, 449.250975).

Acid Hydrolysis. A solution of **3** (15.0 mg, 35.2 μM) in MeOH (2.0 mL) was treated with 5% HCl (100 μL), and the mixture was kept stirring at 60 °C for 24 h. After hydrolysis the solution was evaporated and subjected to semipreparative HPLC. Isocratic HPLC conditions of 90% H_2O/10% MeOH were initially employed for 10 min; then a linear gradient to MeOH was run over 40 min, followed by isocratic conditions of MeOH for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and analyzed by ^{1}H NMR spectroscopy. Fraction 9 yielded **5** (0.2 mg), fraction 16 yielded **6** (0.3 mg), and fraction 19 yielded **7** (0.4 mg). Owing to the low recovery of the hydrolysis products, the semisynthetic derivatives were characterized based on ^{1}H, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and HRESIMS data.

*Methyl 3-O-2′-methylbutyryl-α-fucopyranoside (**5**):* clear oil; ^{1}H NMR (600 MHz, DMSO-d_6) 4.83 (1H, br s, OH-4), 4.79 (1H, dd, J = 10.7, 3.2 Hz, H-3), 4.68 (1H, br s, OH-2), 4.54 (1H, d, J = 3.7 Hz, H-1), 3.82 (1H, m, H-2), 3.79 (1H, q, J = 6.8 Hz, H-5), 3.62 (1H, m, H-4), 3.28 (3H, s, 1-OMe), 2.37 (1H, m, H-2′), 1.60 (1H, m, H-3′A), 1.42 (1H, m, H-3′B), 1.09 (3H, d, J = 6.8 Hz, H-6), 1.07 (3H, d, J = 7.1 Hz, H-5′), 0.86 (3H, t, J = 7.6 Hz, H-4′); HRESIMS m/z [M + Na]$^+$ 285.1316 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

*Methyl 4-O-2′-methylbutyryl-β-fucopyranoside (**6**):* clear oil; ^{1}H NMR (600 MHz, DMSO-d_6) 5.03 (1H, d, J = 4.9 Hz, OH-2), 4.97 (1H, d, J = 5.0 Hz, OH-3), 4.94 (1H, dd, J = 3.9, 0.9 Hz, H-4), 4.07 (1H, d, J = 7.8 Hz, H-1), 3.74 (1H, br q, J = 6.4 Hz, H-5), 3.50 (1H, m, H-3), 3.37 (3H, s, 1-OMe), 3.20 (1H, m, H-2), 2.37 (1H, m, H-2′), 1.59 (1H, m, H-3′A), 1.42 (1H, m, H-3′B), 1.09 (3H, d, J = 7.0 Hz, H-6), 1.00 (3H, d, J = 6.4 Hz, H-5′), 0.88 (3H, t, J = 7.4 Hz, H-4′); HRESIMS m/z [M + Na]$^+$ 285.1313 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

*Methyl 4-O-2′-methylbutyryl-α-fucopyranoside (**7**):* clear oil; ^{1}H NMR (600 MHz, DMSO-d_6) 5.01 (1H, dd, J = 3.5, 1.1 Hz, H-4), 4.83 (1H, d, J = 4.9 Hz, OH-3), 4.68 (1H, d, J = 5.0 Hz, OH-2), 4.57 (1H, d, J = 3.8 Hz, H-1), 3.93 (1H, br q, J = 6.8 Hz, H-5), 3.73 (1H, m, H-3), 3.52 (1H, m, H-2), 3.26 (3H, s, 1-OMe), 2.40 (1H, m, H-2′), 1.61 (1H, m, H-3′A), 1.43 (1H, m, H-3′B), 1.10 (3H, d, J = 7.0 Hz, H-6), 1.97 (3H, d, J = 6.8 Hz, H-5′), 0.88 (3H, t, J = 7.6 Hz, H-4′); HRESIMS m/z [M + Na]$^+$ 285.1317 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

Synthesis of (2′*S*)-Methylbutyryl D-Fucopyranosides. To a stirred solution of methyl D-fucopyranoside (50 mg, 0.28 mmol, 2:1 α/β mixture of anomers) in 1:4 pyridine/CH_2Cl_2 (5 mL) at 0 °C was added (2*S*)-methylbutyryl chloride (20 μL, 0.19 mmol). The reaction mixture was allowed to warm to RT and stirred for 16 h, after which time the mixture was evaporated and the residue was purified by silica gel column chromatography (5:4:1 *n*-hexane/EtOAc/MeOH) to give an impure colorless oil (40 mg). The residue was subjected to purification by semipreparative HPLC. Isocratic HPLC conditions of 90% H_2O/10% MeOH were initially employed for 10 min; then a linear gradient to MeOH was run over 40 min, followed by isocratic conditions of MeOH for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and then analyzed by (+)-LRESIMS and NMR. Fraction 4 yielded (2′*S*)-**8** (3.9 mg), fraction 8 yielded (2′*S*)-**5** (1.9 mg), and fractions 12−17 yielded (2′*S*)-**9** (11.5 mg).

*Methyl 3-O-(2′S)-methylbutyryl-α-fucopyranoside (**5**):* clear oil; $[\alpha]^{25}_D$ = +143 (c 0.1, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) 4.79 (1H, d, J = 5.8 Hz, OH-2), 4.78 (1H, dd, J = 10.9, 3.4 Hz, H-3), 4.64 (1H, d, J = 7.4 Hz, OH-4), 4.53 (1H, d, J = 3.8 Hz, H-1), 3.81 (1H, ddd, J = 10.9, 7.4, 3.8 Hz, H-2), 3.77 (1H, br q, J = 6.8 Hz, H-5), 3.61 (1H, m, H-4), 3.26 (3H, s, 1-OMe), 2.36 (1H, m, H-2′), 1.59 (1H, m, H3′A), 1.40 (1H, m, H3′B), 1.07 (3H, d, J = 6.8 Hz, H-6), 1.08 (3H, d, J = 6.7 Hz, H-5′), 0.86 (3H, t, J = 7.6 Hz, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6) 175.5 (C-1′), 100.0 (C-1), 72.9 (C-3), 68.9 (C-4), 65.5 (C-5), 64.9 (C-2), 54.5 (1-OMe), 40.1 (C-2′), 26.2 (C-3′), 16.0 and 16.1 (C-6 and C-3′), 11.2 (C-4′); HRESIMS m/z [M + Na]$^+$ 285.1318 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

*Methyl 3-O-(2′S)-methylbutyryl-β-fucopyranoside (**8**):* clear oil; $[\alpha]^{25}_D$ = +33 (c 0.1, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) 5.08 (1H, d, J = 5.5 Hz, OH-2), 4.76 (1H, d, J = 6.4 Hz, OH-4), 4.55 (1H, dd, J = 10.1, 3.4 Hz, H-3), 4.10 (1H, d, J = 7.8 Hz, H-1), 3.60 (1H, dq, J = 6.3, 0.6 Hz, H-5), 3.56 (1H, ddd, J = 6.4, 3.4, 0.6 Hz, H-4), 3.49 (1H, ddd, J = 10.1, 7.8, 5.5 Hz, H-2), 3.37 (3H, s, 1-OMe), 2.38 (1H, m, H-2′), 1.60 (1H, m, H3′-A), 1.43 (1H, m, H3′-B), 1.13 (3H, d, J = 6.4 Hz, H-6), 1.08 (3H, d, J = 6.7 Hz, H-5′), 0.86 (3H, t, J = 7.6 Hz, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6) 175.4 (C-1′), 104.0 (C-1), 75.8 (C-3), 69.6 (C-5), 68.3 (C-4), 67.1 (C-2), 55.7 (1-OMe), 39.7 (C-2′), 26.2 (C-3′), 16.3 (C-6), 16.2 (C-3′), 11.2 (C-4′); HRESIMS m/z [M + Na]$^+$ 285.1312 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

*Methyl 2-O-(2′S)-methylbutyryl-α-fucopyranoside (**9**):* clear oil; $[\alpha]^{25}_D$ = +77 (c 0.1, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) 4.78 (1H, dd, J = 10.5, 3.5 Hz, H-2), 4.72 (1H, d, J = 6.9 Hz, OH-3), 4.69 (1H, d, J = 6.9 Hz, OH-4), 4.63 (1H, d, J = 3.5 Hz, H-1), 3.77 (1H, q, J = 6.6 Hz, H-5), 3.74 (1H, m, H-3), 3.53 (1H, m, H-4), 3.21 (3H, s, 1-OMe), 2.34 (1H, m, H-2′), 1.57 (1H, m, H-3′A), 1.40 (1H, m, H-3′B), 1.10 (3H, d, J = 6.6 Hz, H-6), 1.05 (3H, d, J = 7.1 Hz, H-5′), 0.84 (3H, d, J = 7.4 Hz, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6) 175.6 (C-1′), 96.8 (C-1), 71.6 (C-2), 70.6 (C-4), 66.7 (C-3), 65.7 (C-5), 54.5 (1-OMe), 40.2 (C-2′), 26.0 (C-3′), 16.4 (C-5′), 16.2 (C-6), 11.1 (C-4′); HRESIMS m/z [M + Na]$^+$ 285.1319 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

Synthesis of (2′*R*)-Methylbutyryl D-Fucopyranosides. These were synthesized by a procedure equivalent to that for the 2*S* isomers. The residue was then subjected to purification by semipreparative HPLC. Isocratic HPLC conditions of 90% H_2O/10% MeOH were initially employed for 10 min; then a linear gradient to MeOH was run over 40 min, followed by isocratic conditions of MeOH for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and then analyzed by (+)-LRESIMS and NMR. Fractions 3 and 4 yielded (2′*R*)-**8** (11.0 mg), fractions 6 and 7 yielded (2′*R*)-**5** (3.1 mg), and fractions 10−18 yielded (2′*R*)-**9** (14.3 mg).

*Methyl 3-O-(2′R)-methylbutyryl-α-fucopyranoside (**5**):* clear oil; $[\alpha]^{25}_D$ = +231 (c 0.1, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) 4.81 (1H, dd, J = 10.6, 3.5 Hz, H-3), 4.77 (1H, d, J = 6.2 Hz, OH-2), 4.67 (1H, d, J = 7.5 Hz, OH-4), 4.55 (1H, d, J = 3.8 Hz, H-1), 3.84 (1H, ddd, J = 10.6, 7.5, 3.8 Hz, H-2), 3.77 (1H, br q, J = 6.4 Hz, H-5), 3.61 (1H, m, H-4), 3.28 (3H, s, 1-OMe), 2.36 (1H, m, H-2′), 1.62 (1H, m, H3′-A), 1.41 (1H, m, H3′-B), 1.08 (3H, d, J = 6.4 Hz, H-6), 1.09 (3H, d, J = 6.3 Hz, H-5′), 0.87 (3H, t, J = 7.6 Hz, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6) 175.5 (C-1′), 100.0 (C-1), 72.8 (C-3), 69.0 (C-4), 65.5 (C-5), 64.9 (C-2), 54.5 (1-OMe), 40.2 (C-2′), 26.0 (C-3′), 16.3 and 16.1 (C-6 and C-3′), 11.2 (C-4′); HRESIMS m/z [M + Na]$^+$ 285.1317 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

*Methyl 3-O-(2′R)-methylbutyryl-β-fucopyranoside (**8**):* clear oil; $[\alpha]^{25}_D$ = +36 (c 0.1, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) 5.09 (1H, d, J = 5.5 Hz, OH-2), 4.73 (1H, d, J = 6.7 Hz, OH-4), 4.57 (1H, dd, J = 10.3, 3.4 Hz, H-3), 4.10 (1H, d, J = 7.7 Hz, H-1), 3.60 (1H, dq, J = 6.3, 0.9 Hz, H-5), 3.55 (1H, ddd, J = 6.7, 3.4, 0.9 Hz, H-4), 3.51 (1H, ddd, J = 10.3, 7.7, 5.5 Hz, H-2), 3.37 (3H, s, 1-OMe), 2.37 (1H, m, H-2′), 1.62 (1H, m, H3′A), 1.42 (1H, m, H3′B), 1.13 (3H, d, J = 6.4 Hz, H-6), 1.08 (3H, d, J = 6.7 Hz, H-5′), 0.87 (3H, t, J = 7.6 Hz, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6) 175.3 (C-1′), 104.0 (C-1), 75.8 (C-3), 69.7 (C-5), 68.5 (C-4), 67.1 (C-2), 55.7 (1-OMe), 40.1 (C-2′), 26.1 (C-3′), 16.29 (C-3′), 16.25 (C-6), 11.2 (C-4′); HRESIMS m/z [M + Na]$^+$ 285.1317 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

*Methyl 2-O-(2′R)-methylbutyryl-α-fucopyranoside (**9**):* clear oil; $[\alpha]^{25}_D$ = +118 (c 0.1, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) 4.78 (1H, dd, J = 10.7, 3.7 Hz, H-2), 4.75 (1H, d, J = 6.7 Hz, OH-3), 4.70 (1H, d, J = 6.9 Hz, OH-4), 4.65 (1H, d, J = 3.7 Hz, H-1), 3.77 (1H, m, H-5), 3.76 (1H, m, H-3), 3.55 (1H, m, H-4), 3.22 (3H, s, 1-OMe), 2.38 (1H, m, H-2′), 1.56 (1H, m, H-3′A), 1.44 (1H, m, H-3′B), 1.11

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(3H, d, J = 6.5 Hz, H-6), 1.06 (3H, d, J = 6.9 Hz, H-5′), 0.85 (3H, d, J = 7.7 Hz, H-4′); ^{13}C NMR (125 MHz, DMSO-d_6) 175.7 (C-1′), 96.8 (C-1), 71.5 (C-2), 70.7 (C-4), 66.7 (C-3), 65.7 (C-5), 54.5 (1-OMe), 39.9 (C-2′), 26.3 (C-3′), 16.19 and 16.22 (C-5′/C-6), 10.9 (C-4′); HRESIMS m/z [M + Na]$^+$ 285.1318 (calcd for $C_{12}H_{22}O_6Na$, 285.1314).

LAT Assay. High-throughput screening for LAT inhibitors has been conducted as previously described.[8] Briefly, cells were cultured in six-well plates in RPMI media. After collecting and counting, cells (3×10^4/well) were incubated with 0.3 μCi [^{3}H]-L-leucine (200 nM; PerkinElmer) in leucine-free RPMI media (Invitrogen) with 10% (v/v) dialyzed fetal bovine serum (FBS) for 15 min at 37 °C. For high-throughput screening of Nature Bank fractions, LNCaP cells (10^4/well) were incubated with 0.3 μCi [^{3}H]-L-leucine (200 nM) in Hank's balanced salt solution (HBSS) with 10% (v/v) dialyzed FBS and 50 mM L-glutamine. Cells were directly added into 96-well plates containing preloaded fractions from Nature Bank. DMSO 0.5% (v/v) was used as the negative control, and 10 mM BCH (LAT family inhibitor) was used as the positive control. Cells were collected, transferred to filter paper using a 96-well plate harvester (Wallac PerkinElmer), dried, and exposed to scintillation fluid, and counts were measured using a liquid scintillation counter (PerkinElmer).

■ ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra for compounds **3** and **4**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/np500968t.

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Notes

The authors declare no competing financial interest.

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