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Isolation and total synthesis of gymnastatin N, a POLO-like kinase 1 active constituent from the fungus *Arachniotus punctatus*

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Abstract—A high throughput screen against POLO-like kinase 1 (Plk1), an anti-cancer target, identified an active extract from the fungus *Arachniotus punctatus*. Bioassay guided fractionation led to the isolation of the new natural product, gymnastatin N (**1**) and the known compound aranorosinol A (**2**) with IC_{50} values of 13 and 118 μM, respectively. A 12′-hydroxy analog of gymnastatin N, **3**, was also isolated as a minor component. Gymnastatin N (**1**) was found to be a 52:48 mixture of (1*S*,6′*R*) and (1*R*,6′*R*) diastereomers, by synthesis of the four possible diastereomers and comparison of the optical rotation and chiral HPLC profile of each diastereoisomer with the natural product. Analogues of **1** were synthesized and evaluated against the Plk1 assay and these SAR studies suggested that the diene and free carboxylic acid moieties might be responsible for its bioactivity.
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1. Introduction

POLO-like kinases play a pivotal role in various stages of cell division. Recent studies on this novel family of enzymes have unraveled their functions in centrosome maturation and bipolar spindle formation at the onset of mitosis.[1] They are also involved in the activation of cyclin-dependent kinase (Cdk)1-cyclin B.[2] In addition, they have been implicated in the activation of anaphase-promoting complex (APC) and the inactivation of Cdk1 at the point of mitotic exit.[1] POLO-like kinase 1 (Plk1) is a highly conserved mitotic serine/threonine kinase which has been shown to be commonly overexpressed in cancer cell lines.[3] Its expression could reflect the degree of malignancy and proliferation in these cells. Due to its essential roles in cell-cycle regulation, Plk1 could serve as a suitable diagnostic and prognostic marker for tumour progression and as a target for anti-cancer therapy. We have initiated a screening programme of our natural product collection, directed towards the identification of potent Plk1 inhibitors as anti-tumour drugs.

A high throughput screen against Plk1 identified an active extract from the fungus *Arachniotus punctatus*. Bioassay guided fractionation led to the isolation of the new natural product, gymnastatin N (**1**) and the known compound aranorosinol A (**2**) (Fig. 1),[4] with IC_{50} values of 13 and 118 μM, respectively. A 12′-hydroxy analog of gymnastatin N, **3**, was also isolated as a minor component (Fig. 1). This class of fungal derived natural products contains a 4,6-dimethyl-dodecadien-2*E*,4*E*-oic acid unit connected to a tyrosine unit through an amide linkage. The tyrosine unit can have various degrees of oxygenation, halogenation, cyclisation and esterification, as found in aranorosin (**4**),[5] aranorosinol A (**2**), aranorosinol B,[4] aranochlor A and aranochlor B from *Pseudoarachniotus roseus*,[6] gymnastatin A (**5**) to E,[7] F to H (**6**)[8] and I (**7**) to M[9] from *Gymnastella dankaliensis* (Fig. 1). These compounds have been reported to have antibacterial and anti-tumour activity. The total synthesis of aranorosin (**4**),[10,11] gymnastatin A (**5**),[12] gymnastatin H (**6**)[8] and gymnastatin I (**7**),[9] along with various interconversions between compounds, have enabled the absolute stereochemistry of most compounds in this class to be determined. To date, all of these compounds and gymnasterone A from *G. dankaliensis*,[13] have a (6*R*) configuration at the 4,6-dimethyl-dodecadien-2*E*,4*E*-oic acid unit. The occurrence of the (6*S*) configuration is rare: it has only been reported in the isolation of (6*S*)-4,6-dimethyl-dodecadien-2*E*,4*E*-oic acid and its phomalactone ester derivative, from a *Phomopsis* sp.[14]

2. Results and discussion

Approximately 300 out of the 90,000 extracts screened showed more than 40% inhibition against the Plk1 assay at a

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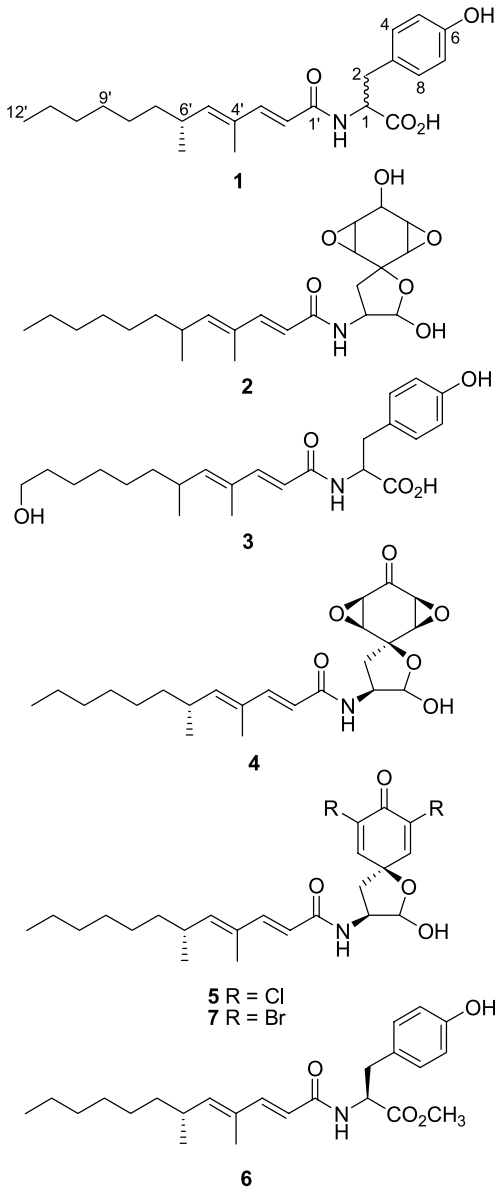


Figure 1. Gymnastatins and aranorosinol A.

concentration of 250 μg/mL. These extracts were tested against Cdk2, a serine/threonine kinase, to eliminate common serine/threonine kinase inhibitors and the 20 samples that had more than 5 fold potency against Plk1 versus Cdk2 were selected for further progression. One of these samples was the CH_3OH extract of the fungus *A. punctatus* and bioassay guided fractionation led to the isolation of gymnastatin N (**1**) and aranorosinol A (**2**) as the active components. Aranorosinol A (**2**) was identified by comparison of the spectroscopic data with that previously reported.[4] A 12′-hydroxy analogue of gymnastatin N, **3**, was isolated as a minor component.

Gymnastatin N (**1**) was isolated as a colourless oil that gave an $[M-H]^-$ ion peak at *m/z* 386.2335 and an $[M+H]^+$ ion peak at *m/z* 388.2470 in the (−)- and (+)-HR-ESIMS respectively, which were consistent with a molecular formula of $C_{23}H_{33}NO_4$. Analysis of the NMR data of **1** (^1H, ^{13}C, COSY, HSQCED and HMBC experiments) established the presence of a 4,6-dimethyl-dodecadien-

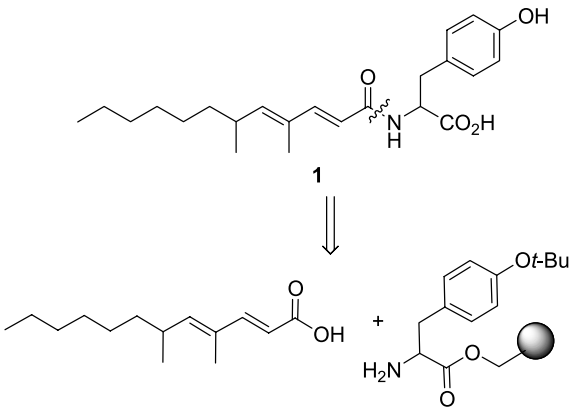


Figure 2. Retrosynthetic analysis of gymnastatin N.

2*E*,4*E*-oic acid derived unit, which was identical with that previously reported,[4–14] and a tyrosine unit. A HMBC correlation from the tyrosine NH (δ_H 8.14) to the carbonyl carbon (δ_C 165.3) of the 4,6-dimethyl-dodecadien-2*E*,4*E*-oic acid derived unit enabled the structure of **1** to be determined as the acid derivative of gymnastatin H (**6**).[8] Although gymnastatin H (**6**) has been synthesized, no optical rotation or NMR data has been reported.

Gymnastatin N (**1**) possesses two chiral centers, one residing at the tyrosine unit, the other at the aliphatic side chain and a total synthesis of each diastereoisomer was undertaken to establish its absolute stereochemistry. A retrosynthetic analysis (Fig. 2) shows that **1** can be disconnected at the amide bond to give the tyrosine unit and the long chain aliphatic acid. The acid component was synthesized using the protocols reported in literature with slight modifications.[10,11] For ease of purification, the assembly of the molecule was performed on solid phase at the final stage.

The preparations of both enantiomers of the acid side chain **8** were reported by Wipf et al.[10] The (*S*)-isomer was obtained by methylating the acylated (*S*)-4-benzyloxazolidinone while the (*R*)-isomer was obtained by alkylating the propionylated (*S*)-4-benzyloxazolidinone. However, the alkylation of the corresponding potassium, sodium, or lithium enolate with hexyl iodide failed to give the desired product and the problem was circumvented by employing the more reactive reagent hexyl triflate. The exact outcome of the earlier failures was not reported in detail.

In our study, alkylation of the (*S*)-propionylated oxazolidinone using sodium enolate and hexyl bromide gave no conversion at −78 °C. However, by raising the reaction temperature to 4 °C, self-acylation of the propionylated oxazolidinone was observed, giving the acylated isomers **11**

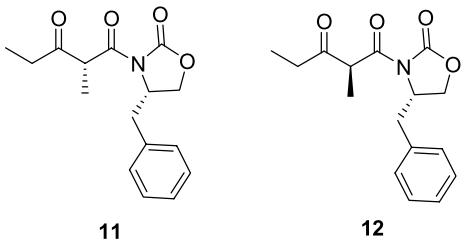
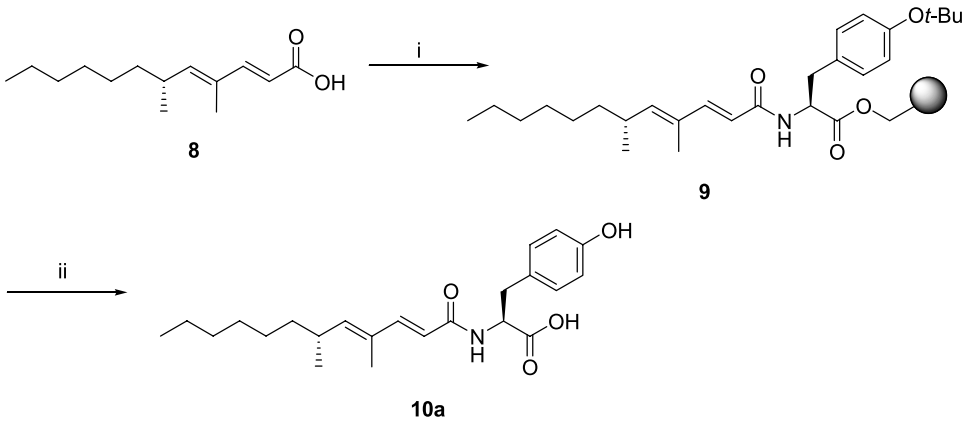


Figure 3. Self-acylated isomers.



Scheme 1. Synthesis of gymnastatin N. Reagents and conditions: (i) Wang resin-bound L-Tyr(*t*-Bu), PyBOP, HOBt, diisopropylethylamine, anhydrous DMA, rt, 18 h; (ii) TFA–CH_2Cl_2 1:1, rt, 2 h (93% from step i).

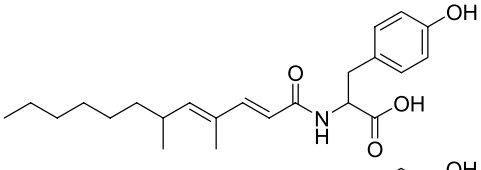
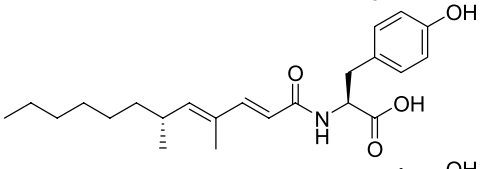
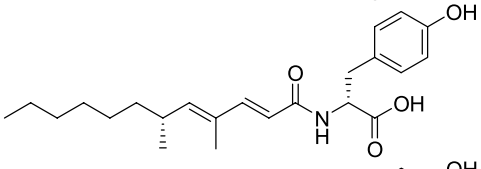
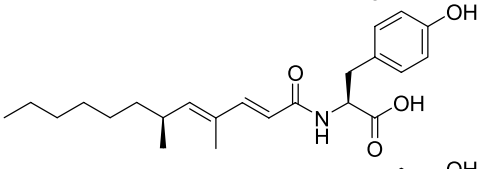
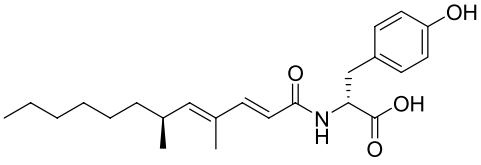
and **12** in a ratio of 61:39 (Fig. 3). The alkylation was also attempted using $TiCl_4$, but this was unsuccessful. There was no self-acylation and the starting material was recovered.

These initial failures led us to attempt the alternative synthetic route by first acylating the (*R*)-4-benzyloxazolidinone with octanoyl chloride following by methylation. This route gave a higher yield and was more appropriate for our purpose. Subsequent reductive cleavage of the methylated imide, oxidation, iterative Horner–Emmons–Wittig reactions and hydrolysis, afforded the (*R*)-acid component **8** in an overall 23% yield, starting from oxazolidinone. Adopting the same approach, the (*S*)-acid component was also obtained in an overall 20% yield from (*S*)-4-benzyloxazolidinone.

PyBOP-mediated coupling of the resin-bound L-tyrosine with the (*R*)-acid side chain **8** gave the amide **9** (Scheme 1). The resultant resin **9** had to be washed extensively with DMF to remove any impurities. Subsequent treatment with 50% TFA, to both remove the *t*-Bu protecting group and execute resin cleavage, gave the final product **10a** in a 93% yield over the two steps. The other three stereoisomers **10b–d** were also prepared by reacting the (*R*)-side chain with D-tyrosine, and (*S*)-side chain with L- or D-tyrosine respectively (Table 1).

The absolute stereochemistry of gymnasatin N (**1**) could not be ascertained by direct comparison with the ^1H or ^{13}C NMR spectra of the synthetic products as the four

Table 1. Structures, optical rotations, and HPLC retention times of gymnastatin N and isomers

Entry	Structure	$[\alpha]_D^{30}$ (*c* 0.2, EtOH)	R_t (min)
Gymnastatin N		−32.5	20.8, 21.3
10a		+27.5	21.4
10b		−94.0	20.8
10c		+87.5	21.3
10d		−20.0	20.7

stereoisomers **10a–d** had identical NMR data. This phenomenon was also encountered by Wipf and co-workers during their synthesis of aranorosin where they had to resort to comparison of optical rotations for each diastereomer to establish the absolute configuration of the natural product.[10] However, none of the optical rotations of the synthetic stereoisomers **10a–d** matched that of the gymnastatin N (Table 1).

Therefore, gymnastatin N was probably a mixture of enantiomers or diastereomers. To determine whether this was the case, the four synthetic stereoisomers and natural product were analyzed by HPLC using a ChiralCel®OD-R column (Fig. 4) and the retention times are shown in Table 1. The HPLC analysis of gymnastatin N showed that it was not a single compound, but instead two peaks that appeared at the retention times 21.3 and 20.8 min in a ratio

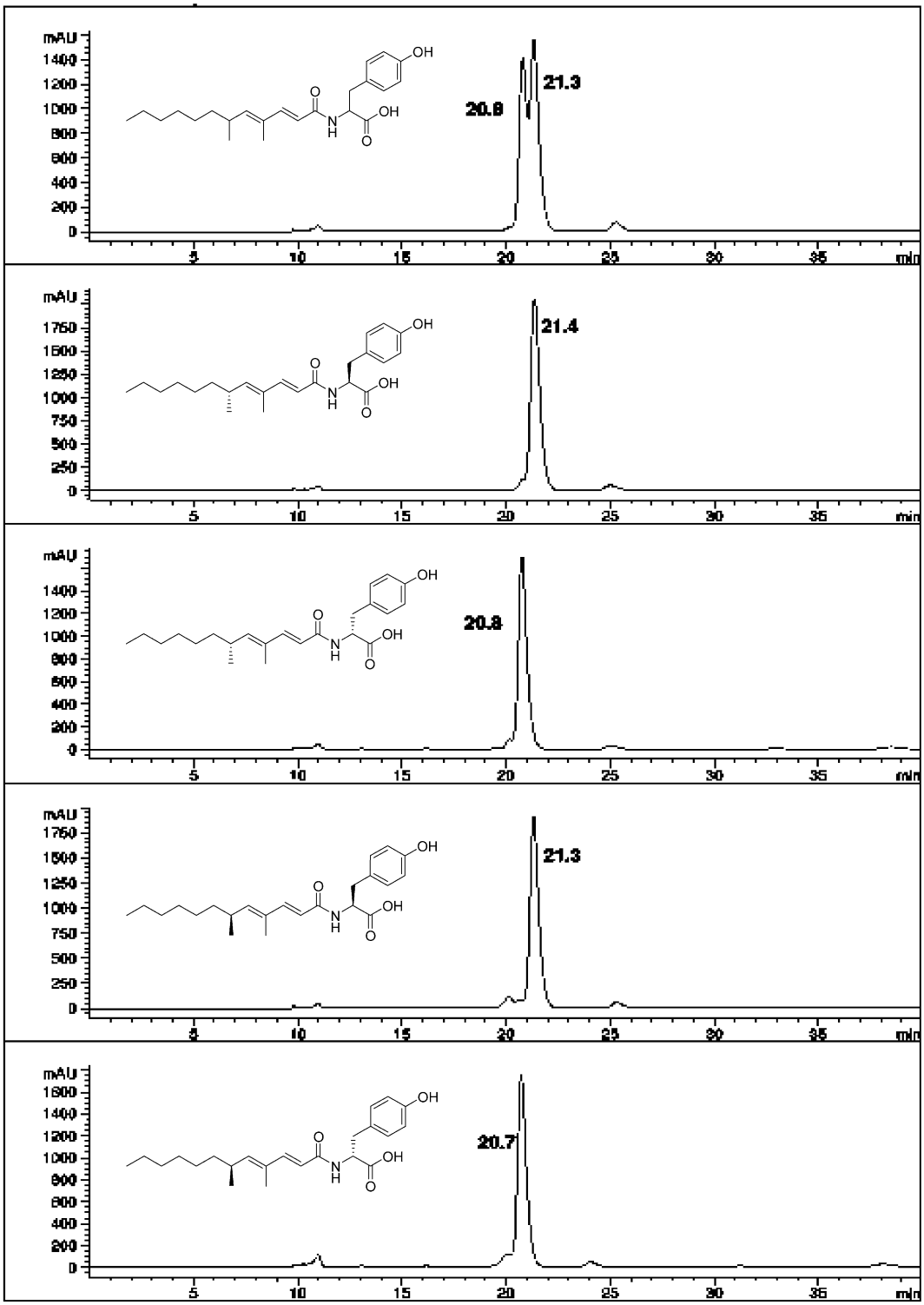


Figure 4. HPLC chromatograms of gymnastatin N and compounds **10a–d**.

of 58:42. Considering the optical rotation and HPLC profile, it was clear that gymnastatin N was a mixture of the diastereomers **10a** and **10b**. This was confirmed by obtaining the optical rotation of a mixture of isomers **10a** and **10b** at a ratio of 58:42 ($[\alpha]_D^{30}$ −35.5), which is similar to that obtained for gymnastatin N ($[\alpha]_D^{30}$ −32.5).

Compound **3** (Fig. 1) displayed an $[M-H]^-$ ion peak at *m/z* 402.2178 in the (−)-HR-ESIMS. These data were consistent with a molecular formula of $C_{23}H_{33}NO_5$ for **3**, which was 16 mass units more than **1**. The NMR data of **3** was almost identical to **1** except that the terminal methyl group on the aliphatic chain was replaced with a primary alcohol (δ_H 3.31; δ_C 60.2). Hence, the structure of **3** was established as the 12′-hydroxy derivative of gymnastatin N.

The IC_{50} values of gymnastatin N (**1**) and aranorosinol A (**2**) were 13 and 118 μM in the Plk1 assay, 45 μM and inactive in Cdk2 assay, respectively (Table 2). To improve the activity profile of **1**, a series of SAR studies was conducted. The importance of the carboxylic acid group and the conjugated diene was firstly assessed by preparing the methyl ester **13** and the fully saturated product **14** (Fig. 5). Both derivatives showed a decrease of activity against Plk1, indicating that the free acid and the conjugated diene functionalities might be essential for bioactivity.

A range of analogues with variations in amino acid moiety and aliphatic chain were also prepared. They were synthesized on solid support either through the reaction of free amino groups with acid chlorides, or PyBOP-mediated

Table 2. Yields, Plk1 and Cdk2 IC_{50} of gymnastatin N, its analogues, and aranorosinol A

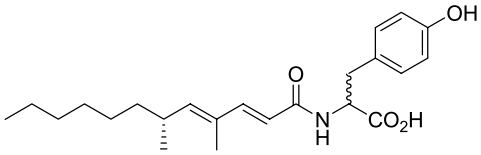
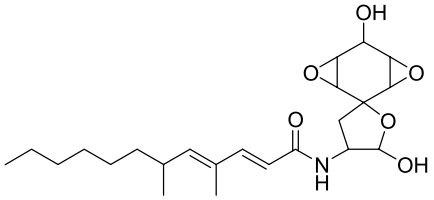
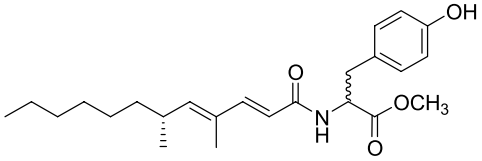
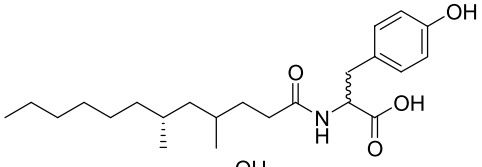
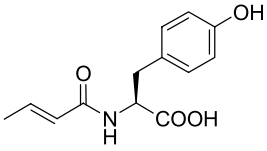
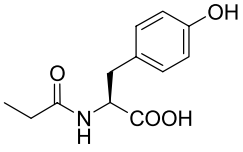
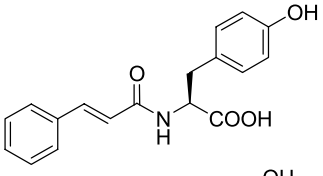
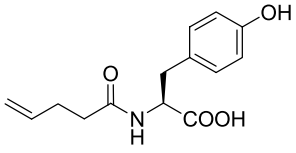
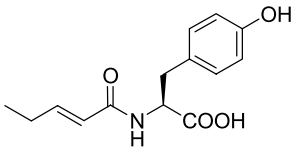
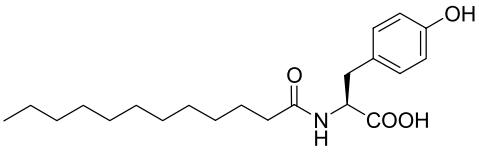
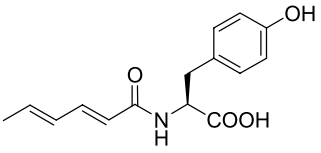
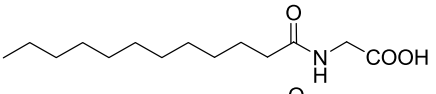
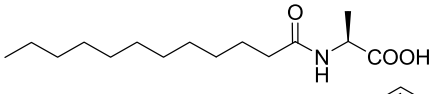
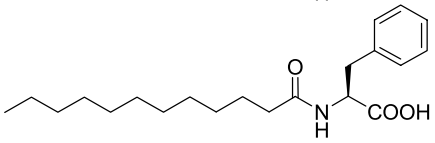
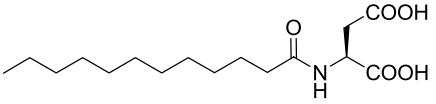
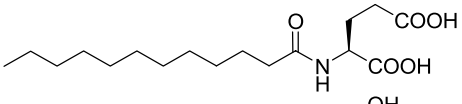
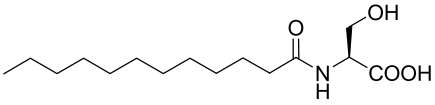
Entry	Structure	Yield (%)	Plk1[a] IC_{50} (μM)	Cdk2[b] IC_{50} (μM)
1		—	13	45
2		—	118	>208
13		92	>208	>208
14		89	82	254
15a		98	>334	>334
15b		96	>351	>351
15c		98	>268	>268
15d		98	>317	>317

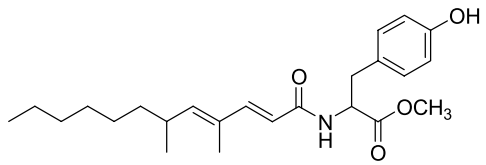
Table 2 (*continued*)

Entry	Structure	Yield (%)	Plk1[a] IC_{50} (μM)	Cdk2[b] IC_{50} (μM)
15e		98	73	>332
15f		99	63	>229
15g		99	2.2	17
15h		98	87	>324
15i		98	121	>307
15j		99	65	>240
15k		98	61	>264
15l		96	144	>253
15m		94	>290	>290

[a] The Plk1 assay was carried out in triplicates.
[b] The Cdk2 assay was carried out in duplicates.

coupling with acids (Scheme 2). The products **15a**–**m** were cleaved off resin in high yields (Table 2). The purities of the compounds are greater than 95% as assessed by ^{1}H NMR spectroscopy. Among all the analogues screened, the compound **15g**, which contained the tyrosine moiety and the conjugated diene, exhibited the most potent activity against Plk1.

In conclusion, the new natural product gymnastatin N (**1**) and the known compound aranorosinol A (**2**) were identified as Plk1 inhibitors. To our knowledge, this is the first report



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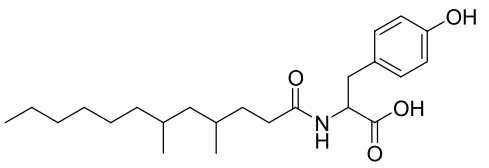

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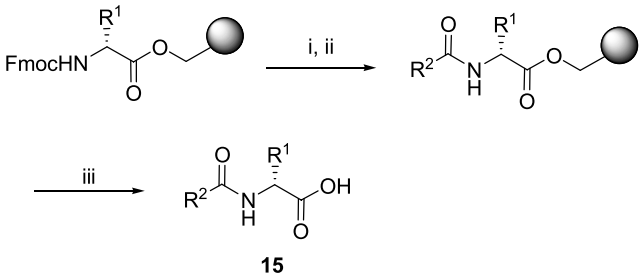
Figure 5. Derivatives of gymnastatin N.



15

Scheme 2. Solid phase synthesis of gymnastatin N analogues. Reagents and conditions: (i) 20% piperidine/DMF, rt, 20 min, 2 cycles; (ii) a cid chloride, diisopropylethylamine, anhydrous CH_2Cl_2, rt, 1 h or acid, PyBOP, HOBt, diisopropylethylamine, anhydrous DMA, rt, 18 h; (iii) TFA–CH_2Cl_2 1:1, rt, 18 h.

on the natural product inhibitors of Plk1. Total syntheses of all four stereoisomers of **1** showed that it is a mixture of two diastereomers **10a** and **10b**. We believe that the presence of both diastereomers is not an artifact as neither heating nor base was employed in the fermentation or the isolation process. Preliminary SAR studies indicated that the free acid and the conjugated diene groups might be responsible for the activity.

3. Experimental

3.1. General experimental procedures

Specific optical rotation was determined using a Jasco DIP-1000 Digital Polarimeter. ^{1}H and ^{13}C NMR spectra of the natural products were acquired on a Bruker Avance DRX-500 NMR spectrometer, using 5 mm inverse (^{1}H, G-COSY, multiplicity-edited G-HSQC, G-HMBC and G-ROESY spectra) or normal (^{13}C spectra) probeheads equipped with *z*-gradients, while NMR data on the synthetic compounds was acquired using a Bruker AVANCE-400 NMR. Spectra were calibrated to residual protonated solvent signals and all chemical shift values (δ) are given in ppm. Infrared spectra were taken on a Bio-Rad Excalibur Series FT-IR spectrometer. Melting points were determined by a Büchi 535 melting point apparatus. HPLC was performed on a Waters system equipped with a Waters 996 PDA detector, Waters 600 gradient controller and pump, Waters 717plus autosampler, Millennium software and a Waters fraction collector II. Preparative HPLC was done using a Waters Delta-Pak™ C_{18} (40 mm I.D.$\times$ 100 mm, 15 μ) column, while semi-preparative HPLC was done using either a Phenomenex Luna C18(2) (10 mm I.D.$\times$150 mm, 5 μ) or Phenomenex Phenyl-Hexyl (10 mm I.D.$\times$150 mm, 5 μ) columns for separation of the natural products. HR-ESIMS data was collected on an Applied Biosystems Mariner TOF mass spectrometer, using sodium trifluoroacetate as an internal standard for both positive and negative ionization modes. Chiral HPLC analyses were carried out on a ChiralCel®OD-R column (4.6 mm I.D.$\times$ 250 mm): isocratic elution with 55% (0.04% TFA/CH_3CN), 45% (0.04% TFA/H_2O); flow rate of 0.5 mL/min over 35 min. The SPA beads and ^{33}P-ATP were purchased from Amersham Biosciences, while the enzymes and peptide substrates were provided by GlaxoSmithKline. The chemical reagents and resins were purchased from Sigma-Aldrich, Novabiochem, and Bachem. Anhydrous CH_2Cl_2 was freshly distilled over calcium hydride. Fritted polyethylene filtration tubes and teflon taps were bought from Jones Chromatography.

3.2. Microorganism and Fermentation

The fungal strain *A. punctatus* is deposited in the MerLion Pharmaceuticals culture collection (F31134). The strain was sub-cultured on Malt Extract Agar (MEA) for 14 days. An aqueous seed medium (50 mL) containing 0.4% yeast extract, 1% malt extract and 0.4% glucose was placed in 250 mL Erlenmeyer flasks and was sterilized at 121 °C for 30 min. Five agar plugs were used for inoculation of seed culture and the inoculated flasks were shaken on a rotary shaker (200 rpm) at 24 °C for 5 days. Seed culture (5 mL) was transferred to each of the twenty 250 mL Erlenmeyer flasks with solid fermentation medium (containing 50 mL of 4% maltose, 12% Glucidex, 1% bacteriological peptone, 1.5% cotton seed flour, 1.5% cane molasses, 0.5% $MgSO_4\cdot 7H_2O$, 0.5% $CaCO_3$, 0.2% $FeSO_4\cdot 7H_2O$, 0.002% $ZnSO_4\cdot 7H_2O$, 0.2% L-tryptophan and 6 g of vermiculite). Fermentation was carried out at 24 °C for 14 days.

3.3. Extraction and isolation

The biomass from the 20 Erlenmeyer flasks were extracted with CH_3OH, and evaporated to dryness under vacuum. The crude CH_3OH extract (130 g) was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ extract (3.5 g) was separated using a C18 column using a 20% stepwise gradient from H_2O to CH_3OH. The 80% CH_3OH active fraction was separated using C18 preparative HPLC (gradient elution from 0 to 100% [0.1% HCOOH/CH_3CN and 0.1% HCOOH/H_2O] in 25 min, 18 mL/min) and C18 semi-preparative HPLC (gradient elution from 60 to 75% [0.1% HCOOH/CH_3CN and 0.1% HCOOH/H_2O] in 25 min, 4 mL/min) to afford gymnastatin N (**1**) (5 mg). Adjacent fractions were also found to be active and was purified by HPLC using a phenylhexyl column (gradient elution from 43 to 50% [0.1% HCOOH/CH_3CN and 0.1% HCOOH/H_2O] in 20 min, 4 mL/min) gave aranorosinol A (**2**) (7 mg). During the process of isolation, another compound more polar than **1** that had a similar UV characteristics was observed, and was purified on a semi-preparative HPLC using phenylhexyl column (isocratic 40% [0.1% HCOOH/CH_3CN and 0.1% HCOOH/H_2O], 4 mL/min) and Sephadex LH-20 (eluent CH_3OH) to give 12′-hydroxy gymnastatin N (**3**) (0.5 mg).

3.3.1. Gymnastatin N (1). Colourless oil; $[\alpha]_D^{30}$ −32.5° (*c* 0.2, EtOH); ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.83 (t, 3H, J=6.6 Hz, H-12′), 0.92 (dd, 3H, J=6.6, 1.9 Hz, 6′-CH_3), 1.21 (br m, 10H, H-7′ to H11′), 1.71 (s, 3H, 4′-CH_3), 2.49 (1H, under DMSO, H-6′), 2.74 (dd, 1H, J=13.9, 9.5 Hz, H-2a), 2.94 (dd, 1H, J=13.9, 4.7 Hz, H-2b), 4.39 (m, 1H, H-1) 5.58 (d, 1H, J=9.8 Hz, H-5′), 5.97 (d, 1H, J=15.4 Hz, H-2′), 6.63 (d, 2H, J=8.2 Hz, H-5 and H-7), 6.95 (d, 1H, J=15.4 Hz, H-3′), 6.99 (d, 2H, J=8.2 Hz, H-4 and H-8), 8.14 (d, 1H, J=8.2 Hz, NH), 9.19 (br. s, 1H, 1-COOH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 12.4 (4′-CH_3), 13.9 (C-12′), 20.5 (6′-CH_3), 22.0 (C-11′), 26.8 (C-9′*), 28.7 (C-8′*), 31.2 (C-10′), 32.4 (C-6′), 36.1 (C-2), 36.7 (C-7′), 54.1 (C-1), 114.9 (C-5, C-7), 119.3 (C-2′), 127.8 (C-3), 129.9 (C-4, C-8), 130.9 (C-4′), 144.1 (C-3′), 145.5 (C-5′), 155.8 (C-6), 165.3 (C-1′), 173.3 (1-COOH), * interchangeable; (−)-HR-ESIMS m/z 386.2335 $[M-H]^-$ (calcd for $C_{23}H_{32}NO_4$, 386.2331); (+)-HR-ESIMS m/z 388.2470 $[M+H]^+$ (calcd for $C_{23}H_{34}NO_4$, 388.2488).

3.3.2. 12′-Hydroxy gymnastatin N (3). Colourless oil; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.92 (dd, 3H, J=6.62, 1.9 Hz, 6′-CH_3), 1.22 (br. m, 10H, H-7′ to H11′), 1.70 (s, 3H, 4′-CH_3), 2.49 (1H, under DMSO, H-6′), 2.77 (dd, 1H, J=13.6, 7.2 Hz, H-2a), 2.95 (br d, 1H, H-2b), 3.31 (under H_2O, 1H, H-12′), 4.14 (m, 1H, H-1) 5.55 (d, 1H, J=9.8, H-5′), 5.98 (d, 1H, J=15.4, H-2′), 6.56 (d, 2H, J=8.2 Hz, H-5 and H-7), 6.91 (br d, 3H, H-3′, H-4 and H-8), 7.56 (br s, 1H, NH), 9.19 (br s, 1H, 1-COOH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 12.4 (4′-CH_3), 20.5 (6′-CH_3), 25.4 (C-11′), 26.9 (C-9′*), 29.0

(C-8′*), 32.2 (C-10′), 32.5 (C-6′), 36.5 (C-2), 36.7 (C-7′), 55.1 (C-1), 60.65 (C-12′), 114.6 (C-5, C-7), 120.3 (C-2′), 128.9 (C-3), 130.1 (C-4, C-8), 131.0 (C-4′), 143.2 (C-3′), 144.8 (C-5′), 155.4 (C-6), 164.5 (C-1′), 172.7 (1-COOH), * interchangeable; (−)-HR-ESIMS *m/z* 402.2278 $[M-H]^-$ (calcd for $C_{23}H_{32}NO_5$, 402.2280).

3.4. Biological assays

The Plk1 assay was performed in 384-well plates. Fractions and compounds were incubated with Plk1 in a reaction mixture containing 25 nM Plk1 enzyme, 1 μM peptide substrate, 0.15 mg SPA beads, 1 μM ATP, 0.3 mg/mL heparin, 0.25 mg/mL bovine serum albumin, 7.5 mM $MgCl_2$, and 12.5 mM HEPES pH 7.5. After 90 min of incubation at rt, the reaction was terminated by the addition of 50 μL stop reagent containing 2.5 mg/mL SPA beads and 100 mM EDTA in PBS buffer. Radioactivity was measured using a Microbeta Counter (PerkinElmer) after an overnight bead settling period. The assay procedure for Cdk2 assay is similar to that for Plk1 assay except that it involves a 30 min incubation at room temperature with the final reaction condition containing 0.64 μg/mL Cdk2 enzyme, 2.5 μM peptide substrate, 1.4 μM ATP, 0.1 mg/mL bovine serum albumin, 10 mM $MgCl_2$, and 100 mM HEPES pH 7.5.

3.5. Synthetic procedure for compounds 10

To the Wang resin-bound Fmoc-L- or D-Tyr(*t*-Bu) (0.63–0.74 mmol/g; 80 mg, 0.05–0.059 mmol) contained in a fritted polyethylene filtration tube was added 20% piperidine/DMF (2 mL). The resin suspension was stirred gently at rt for 20 min and then filtered. The Fmoc deprotection was carried out again. The resin was then washed with CH_2Cl_2 (3×5 mL), followed by drying under vacuum. To the dried resin was added acid **8** (0.1–0.18 mmol), PyBOP (0.1–0.18 mmol) and HOBt (0.1–0.18 mmol). This mixture was suspended in anhydrous DMA (1–1.2 mL), and distilled diisopropylethylamine (0.2–0.36 mmol) was added. The suspension was stirred gently at rt for 18 h, then filtered. The resin was washed successively with DMF (6×5 mL), THF–H_2O 3:2 (3×5 mL), THF (3×5 mL), CH_2Cl_2 (3×5 mL), then dried under vacuum. The derivatised resin **9** was transferred into a round bottom flask, and treated with TFA–CH_2Cl_2 (1:1, 2 mL) at rt for 2 h. Following that, the cleavage suspension was filtered and the resin was washed with CH_2Cl_2 (3×5 mL). The filtrate and collected washings were concentrated under reduced pressure to afford the product **10**.

3.5.1. (1*S*,6′*R*)-2-[(2*E*,4*E*)-4,6-Dimethyldodeca-2,4-dienamido]-3-(4-hydroxy-phenyl)propanoic acid (10a). Yield: 18.1 mg (93%). Colourless oil. ν_{max} (thin film) 3600–3100, 3313, 1748, 1640 cm^{-1}.

3.5.2. (1*R*,6′*R*)-2-[(2*E*,4*E*)-4,6-Dimethyldodeca-2,4-dienamido]-3-(4-hydroxy-phenyl)propanoic acid (10b). Yield: 17.3 mg (85%). White solid, mp 158–160 °C (decomposed). ν_{max} (Nujol) 3600–3100, 3323, 1754, 1639 cm^{-1}.

3.5.3. (1*S*,6′*S*)-2-[(2*E*,4*E*)-4,6-Dimethyldodeca-2,4-dienamido]-3-(4-hydroxy-phenyl)propanoic acid (10c). Yield: 19.0 mg (96%). White solid, mp 158–160 °C (decomposed). ν_{max} (Nujol) 3600–3100, 3328, 1754, 1639 cm^{-1}.

3.5.4. (1*R*,6′*S*)-2-[(2*E*,4*E*)-4,6-Dimethyldodeca-2,4-dienamido]-3-(4-hydroxy-phenyl)propanoic acid (10d). Yield: 20.0 mg (92%). Colourless oil. ν_{max} (thin film) 3600–3100, 3313, 1748, 1640 cm^{-1}.

3.5.5. Methyl 2-[(2*E*,4*E*)-4,6-dimethyldodeca-2,4-dienamido]-3-(4-hydroxyphenyl)-propanoate (13). The product was synthesized according to the literature procedure with modifications in the amounts of reagents used:[15] Gymnastatin N (2.2 mg, 5.68 μmol) was dissolved in diethyl ether (1 mL); Diazald® (100 mg, 0.47 mmol), Carbitol® (0.5 mL, 3.72 mmol) was dissolved in diethyl ether (1 mL); 37% KOH (1 mL). The reaction mixture was left at 0 °C for 3 h with occasional shaking. Excess diazomethane was quenched by adding acetic acid (2–3 drops). The solvent was then removed under reduced pressure to afford the product as colourless oil (2.0 mg, 92%). R_f (50% EtOAc/hexane) 0.71; ^1H NMR (400 MHz, $CDCl_3$) δ 0.86 (3H, td, J=7.0, 2.6 Hz), 0.96 (3H, d, J=6.6 Hz), 1.22–1.26 (10H, m), 1.74 (3H, d, J=1.0 Hz), 2.49 (1H, m), 3.06 (1H, dd, J=13.9, 5.4 Hz), 3.12 (1H, dd, J=13.9, 6.5 Hz), 3.73 (3H, s), 4.95 (1H, m), 5.63 (1H, br d, J=9.7 Hz), 5.72 (1H, d, J=15.5 Hz), 5.94 (1H, br dd, J=7.4, 2.5 Hz), 6.74 (2H, d, J=7.9 Hz), 6.95 (2H, d, J=7.9 Hz), 7.23 (1H, dd, J=15.5, 2.5 Hz); (+)-HR-ESIMS *m/z* 402.2641 $[M+H]^+$ (calcd for $C_{24}H_{35}NO_4$, 402.2644).

3.5.6. 2-(4,6-Dimethyldodecanamido)-3-(4-hydroxyphenyl)propanoic acid (14). To a solution of gymnastatin N (2.9 mg, 7.49 μmol) in ethanol (1 mL) was added 10% Pd/C (50 mg). The suspension was bubbled with hydrogen at room temperature for 10 cycles (each cycle took approximately 2 min), then filtered through a pad of Celite. The pad was washed further with ethanol (5×5 mL). The filtrate and washings were combined and concentrated under reduced pressure to give the product as colourless oil (2.6 mg, 89%). R_f (EtOAc) 0.29; ^1H NMR (400 MHz, CD_3OD) δ 0.80–0.97 (9H, m), 1.19–1.52 (16H, m), 2.15 (2H, br tt, J=7.1, 7.1 Hz), 2.86 (1H, dd, J=13.7, 7.6 Hz), 3.10 (1H, dd, J=13.7, 4.6 Hz), 4.45 (1H, m), 6.65 (2H, d, J=8.4 Hz), 7.02 (2H, d, J=8.4 Hz); (+)-HR-ESIMS *m/z* 414.2628 $[M+Na]^+$ (calcd for $C_{23}H_{37}NO_4Na$, 414.2620).

3.6. Synthetic procedure for compounds 15 using acid chloride coupling

To the Wang resin-bound Fmoc-protected amino acid (0.44–0.7 mmol/g; 100 mg, 0.044–0.07 mmol) in a fritted polyethylene filtration tube was added 20% piperidine/DMF (2 mL). The resin suspension was stirred gently at rt for 20 min and then filtered. The Fmoc deprotection was carried out again. The resin was then washed with CH_2Cl_2 (3×5 mL), followed by drying under vacuum. The dried resin was suspended in anhydrous CH_2Cl_2 (2 mL). Distilled diisopropylethylamine (0.44–0.7 mmol) was added. After 5 min of stirring, the acid chloride (0.44–0.7 mmol) was introduced dropwise. The suspension was stirred gently at rt for 1 h, then filtered. The resin was washed with CH_2Cl_2 (3×5 mL), then dried under vacuum. The derivatised resin was transferred into a round bottom flask, and treated with

TFA–CH_2Cl_2 (1:1, 2 mL) at rt for 18 h. Following that, the cleavage suspension was filtered and the resin was washed with CH_2Cl_2 (3×5 mL). The filtrate and collected washings were concentrated under reduced pressure to afford the product. This procedure was employed for the syntheses of compounds **15a–d**, **f**, **h–m**. Sasrin™ resin-bound Fmoc-L-Ser(*t*-Bu) was used for the preparation of compound **15m**.

3.7. Synthetic procedure of compounds 15 using PyBOP coupling

To the Wang resin-bound Fmoc-amino acid (0.45–0.63 mmol/g; 100 mg, 0.045–0.063 mmol) contained in a fritted polyethylene filtration tube was added 20% piperidine/DMF (2 mL). The resin suspension was stirred gently at rt for 20 min, then filtered. The Fmoc deprotection was carried out again. The resin was then washed with CH_2Cl_2 (3×5 mL), followed by drying under vacuum. To the dried resin was added acid (0.23–0.32 mmol), PyBOP (0.23–0.32 mmol) and HOBt (0.23–0.32 mmol). This mixture was suspended in anhydrous DMA (2 mL), and distilled diisopropylethylamine (0.45–0.63 mmol) was added. The suspension was stirred gently at rt for 18 h, then filtered. The resin was washed successively with DMF (3×5 mL), THF–H_2O 3:2 (3×5 mL), THF (3×5 mL), CH_2Cl_2 (3×5 mL), then dried under vacuum. The derivatised resin was transferred into a round bottom flask, and treated with TFA–CH_2Cl_2 (1:1, 2 mL) at rt for 18 h. Following that, the cleavage suspension was filtered and the resin was washed with CH_2Cl_2 (3×5 mL). The filtrate and collected washings were concentrated under reduced pressure to afford the product. This procedure was employed for the syntheses of compounds **15e** and **15g**.

3.7.1. *N*-(*trans*-Crotonyl)-L-tyrosine (15a). Yield: 11.1 mg (98%). Pale yellow gum. ν_{max} (Nujol) 3600–3100, 3286, 1720, 1670 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.83 (3H, dd, J=6.9, 1.7 Hz), 2.89 (1H, dd, J=14.0, 8.7 Hz), 3.11 (1H, dd, J=14.0, 5.2 Hz), 4.64 (1H, dd, J=8.7, 5.2 Hz), 5.96 (1H, dq, J=15.3, 1.7 Hz), 6.68 (2H, dt, J=8.5, 2.0 Hz), 6.75 (1H, dq, J=15.3, 6.9 Hz), 7.03 (2H, dt, J=8.5, 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 17.8, 37.8, 55.6, 116.1, 125.8, 129.2, 131.2, 141.3, 157.3, 168.3, 175.3; (+)-HR-ESIMS m/z 250.1081 $[M+H]^+$ (calcd for $C_{13}H_{16}NO_4$, 250.1079).

3.7.2. *N*-(1-Oxopropyl)-L-tyrosine (15b). Yield: 10.1 mg (96%). Pale yellow oil. ν_{max} (thin film) 3600–3100, 1727, 1646 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.04 (3H, t, J=7.6 Hz), 2.17 (2H, qd, J=7.6, 0.8 Hz), 2.84 (1H, dd, J=14.0, 9.1 Hz), 3.10 (1H, dd, J=14.0, 5.1 Hz), 4.58 (1H, dd, J=9.1, 5.1 Hz), 6.69 (2H, dt, J=8.6, 2.0 Hz), 7.03 (2H, dt, J=8.6, 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 10.3, 29.9, 37.7, 55.3, 116.1, 129.2, 131.3, 157.3, 175.3, 176.8; (+)-HR-ESIMS m/z 238.1072 $[M+H]^+$ (calcd for $C_{12}H_{16}NO_4$, 238.1079).

3.7.3. *N*-(*trans*-Cinnamoyl)-L-tyrosine (15c). Yield: 13.3 mg (98%). Colourless oil. ν_{max} (thin film) 3600–3100, 1729, 1650 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.94 (1H, dd, J=14.0, 8.5 Hz), 3.16 (1H, dd, J=14.0, 5.2 Hz), 4.73 (1H, dd, J=8.5, 5.2 Hz), 6.66 (1H, d, J=15.8 Hz), 6.70 (2H, dt, J=8.6, 2.1 Hz), 7.07 (2H, dt, J=8.6, 2.1 Hz), 7.35–7.40 (3H, m), 7.49 (1H, d, J=15.8 Hz), 7.54 (2H, br dd, J=7.6, 1.6 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 37.8, 55.7, 116.2, 121.4, 128.9, 129.1, 129.9, 130.9, 131.3, 136.2, 142.2, 157.3, 168.3, 175.0; (+)-HR-ESIMS m/z 312.1236 $[M+H]^+$ (calcd for $C_{18}H_{18}NO_4$, 312.1236).

3.7.4. *N*-(4-Pentenoyl)-L-tyrosine (15d). Yield: 11.7 mg (98%). Pale yellow oil. ν_{max} (thin film) 3600–3100, 1727, 1645 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 2.25–2.27 (4H, m), 2.89 (1H, dd, J=13.9, 8.2 Hz), 3.08 (1H, dd, J=13.9, 5.1 Hz), 4.65 (1H, m), 4.89 (1H, dd, J=9.8, 1.6 Hz), 4.98 (1H, br d, J=17.1 Hz), 5.76 (1H, m), 6.74 (2H, d, J=8.4 Hz), 7.07 (2H, d, J=8.4 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 30.8, 36.1, 37.7, 55.3, 115.7, 116.1, 129.2, 131.3, 138.2, 157.3, 175.2; (+)-HR-ESIMS m/z 264.1231 $[M+H]^+$ (calcd for $C_{14}H_{18}NO_4$, 264.1236).

3.7.5. *N*-(*trans*−2-Pentenoyl)-L-tyrosine (15e). Yield: 11.5 mg (98%). Pale yellow oil. ν_{max} (thin film) 3600–3100, 1724, 1667 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.05 (3H, t, J=7.5 Hz), 2.20 (2H, qdd, J=7.5, 6.5, 1.7 Hz), 2.89 (1H, dd, J=14.0, 8.8 Hz), 3.11 (1H, dd, J=14.0, 5.2 Hz), 4.65 (1H, dd, J=8.8, 5.2 Hz), 5.95 (1H, dt, J=15.4, 1.7 Hz), 6.69 (2H, dt, J=8.6, 2.0 Hz), 6.79 (1H, dt, J=15.4, 6.5 Hz), 7.03 (2H, dt, J=8.6, 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 12.8, 26.1, 37.7, 55.5, 116.2, 123.3, 129.1, 131.2, 147.7, 157.3, 168.5, 175.0; (+)-HR-ESIMS m/z 264.1236 $[M+H]^+$ (calcd for $C_{14}H_{18}NO_4$, 264.1236).

3.7.6. *N*-Dodecanoyl-L-tyrosine (15f). Yield: 15.2 mg (99%). Pale yellow solid, mp 122–125 °C. ν_{max} (Nujol) 3600–3100, 3302, 1706, 1643 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.1 Hz), 1.20–1.37 (16H, m), 1.50 (2H, tt, J=7.4, 7.4 Hz), 2.15 (2H, t, J=7.4 Hz), 2.83 (1H, dd, J=14.0, 9.4 Hz), 3.11 (1H, dd, J=14.0, 4.9 Hz), 4.60 (1H, dd, J=9.4, 4.9 Hz), 6.69 (2H, dt, J=8.5, 2.0 Hz), 7.03 (2H, dt, J=8.5, 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.1, 30.5, 30.6, 30.7, 33.1, 36.8, 37.7, 55.2, 116.2, 129.1, 131.2, 157.3, 175.1, 176.2; (+)-HR-ESIMS m/z 364.2485 $[M+H]^+$ (calcd for $C_{21}H_{34}NO_4$, 364.2488).

3.7.7. *N*-(*trans,trans*-2,4-Hexadienoyl)-L-tyrosine (15g). Yield: 17.0 mg (99%). Colourless oil. ν_{max} (thin film) 3600–3100, 1719, 1656 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.82 (3H, d, J=6.4 Hz), 2.90 (1H, dd, J=14.0, 8.6 Hz), 3.11 (1H, dd, J=14.0, 5.2 Hz), 4.66 (1H, dd, J=8.6, 5.2 Hz), 5.92 (1H, dd, J=15.2, 0.4 Hz), 6.06–6.23 (2H, m), 6.69 (2H, dt, J=8.6, 2.0 Hz), 7.03 (2H, dt, J=8.6, 2.0 Hz), 7.08 (1H, dd, J=15.5, 10.5 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 18.6, 37.8, 55.5, 116.2, 122.3, 129.1, 131.1, 131.2, 139.0, 142.7, 157.3, 168.8, 175.0; (+)-HR-ESIMS m/z 276.1237 $[M+H]^+$ (calcd for $C_{15}H_{18}NO_4$, 276.1236).

3.7.8. *N*-Dodecanoylglycine (15h). Yield: 12.5 mg (98%). White solid, mp 112–114 °C. ν_{max} (Nujol) 3600–3100, 3318, 1703, 1644 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.0 Hz), 1.24–1.32 (16H, m), 1.62 (2H, br tt, J=7.5, 7.5 Hz), 2.24 (2H, t, J=7.5 Hz), 3.89 (2H, s); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.8, 30.3, 30.5, 30.6, 30.7, 33.1, 36.8, 41.8, 173.2, 176.7; (+)-HR-ESIMS m/z 258.2067 $[M+H]^+$ (calcd for $C_{14}H_{28}NO_3$, 258.2069).

3.7.9. *N*-Dodecanoyl-L-alanine (15i). Yield: 17.1 mg (98%). White solid, mp 77–79 °C. ν_{max} (Nujol) 3600–3100, 3314, 1705, 1645 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.0 Hz), 1.24–1.32 (16H, m), 1.38 (3H, d, J=7.3 Hz), 1.61 (2H, br tt, J=7.3, 7.3 Hz), 2.22 (2H, br td, J=7.3, 1.3 Hz), 4.37 (1H, q, J=7.3 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 17.6, 23.7, 26.9, 30.3, 30.5, 30.6, 30.7, 33.1, 36.7, 176.1, 176.2; (+)-HR-ESIMS m/z 272.2224 $[M+H]^+$ (calcd for $C_{15}H_{30}NO_3$, 272.2226).

3.7.10. *N*-Dodecanoyl-L-phenylalanine (15j). Yield: 18.2 mg (99%). White solid, mp 95–96 °C. ν_{max} (Nujol) 3600–3100, 3310, 1707, 1606 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.1 Hz), 1.16–1.36 (16H, m), 1.48 (2H, tt, J=7.4, 7.4 Hz), 2.14 (2H, t, J=7.4 Hz), 2.92 (1H, dd, J=13.9, 9.7 Hz), 3.22 (1H, dd, J=13.9, 4.8 Hz), 4.67 (1H, dd, J=9.7, 4.8 Hz), 7.17–7.29 (5H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.1, 30.5, 30.6, 30.7, 33.1, 36.8, 38.4, 54.8, 127.7, 129.4, 130.2, 138.6, 174.8, 176.2; (+)-HR-ESIMS m/z 348.2534 $[M+H]^+$ (calcd for $C_{21}H_{34}NO_3$, 348.2539).

3.7.11. *N*-Dodecanoyl-L-aspartate (15k). Yield: 13.5 mg (98%). Pale yellow solid, mp 101–104 °C. ν_{max} (Nujol) 3600–3100, 3284, 1704, 1659 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.0 Hz), 1.24–1.32 (16H, m), 1.60 (2H, br tt, J=7.6, 7.6 Hz), 2.23 (2H, t, J=7.6 Hz), 2.77 (1H, dd, J=16.8, 7.1 Hz), 2.85 (1H, dd, J=16.8, 5.2 Hz), 4.73 (1H, dd, J=7.1, 5.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.2, 30.5, 30.6, 30.8, 33.1, 36.8, 37.0, 50.3, 174.0, 174.2, 176.1; (+)-HR-ESIMS m/z 316.2123 $[M+H]^+$ (calcd for $C_{16}H_{30}NO_5$, 316.2124).

3.7.12. *N*-Dodecanoyl-L-glutamate (15l). Yield: 22.0 mg (96%). White solid, mp 103–105 °C. ν_{max} (Nujol) 3600–3100, 3329, 1728, 1676 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.1 Hz), 1.24–1.32 (16H, m), 1.62 (2H, br tt, J=7.3, 7.3 Hz), 1.92 (1H, m), 2.18 (1H, m), 2.24 (2H, t, J=7.3 Hz), 2.40 (2H, t, J=7.8 Hz), 4.43 (1H, dd, J=9.3, 5.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 27.9, 30.3, 30.5, 30.6, 30.7, 31.3, 33.1, 36.8, 53.0, 175.1, 176.3, 176.5; (+)-HR-ESIMS m/z 330.2278 $[M+H]^+$ (calcd for $C_{17}H_{32}NO_5$, 330.2281).

3.7.13. *N*-Dodecanoyl-L-serine (15m). Yield: 15.0 mg (94%). White solid, mp 108–113 °C. ν_{max} (Nujol) 3600–3100, 3337, 1738, 1609 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.90 (3H, t, J=7.1 Hz), 1.24–1.36 (16H, m), 1.63 (2H, br tt, J=7.5, 7.5 Hz), 2.28 (2H, td, J=7.5, 1.6 Hz), 3.81 (1H, dd, J=11.2, 4.1 Hz), 3.89 (1H, dd, J=11.2, 4.9 Hz), 4.49 (1H, dd, J=4.9, 4.1 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.3, 30.47, 30.49, 30.6, 30.7, 33.1, 36.9, 56.1, 63.0, 173.5, 176.4; (+)-HR-ESIMS m/z 288.2178 $[M+H]^+$ (calcd for $C_{15}H_{30}NO_4$, 288.2175).

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