Isolation and Structure Elucidation of Punicalagin, a Toxic Hydrolysable Tannin, from *Terminalia oblongata*

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This paper describes the isolation and structure elucidation of two toxic tannins, named α- and β-punicalagin, from *Terminalia oblongata*, an Australian tree. Techniques used in the structure determination included Fast Atom Bombardment (FAB) mass spectrometry, and one- and two-dimensional NMR, UV, and IR spectroscopy. A number of microscale derivatisations were performed, followed by FAB analysis.

Terminalia oblongata (Yellow-Wood) is a bushy tree, 30 to 40 feet in height, which grows over a large area of Queensland, Australia.[1] Its leaves cause Yellow-Wood poisoning when consumed by cattle and sheep. The disease has been characterised by McCosker and Hunt.[2,3] Tannins form 20–29% of the leaf's dry weight and their toxicity has been implicated.[2] Tannins have also been held responsible for toxicity in *Thiloa glaucocarpa*[4] and in oak leaves.[5] This paper reports the identification of the major toxic tannin from *Terminalia oblongata* as punicalagin. Studies on the toxic properties of punicalagin, which causes liver damage, will be reported in a separate paper.[6]

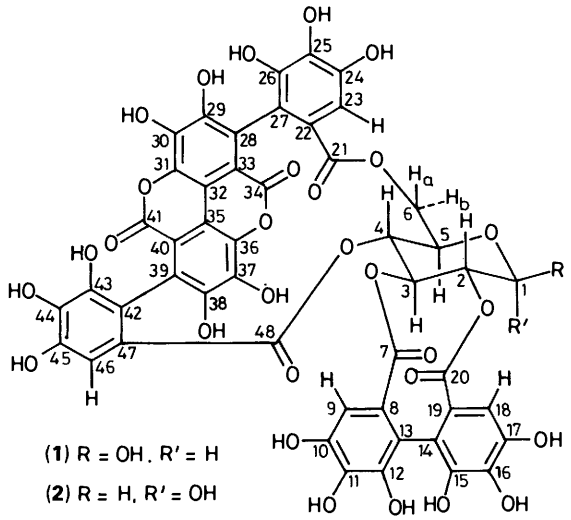
Results and Discussion

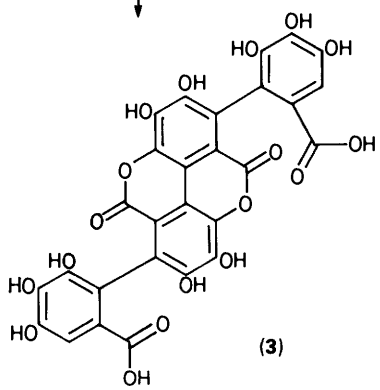
Two main components, labelled A (**1**) and B (**2**), were isolated from the *T. oblongata* tannin extract, by high-performance liquid chromatography (HPLC). HPLC and NMR analysis showed that components A and B interconverted in acidic aqueous solution. The equilibrium constant, K = [B]/[A] was ~1 in methanol and 4 in water. As compound B was present in higher concentration and was somewhat purer after isolation, it received the most attention. Both were found to be highly polar acids, and soluble in water, dimethyl sulphoxide (DMSO), methanol, ethanol, and acetonitrile.

The molecular weight and empirical formula of component B was determined by FAB mass spectrometry. Electron-impact mass spectra (EIMS) gave many low-abundance fragment ions, all below m/z 279. The compound gave a positive ion in FAB-MS at m/z 1 085 when 3-nitrobenzyl alcohol (NOBA) was used as matrix; no molecular ion was seen on using glycerol/thioglycerol, benzyl alcohol, or 1,2,6-trihydroxyhexane. A molecular weight of 1 084 daltons was confirmed by an intense peak at m/z 1 083 in the compound's negative-ion FAB spectrum, corresponding to the $(M - H)^-$ ion. A more abundant negative-ion molecular ion than positive ion is consistent with the presence of one or more acidic groups. In the high-resolution negative-ion FAB spectrum, the molecular ion occurred at m/z 1 083.0604, corresponding to an empirical formula of $C_{48}H_{27}O_{30}$ for $(M - H)^-$ and hence $C_{48}H_{28}O_{30}$ for B. Microanalysis confirmed that no nitrogen was present.

The functional groups present in component B were determined by a number of microscale derivatisations, followed by positive-ion FAB mass spectroscopic analysis in NOBA. The reactions, results, and conclusions were as follows.

Sodium borohydride reduction. No change, indicating no aldehydes or ketones were present.





Hydrogenation. No change, indicating no acetylenes, aldehydes, or alkenes were present.

Esterification with methanol–HCl. No change, indicating no carboxylic acids were present.

Acetylation with acetic anhydride–pyridine. Intense peaks were observed at m/z 1 799, 1 757, and 1 715, corresponding to the addition of 17, 16, and 15 acetyl groups to hydroxy groups.

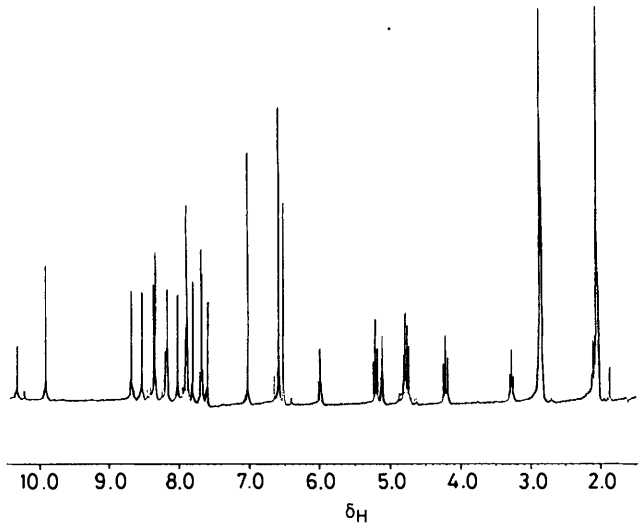


Figure 1. 400 MHz ^{1}H NMR spectrum of α-punicalagin (**2**) in [$^2H_6$]acetone at 20 °C.

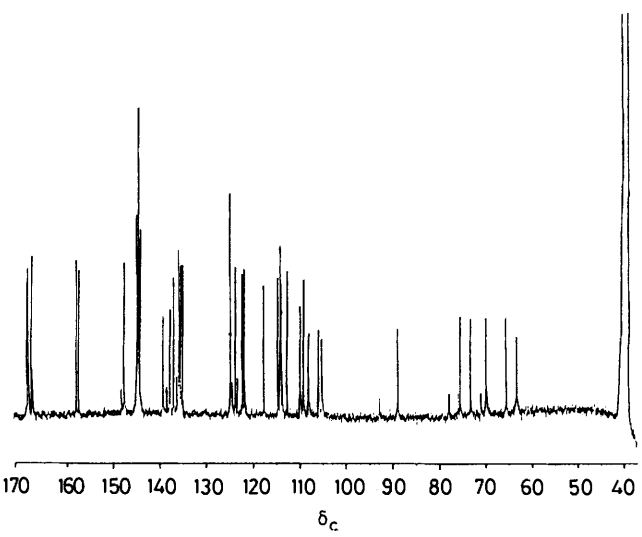


Figure 2. 100 MHz ^{13}C NMR spectrum of α-punicalagin (**2**) in $(CD_3)_2SO$ at 20 °C.

Table 1. ^{1}H NMR data of α-punicalagin (**2**) ([$^2H_6$]acetone).[a]

Proton	δ_H	Multiplicity
1a	5.11	t
1b	5.99	d
2	4.80	dd
3	5.21	t
4	4.76	t
5	3.27	td
6a	4.22	t
6b	2.09	dd
9	6.52/6.58	s
10	8.17/8.52/8.67	s
17	8.17/8.52/8.67	s
18	6.52/6.58	s
23	7.02	s
24	8.35	s
45	8.17/8.52/8.67	s
46	6.52/6.58	s

[a] Proton numbers are defined on structure (**1**) A slash (/) separates two possible chemical shifts for that proton. All signals reported here integrated for 1 H each. Unassigned signals of hydroxy group protons (1 H, s) are as follows.
δ_H: 7.59, 7.67, 7.68, 7.80, 7.88, 7.89, 7.89, 8.01, 8.16, 8.33, 9.90, and 10.31.
Protons: 11, 12, 15, 16, 25, 26, 29, 30, 37, 38, 43, and 44.

Table 2. Proton–proton coupling data of α-punicalagin (**2**).

Protons	$^3J_{HH}$ (Hz)
1a–1b	4
1a–2	4
2–3	9
3–4	9
4–5	10
5–6a	11
5–6b	1.5
	$^2J_{HH}$(Hz)
6a–6b	10

Methylation with diazomethane. Intense peaks were seen at *m/z* 1 281, 1 295, 1 309, 1 323, 1 337, 1 351, 1 365, 1 379, 1 393, 1 407, and 1 421, corresponding to the addition of 14–24 methyl groups. The most intense peak was at *m/z* 1 323, due to the methylation of 17 hydroxy groups. Further methylation was due to insertion reactions adjacent to carbonyls.

Acid hydrolysis. One peak could be identified at *m/z* 593, which could be attributed to $[(3) - CO_2H]^+$.

Basic hydrolysis. One peak was identified at *m/z* 593, as above.

Acetylation with acetic anhydride, water, and 1% triethylamine. No peaks were seen above *m/z* 500, indicating that component B hydrolysed under these conditions.

An IR spectrum of component B, in conjunction with the above results, showed that the only functional groups present were hydroxy groups, ester carbonyls, and aromatic rings [ν_{max} 3 350br s (OH), 1 715s (CO), 1 580m (Ar), and 1 500w cm^{-1} (Ar)]. UV spectroscopy showed the presence of an extended conjugated system [λ_{max}(EtOH) 421 (ε 1 000 dm^3 mol^{-1} cm^{-1}), 382 (2 000), 262 (13 000), and 209 (15 000)], which was also responsible for the bright yellow colour of the compound.

The ^{1}H NMR spectrum of component B is given in Figure 1 and the data are summarised in Table 1. The 17 hydroxy group signals occur at δ_H 5.99 and δ_H 7.59–10.31. In the NOESY[6] spectrum crosspeaks due to exchange were seen between each of these hydroxy protons and from each of these to water. Four aromatic protons were observed at δ_H 6.52–7.02 and there were seven sugar protons with signals at δ_H 2.09–5.21. The DQFCOSY[8,9] spectrum of component B showed all possible two- and three-bond proton–proton couplings with the exception of that from 5-H to 6-H^b. This was due to the small value of $^3J_{CH}$(1.5 Hz). A HOHAHA[10] spectrum confirmed that all the protons 1-H^a, 1-H^b, 2-, 3-, 4-, and 5-H, 6-H^a, and 6-H^b were in the same spin system. Magnetisation was transferred between protons separated by up to 5 bonds in the HOHAHA experiment. Coupling constants were measured from the one-dimensional spectrum and are listed in Table 2. These coupling constants showed that the sugar present was α-glucose. Only one free hydroxy group was attached to the sugar; this was the anomeric hydroxy group, whose proton appeared at δ_H 5.99.

The ^{13}C spectrum (Figure 2, Table 3) showed the presence of six carbonyls (δ_C 157–168), six carbons of a sugar (δ_C 63–89), and 36 aromatic carbons (δ_C 105–147). The total numbers of functional groups present were therefore six esters, one tetra-substituted sugar, six aromatic rings, 16 phenolic hydroxy groups, and one sugar hydroxy group. These carbons were

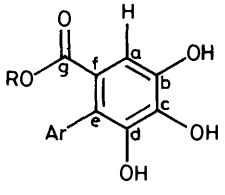
Table 3. ^{13}C NMR data of α-punicalagin (**2**) [$(CD_3)_2SO$].

Carbon	δ_C[a]	Carbon	δ_C[a]
1	89.0	25	137.0
2	70.0	26	144
3	75.6	27	117.7
4	73.3	28	109
5	65.6	29	147
6	63.3	30	137/139
7	168	31	135
8	124	32	112/113
9	105/108	33	121/122
10	144	34	157
11	135	35	112/113
12	144	36	135
13	114	37	137/139
14	114	38	147
15	144	39	109
16	135	40	121/122
17	144	41	157
18	105/108	42	114
19	124	43	144
20	168	44	135
21	167.6	45	144
22	123.8	46	105/108
23	109.2	47	124
24	144	48	168

[a] Chemical shifts recorded to 1 decimal place indicate a fully assigned, unique carbon signal. A slash (/) separates 2 possible chemical shifts for that carbon. A shift to 3 significant figures (*e.g.* 144) means there are a number of signals at that chemical shift which cannot be assigned to individual carbons.

Table 4. Single-bond C–H correlation (HMQC) for α-punicalagin (**2**).

Carbon	δ_C	δ_H	$^1J_{CH}$ (Hz)
1	89.0	5.11	161
2	70.0	4.80	139
3	75.6	5.21	144
4	73.3	4.76	139
5	65.6	3.27	145
6	63.3	4.22	146
9	105/108	6.52/6.58	150
18	105/108	6.52/6.58	150
23	109.2	7.02	150
46	105/108	6.52/6.58	150



(**1**)

Aromatic ring derived from HMBC experiment.

partially assigned by a one-bond carbon–hydrogen correlation[11,12] (Table 4). This two-dimensional NMR experiment gave crosspeaks between directly bonded carbons and protons. Crosspeaks appeared as doublets in the f_2 dimension, with the separation between the peaks giving the carbon–proton coupling constant. With six aromatic rings and only four directly bonded aromatic protons, the most useful experiment for the structural assignment was a two- and three-bond carbon–hydrogen correlation[13] (Table 5). These results enabled four aromatic rings to be constructed as in structure (**I**). Carbon–hydrogen couplings were observed from the proton attached to carbon a to carbons b, c, e, and g. The only possible crosspeak missing was to carbon f, since two-bond carbon–hydrogen couplings in aromatic rings are generally smaller than three-bond couplings. Carbon chemical shifts in these rings agreed closely with published values for identical systems.[14] Two- and three-bond carbon–hydrogen couplings were also detected from protons in the glucose ring. Protons at δ_H 4.22, 4.76, 4.80, 5.11, and 5.21 showed some expected couplings around the ring. The crosspeak between the proton at δ_H 5.11 and the carbon at δ_C 65.6 is a three-bond link across the spectroscopically silent oxygen. Crosspeaks were also seen from protons at δ_H 4.22, 4.76, 4.80, and 5.21 to carbonyl carbons at δ_C 167 and 168. These established the connectivities between the assigned aromatic rings and the glucose, *via* four ester linkages. The remaining groups in component B were two fully substituted aromatic rings, four phenolic hydroxy groups, and two esters. The ester carbonyls were at δ_C 157, which indicated that the ester group was attached to aromatic rings at both ends. The structure could therefore be fully assembled as structure (**2**). The proposed structure was consistent with the carbon chemical shifts (Table 3).

In the NOESY spectrum of α-punicalagin, the only crosspeaks observed were due to exchange between the hydroxy group protons and water. Similarly, no NOEs were seen in a number of one-dimensional NOE difference spectra of α-punicalagin. This can be attributed to a correlation time-effect.[15] Therefore a CAMELSPIN[16] experiment was performed. In the CAMELSPIN spectrum crosspeaks were observed due to Hartmann–Hahn transfer of magnetisation, chemical exchange between hydroxy group protons and water, and due to the nuclear Overhauser effect.[17] NOE crosspeaks (Table 6) were seen around the glucose ring and from the aromatic protons to adjacent hydroxy groups. This enabled four hydroxy groups to be identified (Table 1). It is noteworthy that the geminal protons 6-H^a and 6-H^b show a very large difference in chemical shift (4.22–2.09 = 2.13 ppm). This is readily explained by a CPK model of punicalagin which showed that proton 6-H^b is held over the face of an aromatic ring, causing a large upfield ring-current shift.

Compound B was first identified in pomegranate peel and was named punicalagin.[18] It has also been found in the leaves of *Terminalia catappa*[19] and the bark of *Punica granatum*[20] though its toxicity has not previously been reported. Published chemical shift data[21,22] are in agreement with the experimentally observed results. The location of the hexahydroxydiphenoyl and tetraphenoyl groups was established by Tanaka *et al.*[22]

Compound A was identified as the β-anomer of punicalagin, *i.e.* structure (**1**). Proton chemical shifts and coupling constants agreed with published data.[21] Compound A was shown to contain β-glucose by the one-dimensional ^1H NMR spectrum, which showed a 4 Hz coupling constant between the protons at positions 1 and 2 in the glucose ring. DQFCOSY and HOHAHA spectra confirmed the presence of glucose. The NMR data of component A are given in Tables 7 and 8. The isomerisation between components A and B is explained by interconversion of the two anomers, catalysed by aqueous acid. The phenol groups in punicalagin give rise to an acidic solution when the compound is dissolved in pure water.

Experimental

IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer for Nujol mulls. UV spectra were recorded on a Uvikon 810 spectrophotometer for methanol solutions. EIMS were recorded on Kratos MS30, MS902, or MS50 double-

Table 5. Long-range C–H correlation (HMBC) for α-punicalagin (**2**).

δ_H	δ_C
4.22	65.6, 167.6
4.76	75.6, 65.6, 168
4.80	70.0, 75.6, 168
5.11	65.6, 75.6
5.21	70.0, 168
6.52	168, 144, 135, 114
6.58a	168, 144, 135, 114
6.58b	168, 144, 135, 114
7.02	167.6, 144, 137.0, 117.7

These values correspond to correlations between the following atoms.

Proton	Carbon
6a	5, 21
4	3, 5, 7/20/48
2	2, 3, 7/20/48
1a	5, 3
3	2, 7/20/48
9/18/46	7/20/48, 10/12/15/17/24/26/43/45, 11/16/31/36/44, 13/14/42
9/18/46	7/20/48, 10/12/15/17/24/26/43/45, 11/16/31/36/44, 13/14/42
9/18/46	7/20/48, 10/12/15/17/24/26/43/45, 11/16/31/36/44, 13/14/42
23	21, 10/12/15/17/24/26/43/45, 25, 27

Table 6. CAMELSPIN data for α-punicalagin (**2**).[a]

δ_H	Protons
NOEs observed between	
2.09–3.27	6b–5
2.09–4.22	6b–6a
4.22–4.76	6a–4
3.27–5.21	5–3
3.27–5.99	5–1b
6.52–8.17	9/18/46–10/17/45
6.58–8.67	9/18/46–10/17/45
6.58–8.52	9/18/46–10/17/45
7.02–8.35	23–24
RELAYED crosspeaks observed between	
5.11–5.99	1a–1b
5.11–4.80	1a–2
4.80–4.76	2–4
4.80–5.21	2–3
4.76–5.21	4–3
3.27–4.22	5–6a

[a] Crosspeaks due to chemical exchange were also observed between many hydroxy group protons and water.

Table 7. ^{1}H NMR data of β-punicalagin (**1**) ([$^2H_6$]acetone).[a]

Proton	δ_H	Multiplicity
1a	4.71	d
2	4.63	dd
3	4.87	t
4	4.80	t
5	2.70	td
6a	4.18	t
6b	2.17	dd
9	6.51/6.60/6.66	s
18	6.51/6.60/6.66	s
23	7.01	s
46	6.51/6.60/6.66	s

[a] Hydroxy group protons were not observed. All signals reported here integrated for 1 H.

Table 8. Proton–proton coupling data of β-punicalagin (**1**).

Protons	$^3J_{HH}$ (Hz)
1a–2	8
2–3	9
3–4	9
4–5	9
5–6a	11
5–6b	2
6a–6b	11

focussing spectrometers. Low-resolution FAB mass spectrometry was performed on a Kratos MS50 instrument. For FAB in positive-ion mode, a sample of *ca.* 10 mmol was placed on the probe tip, matrix (1.5 μl) and perchloric acid (1 μl) were added, and the components were mixed before insertion into the spectrometer. In negative-ion mode, NOBA (2 μl) was used as matrix, with no acid. The sample was bombarded with a 4–6 keV beam of Xe atoms and an accelerating potential of 8 kV was employed. High-resolution FAB mass spectrometry was performed on a VG ZAB-2F instrument in the negative-ion mode. The sample was bombarded with a 9 kV beam of Xe atoms with an accelerating potential of 7 kV. The data were obtained using the Upjohn Physical and Analytical Chemistry Data System (UPACS II). For peak-matching an ion of polypropylene glycol was used ($C_{54}H_{109}O_{19}^-$, *m/z* 1 061.75631). The sample was dissolved in dimethylformamide and added to a mixture of 2-hydroxyethyl disulphide and polypropylene glycol until enough signal was apparent to obtain a good mass measurement. Microanalysis was performed by D. Flory and co-workers at the University Chemical Laboratories, Cambridge.

NMR spectroscopy was performed on a Bruker AM400 instrument, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. 5 mm Probes were used, a dedicated proton probe for proton only work and a DUAL probe or VSP probe for work involving carbon. Two samples of component B were prepared for NMR analysis: one containing 5 mg in [$^2H_6$]acetone for proton only work and another containing 22 mg in $(CD_3)_2SO$ for work involving carbon. Two-dimensional DQFCOSY and NOESY experiments were acquired with sweep widths of 3 300 Hz in both dimensions (2K data points in ω_2 and 512 t_1-values zero-filled to 1 K) in ω_1. A one second relaxation delay was used and 64 transients were accumulated for each t_1-value. HOHAHA experiments were recorded with the same parameters as the DQFCOSY and a mixing time of 65 ms. One-bond carbon–hydrogen correlation (HMQC) experiments were acquired with a sweep width of 17 200 Hz (2 K data points in ω_2) and ±1 450 Hz (512 t_1-values zero-filled to 1 K) in ω_1. The relaxation delay was 1.3 s and 48 transients were acquired for each t_1-value. The two- and three-bond carbon–hydrogen correlation (HMBC) used a 1 s relaxation delay, 64 transients for each t_1-value, and the same sweep widths as in the HMQC experiment. The HMQC and HMBC experiments were optimised for a value of $^1J_{CH}$ of 150 Hz. The HMBC was also optimised for values of $^2J_{CH}$ and $^3J_{CH}$ of 10 Hz. The CAMELSPIN experiment used the same parameters as the DQFCOSY and a 200 ms mixing time.

Isolation of the Tannins.—*T. oblongata* leaves were collected, dried, and milled by the method of Filippich *et al.*[6] The leaves (100 g) were then extracted with hot MeOH–water (1:1; 2 l). The extracts were combined, and evaporated to dryness under reduced pressure. The residue was dissolved in water (2 l). $Ca(OH)_2$ (50 g) was added and the mixture was kept for 2 h. The

heavy precipitate which formed was separated (centrifuge) and washed with water (3 × 500 ml). To the washed precipitate was added HCl (2M; 1 l) to decompose the calcium salts. After filtration, the clear aq. solution was added to polyamide (Woehm, 200 g) in a large sinter to absorb the toxins. The polamide was washed with water (1 l) until the eluate was neutral and the toxins were then displaced with MeOH (2 l). The polyamide was regenerated by washing with NH_4OH, followed by water. The MeOH eluate was dried under reduced pressure, redissolved in MeOH (50 ml) and added to an LH-20 column (5 × 50 cm). Fractions (50 ml) were taken and those containing the toxins were combined, dried under reduced pressure, redissolved in water, and added to an XAD-2 (SERVA) (2 × 90 cm) column. The toxins were displaced by use of a gradient of MeOH in water and the toxic fractions were combined. Further purification was achieved by preparative LH-20 (2.5 × 90 cm) column chromatography with MeOH as solvent to give a yield of toxins of *ca.* 1 g. Tannins A and B were separated by use of a 5–10% acetonitrile gradient in water on a Waters μBondapak C-18 reverse-phase semi-preparative HPLC column, with monitoring at 260 nm on a Cecil Instruments CE 212 UV detector and a flow rate of 8 ml min^{-1}. Isolated solutions of components A and B were frozen rapidly and lyophilised to dryness to minimise the time in aq. solution during which they could isomerise.

Toxicity Testing.—Male albino mice (30–40 g) were given a single intraperitoneal injection with sterile aq. solutions of the toxins at dose rates of 0.01–0.1 mg g^{-1} body weight. All the mice were sacrificed and autopsied 48 h after dosing (or sooner if the animals were severely affected) and liver and kidney sections were immediately fixed. Histopathology of the liver[6] showed both the α- and β-isomer of punicalagin caused liver necrosis at a dose rate of 0.04 mg g^{-1} or higher. The liver lesions were identical with those shown in animals dosed orally with the crude plant extract. No kidney lesions were observed in any animals dosed with the α- or β-isomer of punicalagin.

Sodium Borohydride Reduction.—Sodium borohydride (1 mg) was dissolved in sufficient absolute ethanol. The solution was slowly added to component B (0.1 mg) and the mixture was kept for 10 min. The ethanol was then removed under reduced pressure.

Hydrogenation.—Component B (2 mg) was dissolved in absolute methanol in a round-bottomed flask. The flask was evacuated and flushed with argon three times. 10% Palladium/charcoal (4 mg) was added and the flask was evacuated and flushed with hydrogen four times. The solution was stirred at room temperature for 1 h. The Pd/C was removed by filtration through Celite and the ethanol was removed under reduced pressure.

Esterification.—Hydrogen chloride gas was bubbled through conc. sulphuric acid to remove water and then through absolute ethanol (600 μl) for 5 min. Component B (0.1 mg) was added to the acidified solution which was left overnight. Ethanol was then removed under reduced pressure.

Acetylation of Hydroxy Groups.—Pyridine (400 μl) and acetic anhydride (400 μl) were added to component B (0.1 mg) and the solution was left for 5 h. Solvents were removed under reduced pressure and dil. sulphuric acid (600 μl) and chloroform (400 μl) were added. The acetylated product was extracted into chloroform which was then removed on a vacuum centrifuge.

Methylation.—Component B (0.1 mg) was dissolved in diethyl ether (1 ml). A solution of diazomethane in diethyl ether was added dropwise until the yellow colour of unchanged diazomethane persisted. The ether was then removed under reduced pressure.

Acid Hydrolysis.—Component B (0.1 mg) was heated under reflux in HCl (1M; 5 ml) for 10 min. The solution was cooled, frozen, and water was removed by lyophilisation.

Basic Hydrolysis.—Component B (0.1 mg) was heated under reflux for 10 min in water (4.5 ml) containing triethylamine (0.5 ml). The solution was cooled and triethylamine was removed under reduced pressure. Water was then removed by lyophilisation.

Acetylation of Amines.—Component B (0.1 mg) was added to a mixture of acetic anhydride (500 μl), water (500 μl), and triethylamine (10 μl). The solution was sonicated for 1 h until the aqueous and organic layers had thoroughly mixed. Solvents were then removed under reduced pressure.

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