O-Acetyl-O-butyryl-O-carbamoyl-O,O-dimethyl-α-cyclodextrins from the Cyanophyte *Tolypothrix byssoidea*

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The blue-green alga *Tolypothrix byssoidea* contains a 4:2:1 mixture of three *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O,O*-dimethyl-α-cyclodextrins. Extensive one- and two-dimensional proton and carbon NMR studies show that the structure of the major α-cyclodextrin is **1**.

The antineoplastic activity of *Tolypothrix byssoidea*, an epilithic, aerial blue-green alga belonging to the family Scytonemataceae, has been ascribed to tubercidin,[1] an antitumor agent isolated previously from *Streptomyces tubercidicus*.[2] The extract of *T. byssoidea* also exhibits cardiotonic activity (positive chronotropic and inotropic effects) and fungicidal activity. Tubercidin is responsible for part of the antimycotic activity and tyramine accounts for the chronotropic response in isolated mouse atria.[3] Two water-soluble substances, tolypophycins A and B, which have apparent molecular weights of 1717 and 1733, respectively, account for the inotropic activity and the remainder of the fungicidal activity.[3] The extract also contains a mixture of unusual *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O,O*-dimethyl-α-cyclodextrins which blocks the fungicidal activity of the tolypophycins against *Saccharomyces cerevisiae* but not the cardiotonic activity of the tolypophycins in isolated mouse atria. In this report we describe the structure determination of the major *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O,O*-dimethyl-α-cyclodextrin in this mixture.

Isolation. The cyanophyte was collected from the surface of a boulder near Manoa stream on the island of Oahu, HI, and mass cultured in the laboratory. The freeze-dried alga was extracted with 30:70 EtOH–H_2O and the aqueous concentrate was separated into six major fractions by gel filtration on Sephadex G-25. Fraction B (peak 2) contained the tolypophycins and fraction C (shoulder on the slower moving half of peak 2) contained the O-substituted α-cyclodextrins. Tyramine was found in the fraction D (peak 3) and fraction F (peak 5) consisted of essentially pure tubercidin. A 4:2:1 mixture of three α-cyclodextrin analogues was obtained by reverse-phase chromatography of fraction C on C-18 silica gel using 15:85 MeOH–H_2O. This inhibitor mixture could not be resolved by further chromatography. Acetylation of this mixture, however, with acetic anhydride in pyridine produced three tridecaacetate derivatives that could be separated by normal-phase HPLC on silica gel with 85:15 EtOAc–hexane followed by reverse-phase HPLC on C-18 silica gel using 65:35 MeOH–H_2O.

Structure Determination. Fast atom bombardment (FAB) mass spectrometry indicated that the three components in the mixture had molecular weights of 1155. The positive ion FAB mass spectrum showed a M + Na ion at 1178, whereas the negative ion FAB mass spectrum showed a M − H ion at 1154. The molecular weight of the major component **1** in the mixture was confirmed by the FAB mass spectrum of the major tridecaacetate derivative **2** (molecular weight = 1701). The positive ion FAB mass spectrum showed a M + H ion at 1702, whereas the corresponding negative ion FAB mass spectrum showed a M − H ion at 1700.

Table I. ^{1}H NMR Data for **2**[a]

Glc unit	chemical shift,[b] ppm H-1	H-2	H-3	H-4	H-5	H-6
A	5.081	4.743	5.656	3.867	4.34	4.28
						4.40
B	5.037	4.796	5.480	3.896	4.09	3.54
						4.09
C	4.998	4.681	3.96	3.650	4.03	4.29
						4.44
D	4.975	4.703	5.411	3.54	4.09	4.09[c]
						4.40
E	4.971	4.823	5.470	3.780	4.02	4.40
F	4.945	4.703	5.346	3.716	4.22	4.12
						4.536

Glc unit	coupling constant, Hz $J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
A	3.5	10.1	8.7	9.3
B	3.7	10.4	9.3	9.5
C	3.8	10.0	8.7	9.5
D	3.5	10.0	8.0	9.4
E	3.5	10.3	8.7	9.5
F	3.4	10.1	8.6	9.5

[a] Determined at 300 MHz in $CDCl_3$ by using residual $CHCl_3$ as internal reference (7.25 ppm). [b] Methoxyl signals at 3.668 and 3.339 ppm; acetate signals at 2.182, 2.118, 2.105, 2.102, 2.072 (2), 2.068, 2.035 (2), 2.030, 2.015 (2), 1.961, 1.954 ppm; butyrate signals at 2.347 and 2.380 (2 dt, α-CH_2, J = −15.9 and 7.5 Hz), 1.650 (sextet, J = 7.5 Hz, β-CH_2), 0.944 (t, J = 7.5 Hz, Me). [c] Assignment based on carbon–proton CSCM experiment.

Detailed analyses of the 300-MHz ^{1}H and 75-MHz ^{13}C NMR spectra of **2** (Tables I and II) suggested that the molecular formula of **1** was $C_{45}H_{73}NO_{33}$. This was corroborated by high-resolution FAB mass measurements of the 1178 ion (M + Na) of the cyclodextrin mixture (observed, 1178.3924; calculated for $C_{45}H_{73}NO_{33}Na$, 1178.3963) and the M + H ion of **2** (observed, 1702.5495; calculated for $C_{71}H_{100}NO_{46}$, 1702.5517).

Inspection of the 300-MHz ^{1}H and 75-MHz ^{13}C NMR spectra of the inhibitor in dimethyl sulfoxide-d_6 indicated that it was a 4:2:1 mixture of three *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O,O*-dimethyl-α-cyclodextrins. These spectra showed that each of these three compounds were monoacetate esters since there were three methyl proton signals

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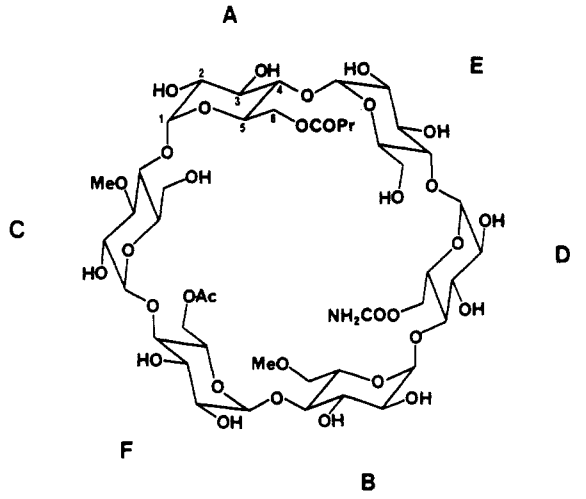

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(singlets) at 2.033, 2.024, and 2.015 ppm in a 4:2:1 ratio, three carbonyl carbon peaks at 172.73, 172.64, and 172.5 (unresolved shoulder) ppm in a 4:2:1 ratio, and three methyl carbon peaks at 20.57, 20.50, and 20.48 (unresolved shoulder) ppm in a 4:2:1 ratio, respectively. The three compounds were also monobutyrate esters. Unlike the acetate chemical shifts, however, the butyrate chemical shifts were essentially identical for the three triesters in the mixture; the proton signals were observed at 0.890 (triplet), 1.552 (1:5:10:10:5:1 sextet), and 2.30 (center of two overlapping doublets of triplets for two nonequivalent methylene protons) ppm and the carbon signals were found at 13.33, 17.92, 35.18, and 170.18 ppm. Carbamate ester functionalities (IR amide II band at 1640 cm^{-1}) were present in the three compounds. The carbamate chemical shifts, viz., 6.38 ppm for the amide protons and 156.45 ppm for the carbonyl carbon, were identical for the three compounds in the mixture. Finally, all three compounds were dimethyl ethers, each showing two methoxyl proton signals at 3.240 and 3.590 ppm. All three triesters had to be oligosaccharides. There were several proton signals in the 3.2–5.8 ppm region and at least five anomeric carbon signals between 101.2 and 102.3 ppm.

The ^1H NMR spectrum of the fully acetylated derivative **2** showed that the inhibitor was composed of six α-glucopyranose units (A–F). The chemical shifts and coupling constants for each α-glucopyranose unit (Table I) were determined by spin–spin decoupling experiments and two-dimensional NMR experiments, in particular COSY16[4] and single and double relayed coherence transfer (RCT) experiments.[5-7] In the contour plot of the conventional COSY16 spectrum of **2**, the signals for the methine protons in each of the six α-glucopyranose units in the molecule could be readily located by cross peaks, but the positions of the protons on C-6 could not be detected unambiguously, even using other solvents. The single and double RCT spectra,[6] however, exhibited additional cross peaks which allowed us to assign all of the intraunit connectivities. For example, two extra cross peaks appeared for the H-3 of unit A signal (5.656 ppm) in the single RCT spectrum, and the positions of the H-6 protons could be located with two of the extra cross peaks

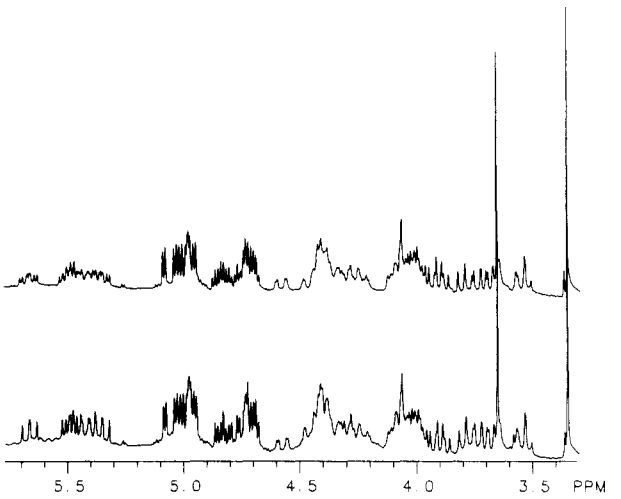

Figure 1. 300-MHz ^1H NMR spectrum (3.3–5.8 ppm) of compound **2** (lower trace) and the corresponding tridecaacetate-*1*-^{13}C **3** (upper trace) in $CDCl_3$.

Table II. ^{13}C NMR Data for 2[a]

types of carbons	chemical shift,[b,c] ppm
butyrate C=O	173.51
acetate C=O	170.93, 170.61, 170.50, 170.33 (2), 170.30, 170.26, 170.22, 170.02, 169.68, 169.38, 169.16, 169.11, 169.04
carbamate	155.93
C-1	98.09 (C), 97.82 (E), 97.39 (B), 96.99 (A), 95.79, 95.75
C-4	80.42 (C), 77.47 (D), 77.28 (A), 77.23 (F), 76.57 (E), 76.47 (B)
C-2	72.14 (C), 71.66, 70.95, 70.92, 70.32 (B), 70.21 (E)
C-3	78.73 (C), 72.13 (D), 71.97 (F), 70.79 (A), 70.50 (E), 69.95 (B)
C-5	71.00, 69.68 (C), 69.50, 69.47, 68.85 (A), 68.23 (F)
C-6	70.32 (B), 64.00 (D), 63.14 (F), 62.70 (C), 62.50, 62.48
OMe	61.49, 58.94
butyrate	35.57 (α-CH_2), 17.95 (β-CH_2), 13.3 (CH_3)

[a] Determined at 75 MHz in $CDCl_3$ by using solvent signal as internal reference (76.9 ppm). [b] Numbers in parentheses refer to numbers of carbons in signal; letters in parentheses refer to α-glucopyranose units. [c] Assignments are based on a carbon–proton CSCM experiment.

for the H-4 signal (3.867 ppm); the double RCT spectrum confirmed the H-6 assignments. The chemical shifts for the C-4 protons suggested that the six α-glucopyranose units were connected to each other 1,4. This meant that the hexasaccharide had to be an α-cyclodextrin.

The sequence of the six α-glucopyranose units could be determined by difference NOE spectroscopy.[8] Irradiation of the signal for H-1 in unit A, for example, produced a significant positive NOE in the signal for H-4 in unit C. Unit A was therefore linked 1,4 to unit C. Similarly selective irradiations of the signals for H-1 in units B, C, and F produced positive NOEs in the signals for H-4 in units D, F, and B, respectively. Units B, C, and F were therefore connected 1,4 to units D, F, and B, respectively. Irradiation of the overlapping signals for H-1 in units D and E produced positive NOEs in the signals for H-4 in units A and E. For the latter NOE experiment, however, it had to be concluded that unit D was linked 1,4 to unit E and unit E was connected 1,4 to unit A.

The sequence of the α-glucopyranose units was corroborated by a long-range COSY16 experiment, using a 400-

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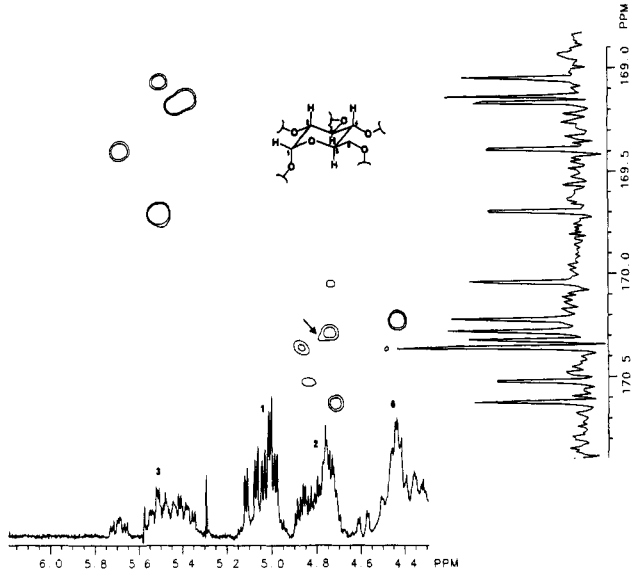

Figure 2. Long-range ^1H–^{13}C chemical shift correlation map (CSCM) of compound **3** in $CDCl_3$. The arrow points to the partially resolved cross peak for the acetate carbonyl carbon signal at 170.30 ppm and the H-2 of glucose unit A signal (4.743 ppm). Only one cross-peak is observed in this spectrum between one of the acetate carbonyl carbon signals at 170.33 ppm and the signals for the nonequivalent H-6 protons in unit F (4.536 and 4.12 ppm).

ms delay before the evolution and detection periods.[9] Inspection of the 2D map showed significant cross-peaks between the anomeric protons of units, A, C, E, and F and the C-4 protons of units C, F, A, and B, respectively. In this delayed COSY16 experiment cross-peaks were also seen between the anomeric and C-5 protons in units A, D, E, and F, providing further proof that these units were α-glucopyranose units.

The chemical shift data for the tridecaacetate indicated that the methoxyl groups were on C-3 in unit C (H-3 at 3.96 ppm) and C-6 in unit B (H-6s at 3.54 and 4.09 ppm).

The free hydroxyl groups in the inhibitor were located by ^1H NMR analysis of the tridecaacetate-*1*-^{13}C (**3**), formed by treating **1** with acetic anhydride-*1,1*-$^{13}C_2$ in pyridine. Each carbon bearing an acetate-*1*-^{13}C group showed a three-bond coupling of the proton(s) on that carbon to the ^{13}C of the acetate carbonyl (3.7 Hz) (Figure 1). Twelve of the 13 hydroxyl groups in **1** were clearly on the C-2 and C-3 positions of the six α-glucopyranose units (except C-3 in unit C which bears a methoxyl group) and C-6 of unit C. These assignments were confirmed by a long-range heteronuclear chemical shift correlation map (CSCM) of **3**,[4] which also showed that the remaining hydroxyl group was on C-6 of unit E (Figure 2).

The acetate, butyrate, and carbamate ester groups of **1** had to be on C-6 of units A, D, and F as these were the only unassigned positions. Their locations were determined by a long-range CSCM spectrum of **2** (Figure 3) as follows:

First of all, examination of the ^1H NMR spectrum of **3** revealed which of the 14 acetate methyl signals in **2** belonged to the naturally occurring acetate group. It was the one at lowest field (2.182 ppm), since this signal was a singlet whereas all of the rest were doublets ($^2J_{CH}$ = 6.9 Hz) in the ^1H NMR spectrum of **3**. In the long-range CSCM spectrum of **2**, the carbonyl carbon signal at 170.93 ppm correlated with the acetate methyl signal at 2.182 ppm. Although the contour plot of our first experiment

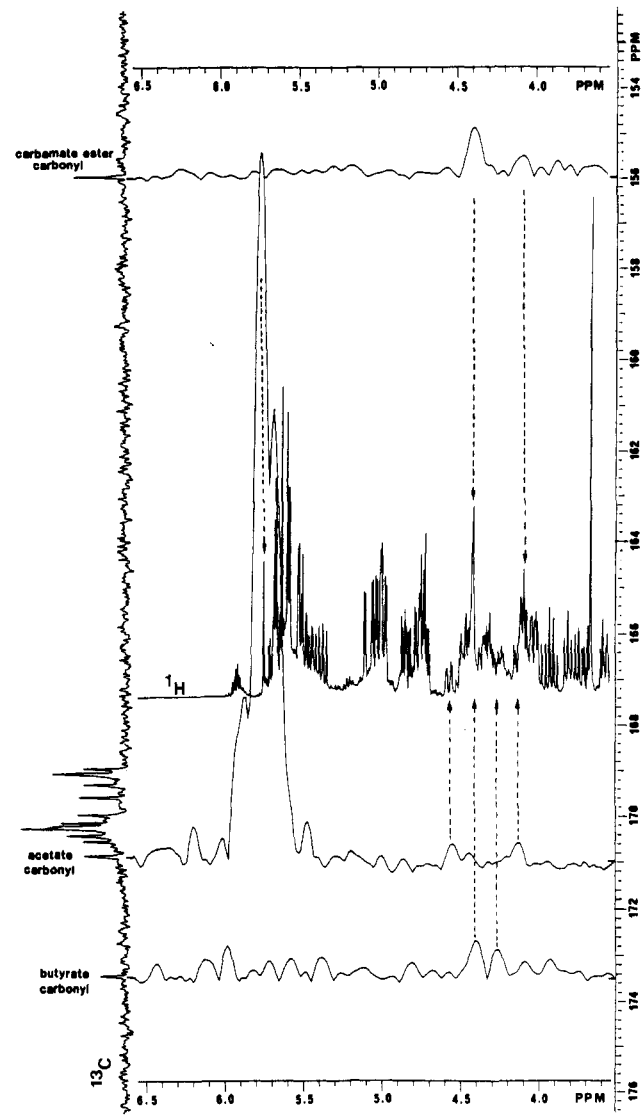

Figure 3. Long-range ^1H–^{13}C CSCM of compound **2** in $CDCl_3$. The proton spectral window, which was narrowed in this 2D experiment to conserve digital resolution, results in a foldover of the high-field signals (0.9–2.4 ppm) onto the signals in the 4.5–5.8 region. F1 slices are shown for the carbonyl carbon signals at 155.93, 170.93, and 173.51 ppm.

showed a relatively strong cross-peak connecting the carbonyl carbon and methyl proton signals, we were not able to see cross-peaks that unambiguously established the connectivity of the acetate carbonyl carbon and the C-6 protons of either unit A, D, or F. In a second experiment, however, in which the delay time was increased to favor detection of the smaller coupling through oxygen between the butyrate carbonyl carbon and the C-6 protons, the one-dimensional proton spectrum (F_1 slice) corresponding to the carbonyl carbon signal at 170.93 ppm showed two very small peaks at 4.54 and 4.12 ppm which were slightly but consistently above noise level in two separate trials (Figure 3). The data suggested that the acetate ester group was on C-6 of unit F.

Second, in the long-range CSCM spectrum of **2**, the carbonyl carbon signal at 173.51 ppm was found to correlate with the signals for the nonequivalent α-methylene protons (2.347 and 2.380 ppm) and the equivalent β-methlene protons (1.650 ppm) of the butyrate ester group. Again the contour plot of our first experiment, which showed significant cross-peaks connecting the carbon signal at 173.51 ppm, now assigned to the butyrate carbonyl

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carbon, and the α- and β-methylene proton signals, did not show unambiguous cross-peaks between the carbonyl carbon signal and signals for the C-6 protons on either unit A or D. In our second experiment, however, the F_1 proton slice corresponding to the carbon signal at 173.51 ppm did show small peaks at 4.28 and 4.40 ppm which were slightly, but consistently above noise level in two separate trials (Figure 3). The butyrate ester group therefore appeared to be on C-6 of unit A.

By process of elimination, the carbamate ester group was placed on C-6 of unit D. This was supported by both of our long-range CSCM experiments on **2**. In the first experiment the contour plot showed cross-peaks connecting the carbamate carbonyl carbon signal at 155.93 ppm and C-6 proton signals at 4.40 and 4.09 ppm. Similarly in the second experiment the F_1 slice corresponding to the carbon signal at 155.93 ppm showed proton signals at 4.40 and 4.09 ppm (Figure 3).

Relative and Absolute Stereochemistry. No experiments were carried out to determine the relative stereochemistry of the six α-glucopyranose units in **1**. The six α-glucopyranose units of **1** appear to be all D on the basis of the optical properties of **2**. α-Cyclodextrin from *Bacillus macerans* is composed of six α-(1→4)-linked D-glucopyranose units. The reported optical rotations[10] of α-cyclodextrin and its octadecaacetate are $[\alpha]_D$ +150.5° (*c* 1.0, H_2O) and $[\alpha]_D$ +105.5° (*c* 1.0, $CHCl_3$), respectively. The optical rotation of compound **2** is $[\alpha]_D$ +112° (*c* 1.1, $CHCl_3$).

Experimental Section

Spectral Analysis. NMR spectra were determined at 300 and 75 Mz for ^1H and ^{13}C, respectively. Proton chemical shifts were referenced in $CDCl_3$ to the residual $CHCl_3$ signal (7.25 ppm) and in dimethyl sulfoxide-d_6 to the residual Me_2SO-d_5 signal (2.52 ppm). Homonuclear ^1H connectivities were determined by using the phase-cycled 16-step COSY experiment as described by Bax[4] and the homonuclear single and double relayed coherence transfer experiments as described by Wagner.[6,11] Confirmatory information and coupling constant data were obtained from normal and difference double-resonance experiments when possible. ^1H–^{13}C connectivities were determined from a phase-cycled 16-step heteronuclear chemical shift correlation experiment.[4]

Qualitative NOEs were determined by using the transient NOE method.[8] The proton signal was inverted using a long (approximately 60 ms) selective pulse from the decoupler in CW mode. The spins were allowed to experience selective inversion for approximately 0.75 s, at which time a nonselective 90° pulse was used to acquire the data. Sets of 600 transients of on- and off-resonance data were collected, processed, and subtracted. The difference spectra were then block averaged for a given proton inversion.

Isolation. *Tolypothrix byssoidea* (strain H-6-2) was cultured as previously described.[1] The freeze-dried alga (0.66 kg) was ground for 2 min with 12 L of 30:70 EtOH–H_2O in a Waring blender. After shaking for 6 h and standing overnight, the mixture was centrifuged at 7500*g*. The clear supernatant was concentrated in vacuo to 2 L, and the concentrate was lyophilized. The freeze-dried extract was reconstituted in 1.4 L of water, and 650 mL of acetone was added. After the mixture was stirred overnight, the precipitate was removed by centrifugation at 7500*g*. The supernatant was concentrated to 1.3 L. Analysis of a small aliquot indicated that 110 g of extract was present. The wet precipitate from the first centrifugation was resuspended in 95% ethanol. After standing overnight, the mixture was filtered and evaporated to dryness to give an additional 36.5 g of extract, which was combined with the supernatant. Water was added to bring the total volume to 2 L.

The extract, in 200-mL portions, was applied to a 3700-mL column of Amberlite XAD-2. After the column was washed with 3.5 L of water, antifungal-active material (total 28.5 g) was eluted with 70:30 EtOH–H_2O. Aliquots (4.5 g) of this 70% ethanol fraction were chromatographed over 4700 mL of Sephadex G-25 packed in a 10 × 100 cm column by using 0.03 M ammonium acetate pH 5.1 buffer. The chromatography was monitored by ultraviolet spectroscopy and several fractions were collected: 1710–2700 (fraction A), 2700–3150 (fraction B), 3150–3600 (fraction C), 3600–4110 (fraction D), 4110–4810 (fraction E), 4810–5810 mL (fraction F). Fractions D and F contained tyramine[3] and tubercidin,[1] respectively. Fraction B, which showed significant antifungal activity against *S. cerevisiae*, contained tolypophycins A and B.[3] Fraction C, which blocked the fungicidal activity of fraction B, contained a 4:2:1 mixture of *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O*,*O*-dimethyl-α-cyclodextrins. Reverse-phase HPLC of fraction C on a Whatman ODS-3 Partisil M9 column in 100-mg portions using 10:90 MeOH–H_2O gave 1.3 g of the α-cyclodextrin mixture.

Compound 2. The 4:2:1 mixture of α-cyclodextrins (20 mg) was acetylated with acetic anhydride–pyridine (2:1) at room temperature. The crude polyacetate was purified by HPLC on silica gel (whatman Partisil) with 85:15 EtOAc–hexane. Three peaks were obtained and the second peak, which was the major one, contained crude **2**. Further purification by reverse-phase HPLC on ODS silica gel with 65:35 MeOH–H_2O gave 16 mg of pure **2**: $[\alpha]_D$ +112° (*c* 1.1, $CHCl_3$); ^1H and ^{13}C NMR data are listed in Tables I and II. Compound **3** was produced in a similar manner by using acetic anhydride-*1,1*-$^{13}C_2$ (99 atom %).

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Supplementary Material Available: Six figures depicting the elution profile of the *T. byssoidea* extract on Sephadex G-25 and the antifungal assay, the 300-MHz ^1H NMR spectrum of the 4:2:1 mixture of *O*-acetyl-*O*-butyryl-*O*-carbamoyl-*O*,*O*-dimethyl-α-cyclodextrins, COSY 16, RCT, and RCT2 spectra of **2**, and comparison of high-field region (0.8–2.5 ppm) of ^1H NMR spectra of **2** and **3** (7 pages). Ordering information is given on any current masthead page.

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