Butyrolactones from *Aspergillus terreus*

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In the process development of lovastatin using *Aspergillus terreus* DRCC 152 in solid state fermentation, we have isolated a new butyrolactone-IV (3) along with the previously reported butyrolactone-I (1) and butyrolactone-II (2) produced under submerged conditions. The structure of compound 3 has been characterized as 3-hydroxy-5-{2-(1-hydroxy-1-methylethyl)-2(*R*)-2,3-dihydro-benzo[*b*]furan-5 ylmethyl}-4-(4-hydroxyphenyl)-5-methoxycarbonyl-(5*R*)-2,5-dihydro-2-furanone on the basis of spectroscopic studies. The absolute stereochemistry has been determined by single crystal X-ray diffraction studies. The cytotoxic and antibacterial activities of these compounds were determined.

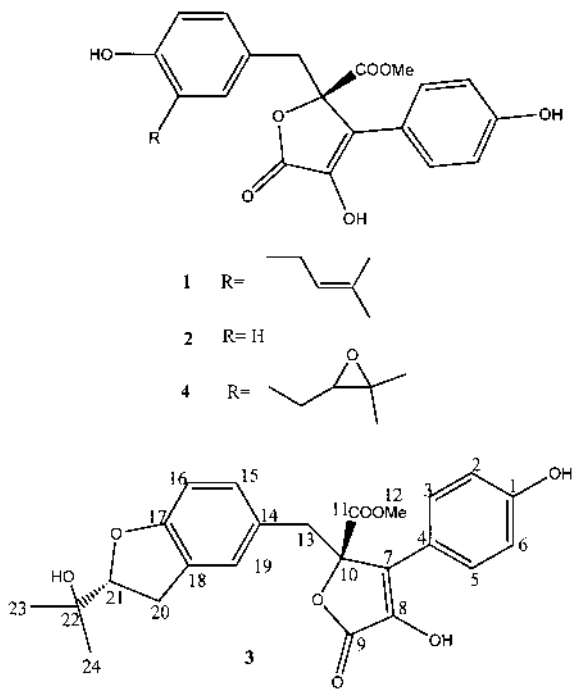
Key words *Aspergillus terreus*; solid state fermentation; butyrolactone; biological activity

We have successfully developed a novel process for the production of lovastatin[1] using *A. terreus* DRCC 152. In the course of our investigations on the secondary metabolites of *A. terreus* in solid state fermentation, we have isolated a new butyrolactones (**3**) along with two known butyrolactones, butyrolactone-I (**1**) and butyrolactone-II (**2**) from the ethyl acetate extract of solid substrate of *A. terreus*. In the present communication we describe the fermentation, isolation and characterization of these butyrolactones (**1**, **2** and **3**) and their biological activities.

Compounds **1** and **2** were known in the literature (with partial NMR data) as butyrolactone I (**1**) and II (**2**), respectively.[2,3] The ^{1}H- and ^{13}C-NMR data of **1** and **2** were unambiguously assigned with the help of 2D-NMR experiments *viz*., double-quantum filtered-correlation spectroscopy (DQF-COSY), nuclear Overhauser enhancement exchange spectroscopy (NOESY), heteronuclear correlation (HETCOR) and long range HETCOR (LRHETCOR) spectra.

Compound **3** was obtained as colorless crystals and showed IR absorption bands at 3425 and 1739 cm^{-1} which indicated the presence of hydroxyl and ester carbonyl groups, respectively. The molecular formula was deduced to be $C_{24}H_{24}O_8$ with 13 unsaturations from MS and ^{13}C-NMR spectral data. The twenty-four carbon signals and their multiplicities were determined by distortionless enhancement by polarization transfer (DEPT).

The ^{1}H-NMR spectrum showed a 4H A_2B_2 system at δ 7.5 (2H, d, J=8.6 Hz) and 6.9 (2H, d, J=8.6 Hz), indicating the presence of a *para*-disubstituted phenyl group in **3**. An AB quartet at δ 6.6 (J=9.0 Hz) integrating for two protons and a singlet at δ 6.5 integrating for one proton, suggested the presence of a tri-substituted benzene ring. The presence of a methylene group and a methoxy group was inferred from the signals at δ 3.4 (2H, s) and δ 3.8 (3H, s). ^{1}H-NMR signal (eventually shown to be that of H-21) at δ 4.5 (1H, t, J=7.6 Hz) was coupled (as shown by COSY) to a multiplet at δ 3.0 and to two overlapped methyl singlets at δ 1.1 (a *gem*-dimethyl group). This is characteristic of a furan ring formed from the prenyl residue with the oxygen function on C-17. Heteronuclear couplings between C-18 with the proton of C-20 (Fig. 2) defined the location of this isoprenoid moiety involving the carbon atom C-18. The ^{1}H-NMR spectrum of **3** displayed a long-range hetero nuclear coupling between a proton at δ 4.5 and the C-24 methyl carbon. On the basis of these results the structure of **3** was determined as 3-hydroxy-5-{2-(1-hydroxy-1-methylethyl)-2,3-dihydro-benzo[*b*]furan-5 ylmethyl}-4-(4-hydroxyphenyl)-5-methoxycarbonyl-2,5-dihydro-2-furanone. The structure was supported by 2D-NMR experiments [DQF-COSY, HETCOR, NOESY (Fig. 1) and LRHETCOR (Fig. 2)].



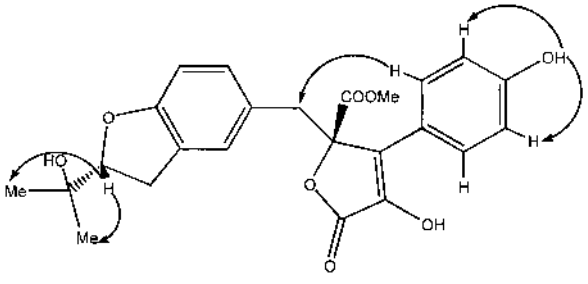


Fig. 1. NOE Correlations

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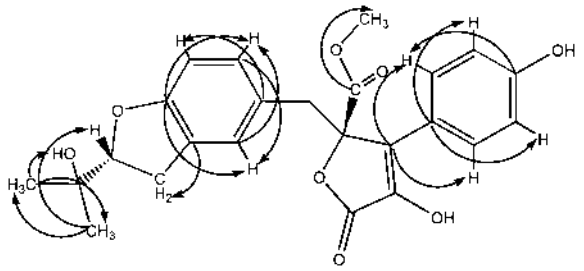

Fig. 2. LRHETCOR Correlations

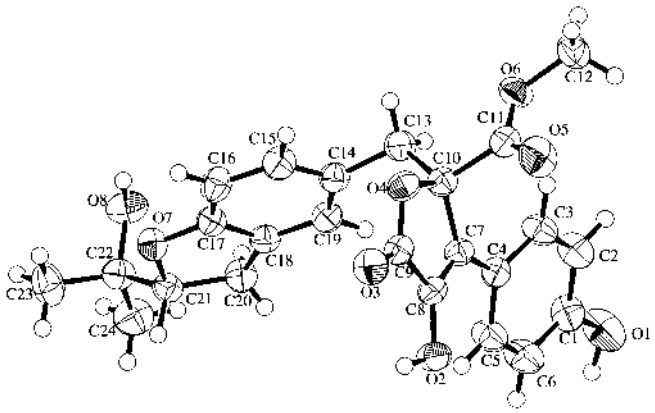

Fig. 3

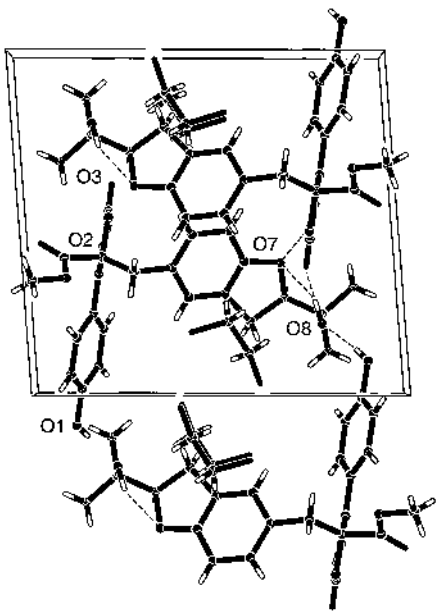

Fig. 4

The unambiguous confirmation of the structure came from single crystal X-ray analysis. The ORTEP representation of the molecular structure of **3** is shown in Fig. 3. The molecule crystallizes as a dichloroethane solvate. The 1 : 1 ratio of molecule to solvent (dichloroethane) has been established by elemental analysis. By collecting the X-ray intensity data of Bijvoet pairs and from using the anomalous scattering of the chlorine atoms, the absolute stereochemistry of both chiral centres in butyrolactone IV are determined to be *R*. The hydrogen bond interactions (Table 1) stabilizes the molecule in the lattice (Fig. 4).

Table 1. The Hydrogen Bond Interactions of **3**

Donor	H	Acceptor	Sym. code of acceptor	D…A	D-H	H…A	D-H…A
O(1)	H(26)	O(8)	54602	2.703(4)	1.00	1.75	158.9
O(2)	H(27)	O(7)	64602	2.912(3)	0.97	2.04	149.0
O(8)	H(28)	O(3)	65602	2.917(4)	1.01	2.01	148.9
O(8)	H(28)	O(7)	1	2.838(3)	1.01	2.33	110.0

Table 2. Cytotoxic Activities of **1**—**4** on Human Cancer Cell Lines

Cell line	Percentage growth (10^{-5} M)			
	1	**2**	**3**	**4**
MCF-7/ADR	60.9	100	46.7	83
U251	57.6	100	67.3	91
SW620	96.4	75	76.7	54
H522	100	100	100	38
M14	100	100	77.7	100
SKOV3	100	70.3	53.6	100
DU145	94.6	74	47.4	90
A498	73	62.6	62.6	16

Data are mean±S.D. from three separate experiments.
Cell lines: MCF-7/ADR, human breast cancer; U251, human CNS cancer; SW620, human colon cancer; H522, human lung cancer; M14, human melanoma; SKOV3, human ovarian cancer; DU145, human prostate cancer; A498, human renal cancer.

Butyrolactone III (**4**), an isomer of **3** was synthesized from **1** and the spectral data was compared. The biological activities of **4** were also determined.

Cytotoxicity of **1**—**4** was measured *in vitro* using both sensitive and multi-drug resistant (MCF7/ADR) cell lines. As shown in Table 2, all the compounds exhibited mild cytotoxic activity. No detectable antibacterial activity was noticed against Gram (+) and Gram (−) strains at a concentration of 100 μg/disc.

Experimental

General Procedures Melting points (mp) were determined on Buchi micromelting point apparatus without corrections. Optical rotations were determined using JASCO polarimeter. UV spectra were taken in MeOH on a Shimadzu Model 2100S double beam UV-visible spectrophotometer, and FT-IR spectra were on a Perkin Elmer 1650 FT-IR spectrophotometer. Elemental analyses were performed on Perkin Elmer Series II CHN analyser 2400. NMR spectra were recorded on a Varian Gemini 200 NMR spectrometer in dimethyl sulfoxide (DMSO-d_6) using TMS as internal standard. Mass spectra were obtained in the EI mode at 70 eV.

Isolation of butyrolactones (1, 2 and 3) *Aspergillus terreus* DRCC 152, a mutant developed from ATCC 20542, was cultivated on sterilized wheat bran (1 kg) consisting starch (100 g), millet flour (100 g) and ragi flour (100 g) at 27±1 °C for 9 d in stainless steel trays (775 mm×375 mm×50 mm). The moldy bran (1 kg) was extracted with 10 l of EtOAc and the extact was treated with 0.25 M Na_2CO_3. The aqueous layer was extracted with a mixture of hexane–EtOAc (2 : 1) at pH 8.0 and 4.0 to obtain respectively fraction I and fraction II. Fraction I (5 g) was subjected to flash chromatography over silica gel using 1,2-dichloroethane–EtOAc (8 : 2). A total of 50 fractions, each *ca*. 25 ml, were collected. Fractions 9—12, were combined and upon concentration gave the residue (650 mg) and was recrystallized from hexane to give 550 mg of butyrolactone I (**1**). Fractions 23—45 upon removal of solvent followed by recrystallization yielded 600 mg of butyrolactone II (**2**).

Fraction II (7 g) was subjected to flash chromatography over silica gel using 1,2-dichloroethane–EtOAc (8 : 2). A total of 17 fractions, each *ca*. 25 ml, were collected. Combined fractions 7—12 were evaporated and the residue (600mg) recrystallized from chloroform–EtOAc (9 : 1) to give

Table 3. ^{1}H- and ^{13}C-NMR Data of **3** in DMSO-d_6 (200 MHz)

Atom	**3**	
	^{1}H δ (Hz)	^{13}C ppm
1	—	157.89
2	6.9 (d, J=8.6)	115.79
3	7.5 (d, J=8.6)	128.83
4	—	121.00
5	7.5 (d, J=8.6)	128.83
6	6.9 (d, J=8.6)	115.79
7	—	127.64
8	—	138.13
9	—	167.89
10	—	84.76
11	—	169.69
12	3.8 (s)	53.36
13	3.4 (s)	38.26
14	—	124.42
15	6.6 (d, 9.0)	126.61
16	6.6 (d, 9.0)	107.77
17	—	158.79
18	—	126.85
19	6.5 (s)	129.40
20	3.0 (m)	29.75
21	4.5 (t, J=7.6)	89.04
22	—	69.95
23	1.1 (s)	24.84
24	1.1 (s)	25.91
1-OH	10.0 (s)	—
8-OH	—	—
17-OH	—	—
22-OH	4.5 (s)	—

125 mg of butyrolactone IV (**3**).

Butyrolactone I (**1**): Colorless crystals (hexane), mp 95 °C (dec). ^{13}C-NMR (DMSO-d_6) δ: 17.5 (C-24), 25.5 (C-23), 27.6 (C-20), 38.1 (C-13), 53.4 (C-12), 84.8 (C-10), 114.1 (C-16), 115.8 (C-2, 6), 121.1 (C-4), 122.4 (C-21), 123.1 (C-14), 126.5 (C-18), 127.5 (C-7), 128.4 (C-15), 128.8 (C-3, 5), 131.0 (C-19), 131.4 (C-22), 138.1 (C-8), 153.8 (C-17), 157.9 (C-1), 167.9 (C-9), 169.8 (C-11).[4)]

Butyrolactone II (**2**): Colorless crystals (EtOAc), mp 94—96 °C. ^{13}C-NMR (DMSO-d_6) δ: 38.2 (C-13), 53.5 (C-12), 84.9 (C-10), 114.8 (C-16), 116.0 (C-2, 6), 121.2 (C-4), 123.4 (C-14), 114.8 (C-18), 127.7 (C-7), 129.0 (C-3, 5), 131.3 (C-15), 131.3 (C-19), 138.3 (C-8), 156.4 (C-17), 158.1 (C-1), 168.1 (C-9), 169.9 (C-11).[4)]

Butyrolactone IV (**3**): Colorless crystals ($CHCl_3$: EtOAc); mp 110—112 °C; $[\alpha]_D^{27}$ +92° (c=0.7, EtOH); UV λ_{max}^{MeOH} nm 309, 227, 204. IR cm^{-1} 3425, 2974, 1739, 1610, 1519, 1385, 1247, 1180, 1036, 839, 583. ^{1}H- and ^{13}C-NMR: Table 3. (Calcd for $C_{24}H_{24}O_8 \cdot C_2H_4Cl_2$, C, 57.9; H, 5.2% Found: C, 58.6; H, 5.1%). MS *m/z* (%): 440 (M^+, 12), 396 (28), 364 (25), 347 (20), 306 (30), 278 (18), 249 (37), 207 (33), 191 (22), 177 (23), 131 (100), 119 (51), 107 (42), 91 (41).

Single Crystal Analysis of Butyrolactone IV (3) Crystal data: $C_{24}H_{24}O_8 \cdot C_2H_4Cl_2$, M.W.=538, monoclinic, a=12.066(1), b=8.167(1), c=13.299(1) Å, β=94.37(1)° V=1306.8(2) Å^3, space group $P2_1$(#4), Z=2, D_{calc}=1.37 g·cm^{-3}. λ(Cu-K$_\alpha$) 1.5418 Å. μ(Cu-K$_\alpha$) 26.45 cm^{-1}. Intensity data were measured on a Rigaku AFC7S diffractometer up to 2θ of 155.3°. A total of 5721 reflections were collected out of which 2888 reflections were unique (R_{int}=0.03) and 2700 were considered observed [$I>3\sigma(I)$]. Intensity data were corrected for Lorentz, polarization and absorption (psi-scan) effects. The structure was solved by direct methods (SIR92).[5)] The structure was refined by full matrix least squares procedures by TEXSAN[6)] software. In view of the presence of heavy chlorine atoms in the structure, Bijvoet pairs were collected and the absolute configuration was determined. The hydrogens of the hydroxyl groups were located from a difference Fourier map while the remaining hydrogen atoms were placed at the calculated positions based on the geometry. The agreement indices were R(F)=0.049, R_w(F)=0.075 with anisotropic refinement done on all non-hydrogen atoms. Final atomic coordinates are listed in Table 4.

Table 4. Positional Parameters and B (eq) for Butyrolactone IV

Atom	x	y	z	B (eq)
Cl (1)	0.1796(2)	0.6572	0.4192(1)	11.66(7)
Cl (2)	0.0206(1)	0.6585(6)	0.6113(1)	8.72(5)
O (1)	−0.0988(2)	−0.0550(7)	0.0971(2)	5.59(7)
O (2)	0.4354(2)	−0.1988(6)	0.2040(2)	3.64(5)
O (3)	0.6360(2)	−0.0028(6)	0.2464(2)	4.10(5)
O (4)	0.5227(2)	0.2152(6)	0.2306(2)	3.29(4)
O (5)	0.4493(2)	0.2923(7)	0.0343(2)	5.03(6)
O (6)	0.3410(2)	0.4730(6)	0.1065(2)	3.67(4)
O (7)	0.3898(2)	0.1087(6)	0.6892(1)	3.26(4)
O (8)	0.2087(2)	0.2337(6)	0.7898(2)	4.15(5)
C (1)	0.0117(2)	−0.0292(7)	0.1210(2)	3.80(6)
C (2)	0.0554(2)	0.1210(7)	0.0957(3)	3.95(7)
C (3)	0.1662(2)	0.1562(7)	0.1190(2)	3.57(6)
C (4)	0.2372(2)	0.0409(7)	0.1675(2)	2.96(5)
C (5)	0.1930(3)	−0.1106(7)	0.1904(3)	3.88(7)
C (6)	0.0814(3)	−0.1452(7)	0.1691(3)	4.45(8)
C (7)	0.3555(2)	0.0737(6)	0.1909(2)	2.76(5)
C (8)	0.4387(2)	−0.0345(7)	0.2075(2)	2.81(5)
C (9)	0.5437(2)	0.0522(7)	0.2300(2)	2.97(5)
C (10)	0.4046(2)	0.2433(6)	0.2050(2)	2.91(5)
C (11)	0.4015(2)	0.3398(7)	0.1038(2)	3.11(5)
C (12)	0.3325(3)	0.5732(8)	0.0159(2)	4.40(7)
C (13)	0.3594(3)	0.3442(7)	0.2899(2)	3.42(6)
C (14)	0.3730(2)	0.2669(6)	0.3937(2)	3.09(5)
C (15)	0.4713(3)	0.2895(7)	0.4543(2)	3.61(6)
C (16)	0.4834(2)	0.2388(7)	0.5547(2)	3.66(6)
C (17)	0.3926(2)	0.1622(7)	0.5906(2)	2.94(5)
C (18)	0.2956(2)	0.1307(7)	0.5321(2)	2.96(5)
C (19)	0.2852(2)	0.1829(7)	0.4325(2)	3.17(5)
C (20)	0.2141(2)	0.0529(7)	0.5971(2)	3.48(6)
C (21)	0.2863(2)	0.0158(6)	0.6955(2)	3.00(5)
C (22)	0.2364(3)	0.0620(7)	0.7934(2)	3.45(6)
C (23)	0.3175(3)	0.0270(9)	0.8840(2)	5.01(9)
C (24)	0.1276(3)	−0.0284(8)	0.8024(3)	4.70(8)
C (25)	0.2155(5)	0.573(1)	0.5359(5)	7.2(2)
C (26)	0.1222(6)	0.505(1)	0.5848(6)	8.6(2)

Synthesis of butyrolactone III (**4**) from **1**: *m*-Chloroperbenzoic acid (60 mg) was added to a solution of **1** (500 mg) in $CHCl_3$ (25 ml), and the mixture was kept at 4 °C for 24 h. After concentration *in vacuo* the reaction mixture was subjected to flash chromatography on silica gel (230—400 mesh) and eluted with 1,2-dichloroethane–EtOAc (6 : 4) to obtain 400 mg of **4**. Colorless amorphous solid; mp 71—72 °C. $[\alpha]_D^{27}$ +93° (c=0.6, EtOH). ^{13}C-NMR (DMSO-d_6) δ: 20.1 (C-24), 25.7 (C-23), 31.0 (C-20), 38.1 (C-13), 53.5 (C-12), 68.67 (C-21), 77.0 (C-22), 84.8 (C-10), 114.1 (C-16), 115.8 (C-2, 6, 16), 119.6 (C-14), 121.1 (C-4), 124.3 (C-18), 127.7 (C-7), 128.8 (C-15), 128.8 (C-3, 5), 131.6 (C-19), 138.2 (C-8), 151.7 (C-17), 157.9 (C-1), 168.0 (C-9), 169.7 (C-11).[4)] MS *m/z* (%): 440 (M^+, 12), 396 (100), 364 (56), 294 (34), 265 (16), 237 (17), 191 (37), 177 (10), 131 (30), 119 (51), 107 (18), 91 (22).

Biological Activity Cytotoxic activities of **1**—**4** were tested against a panel of 8 solid tumor cell lines (Table 2), and cell growth was measured by the SRB method.[7)] They (**1**—**4**) were also tested for their anti-microbial activity against five Gram-positive (*Staphylococcus aureus* ATCC 6538P, methicillin resistant *S. aureus* ATCC 33591, *Enterococcus faecalis* ATCC 29212 and NCTC 12201, and *Bacillus subtilis* NCIM 2063) and three Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, and of *Escherichia coli*, DRCC 091). The zone of inhibition if any, was measured by following the guidelines of National Committee for Clinical Laboratory Standards (NCCLS).

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