Pergamon

S0031-9422(96)00146-X

Phytochemistry, Vol. 42, No. 5, pp. 1427–1433, 1996

0031-9422/96 $15.00 + 0.00

ACIDIC METABOLITES FROM *PHYCOMYCES BLAKESLEEANUS*

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(*Received in revised form* 17 *January* 1996)

Key Word Index—*Phycomyces blakesleeanus*; Mucorales; *p*-hydroxybenzaldehyde; protocatechuic acid; gallic acid; turbinaric acid; phycodioic acid; GC-MS.

Abstract—The acids of *Phycomyces blakesleeanus* were studied under a variety of incubation conditions. Forty-six metabolites were identified. Among these were *p*-hydroxybenzaldehyde, turbinaric acid and phycodioic acid, the last being a new compound. The levels of fumaric, malic, protocatechuic, gallic and turbinaric acids varied, depending on agitation, illumination and age of the culture.

INTRODUCTION

The zygomycete *Phycomyces blakesleeanus* is a producer of β-carotene [1]. This carotene is essential in mammalian diets as a source of vitamin A. Moreover, diets rich in β-carotene diminish the risks of cancer and cardiovascular diseases [2, 3]. A carotene-rich oil from *Phycomyces* has potential as a nutrient additive [4], but a detailed description of the numerous metabolites from *P. blakesleeanus* is not yet available.

The following compounds have been found in the fungus: trisporic acids [5]; the thiamine derivative, 4-methylthiazole-5-acetic acid [6]; metabolites of the shikimate pathway (catechol and gallic, protocatechuic, shikimic and dehydroshikimic acids [7], chorismic [8], *p*-hydroxybenzoic and *p*-coumaric acids [9]); pimelic acid [10], 8-amino-7-ketopelargonic acid, biotin and biotin vitamers [11]; pyruvic, lactic and glucuronic acids; intermediates of the citric acid cycle (oxaloacetic, citric, isocitric, α-ketoglutaric, succinic, fumaric and malic acids); derivatives of tryptophan (kynurenic, 3-hydroxyanthranilic, nicotinic and indoleactic acids [12]); saturated fatty acids with 12–18, 20–22, 24 and 26 carbons; mono-unsaturated fatty acids with 12–14, 16–19, 24 and 26 carbons; and di- and tri-unsaturated (γ-linolenic) fatty acids with 18 carbons [4, 13]. However, the majority of the analytical methods described required large amounts of sample material or were based on either biological tests or colorimetric methods restricted to specific metabolites.

In this paper, a general method for the analysis of acidic metabolites from *Phycomyces*, by means of extraction, derivatization and GC–mass spectrometry, is described and applied to cultures incubated under a variety of conditions. With this method, chromatograms showing more than 100 different peaks were obtained. Forty-six compounds were identified, 17 not previously described in *Phycomyces*. One of the compounds is described for the first time and another one was previously known only from a brown alga.

RESULTS AND DISCUSSION

Identification of compounds

Aliquots of the acidic extracts obtained from seven different cultures of *P. blakesleeanus* were derivatized and the mixtures of trimethylsilyl (TMSi) derivatives were analysed by GC and GC–mass spectrometry. Identified compounds are shown in Tables 1 (solid medium) and 2 (liquid medium). The amounts of (μg g^{-1} biomass dry wt weight) are only approximative because they were determined on the basis of the acidic extract weight and the area of integration on the corresponding FID-GC chromatogram.

Authentic samples of compounds **2**, **5–10**, **12**, **13**, **16**, **17**, **19**, **20**, **22**, **25** and **34** (commercially available) were used as standards (see Table 1 for names or identities of compounds **1–46**). Compounds **1**, **4**, **6**, **10**, **14**, **15–18**, **19**, **24** and **27–31** were identified by comparison of the mass spectra of their TMSi derivatives with those taken from Wiley's 138.K compendium of mass spectra. Compound **9** was identified by comparison of the mass spectrum of its TMSi derivative with that described in the literature [14]. The fatty acids **18**, **24**, **26–33**, **35–40** and **42–46** were identified by interpretation of the GC-mass spectra of both their TMSi derivatives and their methyl esters [15]; the latter were obtained by treatment of the acid fraction from the SP extract (see Experimental) of culture 1 with diazomethane.

The mass spectrum of the TMSi derivative of **11** was analysed, bearing in mind the corresponding spectrum of malic acid (**10**), a lower homologue. The spectrum of TMSi-**11** showed peaks at *m/z* 377 $[M - Me]^+$, 349 $[M - 43]^+$, 275 $[M - CO_2TMSi]^+$ and 231 [M –

Table 1. Acidic metabolites from *P. blakesleeanus* grown on solid medium

			Culture 1			Culture 2		
			JUI*	SP	MYC	JUI	SP	MYC
	Extract†		67211	57331	62576	41758	56441	34157
	Acid fraction		62088	24518	8538	38858	46083	20395
RR_t‡	Compound	Id§						
0.1340	Lactic acid (**1**)	a	170	77	32	190	65	157
0.1542	Furoic acid (**2**)		90	76	32	70	—	3
0.2205	Benzoic acid (**3**)	a,b	80	36	6	424	10	3
0.2415	H_3PO_4 (**4**)	a	4351	207	—	3837	134	2040
0.2460	Nicotonic acid (**5**)	b	130	4	30	30	10	80
0.2614	Succinic acid (**6**)	a,b	400	146	60	831	87	—
0.2763	Catechol (**7**)	a,b	730	—	—	40	—	—
0.2885	Fumaric acid (**8**)	a,b	978	316	21	2694	420	20
0.3041	*p*-Hydroxy-benzaldehyde (**9**)	a,b	5	40	79	—	—	—
0.4084	Malic acid (**10**)	a,b	199	71	34	1914	29	—
0.4914	2-Hydroxy-heptanedioic acid (**11**)	a	30	28	20	70	80	20
0.5073	α-Ketoglutaric acid (**12**)	a,b	360	6	1	771	7	—
0.5181	*p*-Hydroxybenzoic acid (**13**)	a,b	10	22	40	60	10	20
0.5871	Pentonic acid (γ-lactone) (**14**)	a	202	429	225	292	20	120
0.6524	Nonanedioic acid (**15**)	a	23	15	2	30	15	—
0.6821	Protocatechuic acid (**16**)	a,b	892	40	8	4158	210	—
0.6945	Citric acid (**17**)	a,b	365	57	1	517	—	—
0.6984	14:0 (**18**)¶	a	44	114	43	59	30	30
0.7302	Syringic acid (**19**)	b	—	—	—	269	70	—
0.7655	*p*-Coumaric acid (**20**)	a,b	—	30	6	—	—	—
0.7673	Phycodioic acid (**21**)	a,c,d,e	—	—	—	60	10	—
0.7762	Indoleacetic acid (**22**)**	a,b						
0.7762	3-*O*-Methylgallic acid (**23**)	a	32	36	8	220	30	70
0.7762	15:0 (**24**)	a						
0.7964	Gallic acid (**25**)	a,b	20620	44	37	6419	19	20
0.8337	16:1 (**26**)	a	16	8	8	70	10	94
0.8561	16:0 (**27**)	a	2669	3027	756	512	1568	3843
0.9748	18:3 (**28**)	a	510	2390	462	350	710	1865
0.9930	18:2 (**29**)	a	468	2479	696	167	1471	2727
1.0000	18:1 (**30**)	a	782	1980	606	188	778	2032
1.0247	18:0 (**31**)	a	2136	1562	509	893	444	546
1.1768	20:1 (**32**)		—	53	2	—	—	—
1.2011	20:0 (**33**)	a	217	162	51	—	221	89
1.2283	Biotin (**34**)	b	—	—	—	310	—	-
1.3619	22:1 (**35**)	a	50	119	20	—	892	60
1.3798	22:0 (**36**)	a	282	765	210	—	9581	1152
1.5115	24:2 (**37**)	b	2	10	—	—	25	—
1.5309	24:1 (**38**)	a	200	221	8	—	2818	165
1.5378	24:1 (**39**)	a	178	421	80	—	3978	179
1.5555	24:0 (**40**)	a	309	1076	206	79	9000	1017
1.6717	Turbinaric acid (**41**)	a,c,d,e	—	61	—	—	2544	60
1.7076	26:2 (**42**)	a	220	30	—	—	823	27
1.7160	26:2 (**43**)	b	2	7	—	—	50	—
1.7417	26:1 (**44**)	a	852	801	68	—	7452	5
1.7494	26:1 (**45**)	a	122	630	—	—	1634	—
1.7732	26:0 (**46**)	a	146	296	—	—	1109	—

*JUI: sporangiophone 'juice' extract; SP: sporangiophore wall extract; MYC: mycelium extract.

†Concentration in μg g^{-1} biomass dry wt, based on the integration area (FID).

‡R_t is relative to oleic acid (**30**).

§Identification: a, MS; b, standards; c, ^{1}H NMR; d, ^{13}C NMR: e, IR.

¶Fatty acids are represented by the number of carbons and the number of unsaturated bonds.

Compounds **22–**24** gave the same RR_ts.

Table 2. Acidic metabolites from *P. blakesleeanus* grown in shaken liquid cultures

	Culture 3		Culture 4		Culture 5		Culture 6		Culture 7	
	FIL*	MYC	FIL	MYC	FIL	MYC	FIL	MYC	FIL	MYC
Extract†	26387	253826	40094	180790	44594	188799	13967	155628	26812	130331
Acid fraction	25215	59421	37304	48171	43168	55126	12630	17669	25070	32997
Compound										
1	549	1379	254	220	152	10	24	783	197	36
2	193	60	—	30	94	20	8	27	40	20
3	105	37	31	200	30	30	10	10	60	—
4	16521	291	17230	48	17045	341	4932	102	14132	10
5	—	—	—	109	—	10	—	—	—	—
6	170	632	321	100	243	40	110	129	149	70
7	—	—	—	—	—	—	—	—	—	—
8	1345	680	9673	90	10509	30	2937	107	80	110
9	20	7	19	100	90	30	10	27	—	9
10	122	402	261	—	90	23	46	30	113	13
11	200	50	582	140	347	10	182	10	416	10
12	40	167	—	—	33	—	11	22	—	5
13	110	—	246	38	279	20	245	158	120	4
14	218	40	399	560	439	50	189	125	192	31
15	—	14	—	—	6	10	8	10	—	17
16	446	74	146	30	127	50	745	30	1190	14
17	29	120	168	—	92	—	10	—	106	—
18	9	249	52	299	116	327	10	80	30	160
19	—	—	—	—	30	—	—	—	110	—
20	—	—	—	—	—	—	—	—	—	—
21	—	620	—	132	17	10	74	193	150	146
22‡										
23	11	100	10	30	20	143	—	30	—	48
24										
25	73	144	886	30	878	20	181	20	55	19
26	24	60	—	140	10	80	—	30	—	50
27	80	10352	261	7674	495	12749	162	3030	265	3768
28	15	7887	28	5101	28	3539	27	1906	—	6957
29	112	10660	104	5414	287	5324	48	3074	153	9481
30	40	6762	104	13719	231	16400	215	2950	280	7719
31	216	4639	418	4446	723	5908	168	1026	292	1128
32	—	109	—	217	—	350	—	59	–	188
33	9	497	33	236	70	510	8	106	30	210
34	—	—	—	—	4	10	—	—	—	—
35	—	4	—	—	16	—	—	34	—	—
36	—	1165	39	178	60	758	—	188	36	261
37	—	—	—	—	—	—	—	—	—	—
38	—	130	—	70	—	268	—	48	—	168
39	—	138	42	—	22	—	—	13	—	—
40	—	2242	41	97	132	1575	—	398	110	555
41	—	—	—	—	—	—	—	—	—	—
42	—	—	—	—	—	—	—	—	—	—
43	—	—	—	—	—	—	—	—	—	—
44	—	—	34	40	—	333	—	78	—	176
45	—	—	—	—	—	—	—	46	—	—
46	—	—	—	—	—	374	—	69	—	147

*FIL: broth filtrate extracts; MYC: mycelium extracts.

†Concentration in $\mu g\ g^{-1}$ biomass dry wt, based on the integration area (FID detection).

‡Compounds **22–24** gave the same RR_ts.

$CO_2TMSi - CO_2]^+$ whose intensities matched with those of the corresponding ions of the mass spectrum of TMSi – **10** (whose ions were 42 amu lower). The intensities of the peaks at *m/z* 147 [$Me_3Si - O{=}SiMe_2]^+$ and 73 (base peak) were also coincident in both spectra.

The mass spectrum of the TMSi derivative of **21** showed the molecular ion peak at *m/z* 342 together

with fragment ions at m/z 252 $[M - TMSiOH]^+$, 162 $[M - 2TMSiOH]^+$ and 134 $[M - 2TMSiOH - CO]^+$, pointing to a dicarboxylic acid with the molecular formula $C_{10}H_{14}O_4$. It was possible to isolate the dimethyl ester of **21** by treatment of the acid fraction from the MYC extract (see Experimental) of culture 3 with diazomethane, followed by silica-gel column chromatography. The mass spectrum of this ester showed the parent ion at m/z 226 together with fragments at m/z 194 $[M - MeOH]^+$, 162 $[M - 2MeOH]^+$ and 134 $[M - 2MeOH - CO]^+$, in agreement with a $C_{12}H_{18}O_4$ dimethyl ester. In its IR spectrum carbonyl bands appeared at 1736 (saturated ester) and 1718 cm^{-1} (α,β-unsaturated ester). The ^{13}C NMR spectrum showed four methylenes, four olefinic methynes and two carboxylic carbons. In the ^1H NMR spectrum two singlets appeared at δ 3.65 (OMe) and 3.74 (OMe) together with the signals of four protons of a diene conjugated with an ester group. The coupling constants $J_{2,3} = 15.4$ Hz and $J_{4,5} = 10.7$ Hz indicated the 2*E*,4*Z* configuration of the compound, which was thereby identified as the dimethyl ester of deca-2*E*,4*Z*-dienoic acid. The free diacid **21** is a new compound which we have named phycodioic acid. Dimethyl phycodioate was saponified, treated with *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and analysed by GC–mass spectrometry, giving a RR_t (0.7673) and a mass spectrum identical to that of the TMSi derivative of the diacid from cultures 2–7.

In the mass spectrum of the TMSi derivative of **23**, in addition to the molecular ion at m/z 400, the fragment at m/z 223 $[M - TMSiO - C_3H_8OSi]^+$ stood out. This is a feature characteristic of trimethylsilylated methoxyphenolic acids [14]. The mass spectral data and biogenetic considerations (**23** could be the metabolic intermediate between gallic and syringic acids) pointed to the structure of 3-*O*-methylgallic acid for the free acid.

The mass spectrum of the TMSi derivative of **41** showed the molecular ion at m/z 472 together with fragment ions at m/z 403 $[M - C_5H_9]^+$, 335 $[M - C_{10}H_{17}]^+$, 267 $[M - C_{15}H_{25}]^+$, 199 $[M - C_{20}H_{33}]^+$ and 69 $[C_5H_9]^+$ (base peak), suggesting the TMSi derivative of a $C_{27}H_{44}O_2$ polyprenic carboxylic acid. The methyl ester of **41** was isolated by $AgNO_3$–silica gel column chromatography of the methylated acid fraction from the SP extract of culture 2. The HR mass spectrum of this compound gave the molecular formula $C_{28}H_{46}O_2$ ($[M]^+$ at m/z 414.3493). The LR mass spectrum displayed the parent ion together with fragment ions at m/z 345 $[M - C_5H_9]^+$, 277 $[M - C_{10}H_{17}]^+$, 209 $[M - C_{15}H_{25}]^+$, 141 $[M - C_{20}H_{33}]^+$ and 69 $[C_5H_9]^+$ (base peak), corresponding to a polyprenic methyl ester. The ^1H NMR spectrum showed five olefinic protons (*m*, δ 5.03–5.13), a triplet at δ 2.37 ($J = 8.4$ Hz, H-2), a broad triplet at δ 2.25 ($J = 8.4$ Hz, H-3) and finally a doublet (δ 1.64, 3H, $J = 0.9$ Hz) together with a broad singlet (δ 1.57, 15H) corresponding, respectively, to an 'in-chain' allyic methyl group and five 'out-of-chain' allylic methyl groups of a polyprenoid system [16]. In the ^{13}C NMR spectrum the 'out-of-chain' methyl groups resonated at δ 15.98, 16.03, 16.07, 16.10 and 17.76 indicating the *E* stereochemistry of the five trisubstituted double bonds, while C-22 appeared at δ 25.8, confirming its 'in chain' position [17]. The spectroscopic data and biogenetic considerations (the compound might be derived from the oxidative degradation of 2,3-oxidosqualene) resulted in the proposal of structure **41** for the natural acid. On examination of the literature, turbinaric acid, a metabolite isolated from the brown alga *Turbinaria ornata* [18], was found to have the same structure. The ^1H and ^{13}C NMR (benzene-d_6) spectra of methyl turbinarate match with those of **41**. Turbinaric acid could be involved in the regulation of sterol biosynthesis of *Phycomyces* because it is a moderate inhibitor of squalene epoxidase [19] and a potent inhibitor of oxidosqualene cyclase [18]. This is the second time that **41** has been described in nature.

More than 80% of the acidic content of the cultures 2–7 of *P. blakesleeanus* was identified in this study. Not one of the acids identified is listed in the Cole and Cox mycotoxins hand book [20]. This is one more fact supporting the utility of an oil from *Phycomyces* as a nutrient additive.

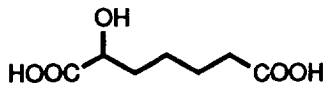
Acidic metabolite production

The metabolites **2–4**, **9**, **11**, **14**, **15**, **18**, **21**, **23**, **33**, **35**, **37**, **39**, **41–34** and **45** are described for the first time in *Phycomyces*. The presence of the aldehyde **9** in the fungus points to the biosynthesis of *p*-hydroxybenzoic acid from *p*-coumaric acid via a non-oxidative degradation mechanism, as occurs in *Lithospermum erythrorhizon* [21].

The main metabolite in culture 1 (solid medium, five days old) was gallic acid, which accumulated in the vacuole of the sporangiophore (JUI extracts) (see Experimental). Phosphoric, fumaric and free fatty acids with 16 and 18 carbons also accumulated in the fungus at concentrations greater than 10^3 ppm. In culture 2 (solid medium, 14 days old), a noticeable increase in the protocatechuic acid content was observed (four times greater than in culture 1), together with a decrease in gallic acid (four times less than in culture 1). Increases in the production of fumaric, malic and long chain fatty acids (22, 24 and 26 carbons) were also observed in culture 2.

Some aspects of acid biosynthesis in shaken liquid cultures differed from those produced on solidified medium. As in cultures grown on solidified medium, free fatty acids formed a large part of the acids produced in liquid medium. However, in shaken liquid cultures neither catechol (**7**), *p*-coumaric (**20**), turbinaric acid (**41**) nor the di-unsaturated fatty acids with 24 (**37**) and 26 carbons (**42** and **43**) were detected. Moreover, both the overall production of phenolic acids and the uptake of H_3PO_4 from the medium decreased.

In shaken liquid cultures, the levels of some metabolites underwent noticeable changes between the fourth (culture 3) and fifth days (culture 4) of culture. Fumaric



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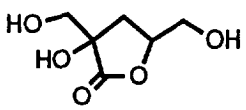

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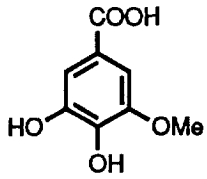

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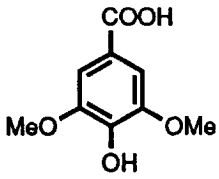

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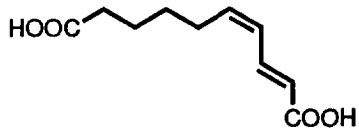

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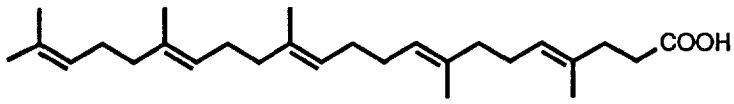


41

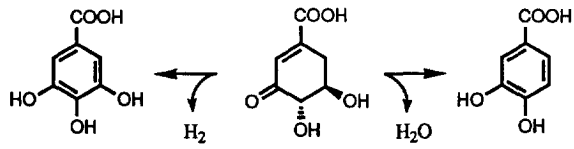
(**8**) and gallic (**25**) acids increased by five and four times, respectively. Conversely, protocatechuic acid (**16**) decreased by two-thirds in the same period. Therefore, protocatechuic and gallic acids levels increased and decreased in the same way as observed by Sandmann and Hilgenberg in cultures of similar ages [22].

Phycomyces is an organism which exhibits a great variety of physiological responses to light [1, 23, 24]. Thus, a slight inhibiting effect of light on the accumulation of gallic acid in five-day-old cultures of *P. blakesleeanus* has been reported [22]. In the experiments reported here, light had little effect on acid production in growing cultures (four days old, cultures 3 and 6), but had marked effects on the metabolism of some acids in the mature fungus. In the culture illuminated for seven days, the levels of fumaric and gallic acids were diminished markedly, while protocatechuic acid increased (culture 7 compared with 5). The light promoted decrease of gallic acid level is interesting because this substance has been related to the phototropisms shown by the sporangiophore of *Phycomyces* [23].

Variations in gallic acid concentration were accompanied by inverse variations in protocatechuic acid concentration in all the cases described in this paper. This fact supports the biogenetic relationship suggested by the chemical structures of these acids. Gallic acid is not biosynthesized from protocatechuic acid in *Phycomyces* [7], but both compounds could be derived from the same biogenetic precursor. In *P. blakesleeanus* gallic acid is synthesized by dehydrogenation of dehydroshikimic acid (DHS) [7]. Formation of protocatechuic acid (**16**) from 4-hydroxybenzoic acid has been reported before [9], but **16** could also be obtained by dehydration of DHS (Fig. 1), as in other fungi [25]. This possibility would explain the effect of light on gallic and protocatechuic acid production, either by inhibition of DHS dehydrogenation or by stimulation of DHS dehydration.

EXPERIMENTAL

Culture conditions and extraction. Phycomyces blakesleeanus strain NRRL1555 was provided by Prof.



25 DHS **16**

Fig. 1.

Table 3. Solid medium cultures

Culture	Vol (l)	Dry wt (g) Sp*	Myc	Biomass	Extract wt (g)† JUI Neut. fr.	JUI Acid fr.	SP Neut. fr.	SP Acid fr.	MYC Neut. fr.	MYC Acid fr.
1	1.0	3.05	3.70	6.75	0.04	0.51	0.27	0.20	0.44	0.07
2	5.0	14.82	17.94	32.76	0.09	1.27	0.34	1.51	0.45	0.67

*Sp: sporangiophores; Myc: mycelium; Biomass: Sp + Myc.
†JUI: sporangiophore 'juice'; SP: sporangiophore: MYC: mycelium extracts.

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Solid medium cultures. Solid minimal medium [26] was distributed on plastic trays of 800 cm^2 surface area (500 ml $tray^{-1}$) and inoculated with 4×10^5 heat-activated [26] spores l^{-1}. Cultures were grown at 23° in darkness for 5 (culture 1) and 14 days (culture 2). After incubation the sporangiophores were harvested and squeezed to obtain their aq. vacuole contents. This aq. sporangiophore juice was extracted with EtOAc at pH 10 (JUI neutral fraction) and subsequently with EtOAc and *n*-BuOH at pH 2 (JUI acid fr.). The cellular remains of the sporangiophores were freeze-dried, ground and extracted by vigorous shaking with hexane–Et_2O (1:1) (200 ml l^{-1} of culture, 8 hr, XZ) and MeOH (200 ml l of culture, 8 hr, X2) (SP extracts). The mycelia were sepd from the agar layer, freeze-dried, ground and extracted as above (MYC extracts). The SP extracts as well as the MYC extracts were dissolved in Et_2O and washed with 10% aq. NaOH. The aq. layers were acidified to pH 2 and extracted with EtOAc (acid frs). The results are presented in Table 3.

Shaken liquid culture. Cultures were grown in minimal medium [26] in 500 ml flasks, each containing 200 ml medium. The cultures were inoculated with 7×10^5 spores l^{-1} and incubated in an orbital shaker (180 rpm) for 4, 5 and 7 days in darkness (cultures 3–5) and for 4 and 7 days under illumination provided by four × 40 W fluorescent tubes (cultures 6 and 7). After incubation, the broths were filtered and the filtrates extracted with EtOAc at pH 10 (FIL neutral fr.) and subsequently with EtOAc and *n*-BuOH at pH 2 (FIL acid fr.). The mycelia were freeze-dried, ground, extracted with organic solvents and fractionated as described for the solid medium cultures (Table 4).

Preparation of TMSi derivatives and analysis by GC and GC–MS. Pyridine (20 μl) and BSTFA (40 μl) were added to 2 mg of each acid extract and the mixt. was heated at 110° for 30 min. The samples (1 μl) were injected on to a 25 m × 0.32 mm i.d. HP-1 methylsilicone capillary column (He at 25 ml min^{-1}; injector temp. 260°; detector temp. 290°, FID), temp. programmed from 120° to 220° at 5° min^{-1}, 220° to 280° at 3° min^{-1} and 10 min hold at 280°. For GC–MS analysis the conditions were the same. EIMS were obtained at 70 eV.

2-*Hydroxyheptanedioic acid* (**11**) (*TMSi derivative*). GC–MS, 70 eV, *m/z* (rel. int.): 377 $[M - Me^+$ (6), 349 $[M - 43]^+$ (6), 275 $[M - CO_2TMSi]^+$ (42), 231 $[M - CO_2TMSiCO_2]^+$ (8), 147 $[C_5H_{15}OSi_2]^+$ (50), 117 $[CO_2TMSi]^+$ (9), 73 (100).

*Deca-*2E,4Z*-dienoic acid* (*phycodioic acid,* **21**) *derivatives.* The acid fr. from the MYC extract of culture 3 (3.55 g) was dissolved in MeOH and washed with hexane. The MeOH fr. (0.98 g) was methylated with CH_2N_2 excess at −10° and subjected to CC on silica gel using a hexane–Et_2O gradient. The hexane-Et_2O (3:1) fr. yielded 20 mg of the dimethyl ester of phycodioic acid. Oil, IR λ_{max} cm^{-1}: 1736 (C=O), 1718 (C=O), 1638; EIMS (probe) 70 eV, *m/z* (rel. int.): 226 $[M]^+$ (4), 195 $[M - MeO]^+$ (22), 194 $[M - MeOH]^+$ (32), 163 $[M - MeO - MeOH]^+$ (29), 162 $[M - 2MeOH]^+$ (43), 135 $[M - MeOH - MeO - CO]^+$ (35), 134 $[M - 2MeOH - CO]^+$ (37), 117 (65), 111

Table 4. Shaken liquid cultures

Culture	Vol (l)	Dry wt Myc (g)	Extract wt (g) FIL* Neut. fr.	FIL* Acid fr.	MYC Neut. fr.	MYC Acid fr.
3	7.8	59.726	0.07	1.51	11.61	3.55
4	1.0	6.809	0.02	0.25	0.90	0.33
5	2.6	18.231	0.03	0.79	2.44	1.01
6	7.1	48.616	0.06	0.61	6.71	0.86
7	5.0	32.760	0.34	1.51	0.45	0.67

*Fil: broth filtrate; MYC: mycelium extract.

$[C_6H_7O_2]^+$ (96), 107 (78), 79 (100), 59 (89); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.44 (2H, *m*, H-7), 1.64 (2H, *m*, H-8), 2.31 (2H, *m*, H-6), 2.31 (2H, *t*, J = 7.4 Hz, H-9), 3.65 (3H, *s*, OMe), 3.74 (3H, *s*, OMe), 5.82 (1H, *br td*, $J_{4,5}$ = 10.7 Hz, $J_{5,6}$ = 7.8 Hz, H-5), 5.86 (1H, *d*, J = 15.4 Hz, H-2), 6.12 (1H, *br dd*, $J_{3,4}$ = 11.7 Hz, $J_{4,5}$ = 10.7 Hz, H-4), 7.57 (1H, *ddd*, $J_{2,3}$ = 15.4 Hz, $J_{3,4}$ = 11.7 Hz, $J_{3,5}$ = 1.1 Hz, H-3); ^{13}C NMR (75 MHz, $CDCl_3$); δ 24.5 (*t*, C-8), 27.9 (*t*, C-6), 28.9 (*t*, C-7), 33.9 (*t*, C-9), 51.6 (*q*, OMe), 121.1 (*d*, C-3), 126.9 (*d*, C-4), 139.5 (*d*, C-5), 140.9 (*d*, C-2), 167.7 (*s*, C-1), 174.0 (*s*, C-10). TMSi-**21**. GC–MS, 70 eV, *m/z* (rel. int.): 342 $[M]^+$ (1), 327 $[M-Me]^+$ (13), 252 [M-TMSiOH]$^+$ (4), 169 $[C_8H_{13}O_2Si]^+$ (43), 162 [M − 2TMSiOH]$^+$ (51), 147 $[C_5H_{15}OSi_2]^+$ (33), 134 [M − 2TMSiOH − CO]$^+$ (32), 117 (82), 75 (98), 73 (100).

3-O-*Methylgallic acid* (**23**) (*TMSi derivative*). GC–MS, 70 eV, *m/z* (rel. int.): 400 $[M]^+$ (19), 385 $[M-Me]^+$ (10), 312 $[M-C_3H_8OSi]^+$ (3), 311 [M − TMSiOH]$^+$ (5), 223 $[M-TMSiOH-C_3H_8OSi]^+$ (100), 73 (75).

Turbinaric acid (**41**) *derivatives*. The acid fr. (1.51 g) from the SP extract of culture 2 was treated with CH_2N_2 in excess at −10° and subjected to CC on silica gel using a hexane–Et_2O gradient. The Me ester fatty acid fr. obtained was subjected to CC on 10% $AgNO_3$–silica gel with a hexane–Et_2O gradient. The hexane–Et_2O (2:3) fr. gave 30 mg methyl turbinarate. Oil, HREIMS: *m/z* 414.3493 $[M]^+$, calc. for $C_{28}H_{46}O_2$: 414.3498; EIMS (probe) 70 eV, *m/z* (rel. int.): 414 $[M]^+$ (1), 345 $[M-C_5H_9]^+$ (1), 277 $[M-C_{10}H_{17}]^+$ (1), 209 $[M-C_{15}H_{25}]^+$ (1), 141 $[M-C_{20}H_{33}]^+$ (20), 81 $[C_6H_9]^+$ (85), 69 $[C_5H_9]^+$ (100); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.57 (15H, *br s*, Me-C-4, Me-C-8, Me-C-13, Me-C-17, Me-C-21), 1.64 (3H, *d*, J = 0.9 Hz, H-22), 1.98 (16H, *m*, CH_2CH_2), 2.25 (2H, *br t*, J = 8.4 Hz, H-3), 2.37 (2H, *t*, J = 8.4 Hz, H-2), 3.62 (3H, *s*, OMe), 5.03–5.13 (5H, *m*, C=CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.98 (*q*, Me-C-4), 16.03 (*q*, Me-C-8), 16.07, 16.10 (*q*, Me-C-13, Me-C-17), 17.76 (*q*, Me-C-21), 25.8 (*q*, C-22), 26.7, 26.8 (*t*, C-6, C-15, C-19), 28.3 (*t*, C-10, C-11), 33.1 (*t*, C-2), 34.7 (*t*, C-3), 39.6, 39.8 (*t*, C-7, C-14, C-18), 51.6 (*q*, OMe), 124.3 (*d*, C-9, C-12), 124.5 (*d*, C-5, C-16), 125.2 (*d*, C-20), 131.3 (*s*, C-21), 133.3 (*s*, C-4), 135.0 (*s*, C-17), 135.2 (*s*, C-8, C-13), 174.0 (*s*, C-1). TMSi-**41**. GC-MS, 70 eV, *m/z* (rel. int.): 472 $[M]^+$ (1), 457 $[M-Me]^+$ (3), 403 $[M-C_5H_9]^+$ (1), 335 $[M-C_{10}H_{17}]^+$ (2), 267 $[M-C_{15}H_{25}]^+$ (1), 199 $[M-C_{20}H_{33}]^+$ (17), 109 (29), 81 $[C_6H_9]^+$ (85), 69 $[C_5H_9]^+$ (100).

Acknowledgements—We thank Prof. E. Cerdá-Olmedo (Departamento de Genética, Universidad de Sevilla, Spain) for providing *P. blakesleeanus* strain NRRL1555 and for his suggestions, W. Taylor for translation of the manuscript into English, the Spanish CICYT for financial support (Research Programme no. BIO90-063) and to the Junta de Andalucía for the award of a grant to J. A. Poyatos.

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