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Papyracillic acid, a New Penicillic Acid Analogue from the Ascomycete *Lachnum papyraceum*[1]

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Abstract: The ascomycete *Lachnum papyraceum* is an efficient producer of bioactive and chlorinated metabolites, in which chlorine to some extent can be exchanged for bromine in fermentations to which bromide has been added. However, large amounts of bromide (100 mM $CaBr_2$) alter the secondary metabolism of the fungus, and it produces papyracillic acid as the main metabolite. Papyracillic acid is a new bioactive 1,6-dioxaspiro[4,4]nonene derivative in equilibrium with the open-chain form, and related to the mycotoxin penicillic acid.

The ascomycete *Lachnum papyraceum* was recently identified as a producer of nematicidal and antimicrobial metabolites,[2] and has during the last years been extensively investigated. Approximately 30 metabolites, most of them new, have been isolated from the culture fluids of various fermentations. During normal fermentation conditions the fungus produces a number of chlorinated metabolites, e.g. lachnumon (**1b**)[3] and mycorrhizin A (**2b**),[4] but if bromide (50 mM $CaBr_2$) is added to the culture medium, in an effort to obtain brominated instead of chlorinated metabolites, mainly 6-hydroxymellein derivatives (e.g. compounds **3a**, **3b** and **3c**) are obtained instead.[5] However, if the $CaBr_2$ is added first after the onset of secondary metabolism the brominated metabolites **1c** and **2c** are obtained, in addition to a number of other metabolites.[6] Larger amounts of $CaBr_2$ (100 mM) apparently shift the secondary metabolism of the fungus completely, and large amounts of a new antibiotic, called papyracillic acid, with no obvious relationship to the previously identified metabolites (e.g. **1**, **2** and **3**, see Figure 1) are produced. Papyracillic acid (**4**) was isolated and characterised, and the structure determination of **4** and some derivatives formed by methylation of **4** is reported. The fermentation conditions and the biological activities of papyracillic acid (**4**) will be reported elsewhere.

Papyracillic acid (**4**) was obtained by silica gel chromatography, and initial spectroscopic characterisation by 1D NMR suggested that it is a mixture of four isomers (approximately 1:1:2:4 in chloroform according to the integrals of the ^1H NMR spectrum). In order to facilitate the structure determination, derivatives of papyracillic acid (**4**) were prepared by its reaction with methanol, producing a

mixture of methyl acetals from which the major isomer **5** could be isolated, and with trimethylsilyldiazomethane, producing the derivatives **6**, **7** and **8** (see Figure 1).

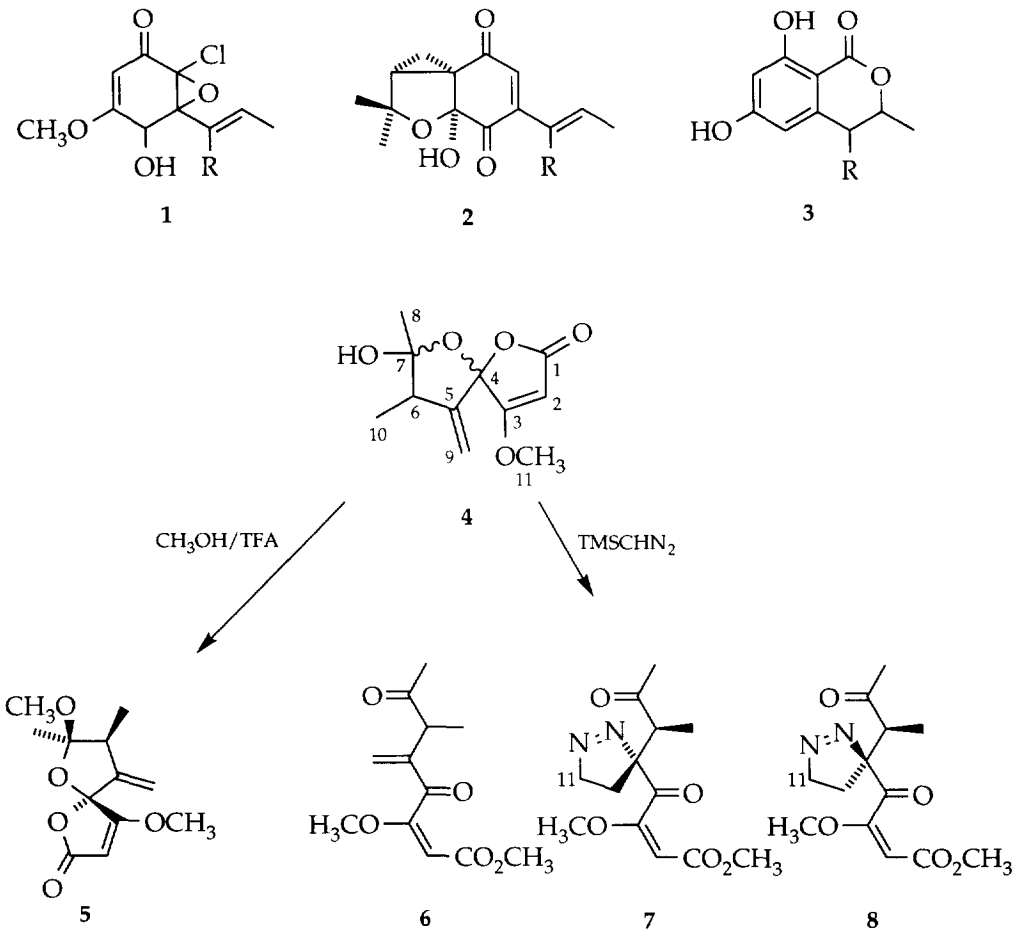

Figure 1. a: R=H, b: R=Cl, c: R=Br.

The methylation of papyracillic acid (**4**) with TMS-diazomethane, which was carried out at room temperature in benzene:methanol 1:1, yielded an unexpected number of products of which most were too unstable to isolate. The methyl ester **6** is formed rapidly, and is the major product as long as the reaction time is short (minutes). The two azo isomers **7** and **8** were formed together with the ester **6** in smaller amounts, but **6** is surprisingly not the precursor of the azo derivatives. The formation of similar cyclic azo products (via a 1,3-dipolar cycloaddition of the reagent to the double bond) after treatment of α,β-unsaturated carbonyl compounds with diazomethane or TMS-diazomethane has been reported, although these normally are

oxidised to pyrazoles or rearranged to pyrazolines.[7]

The molecular ions of papyracillic acid (**4**) and the methyl acetal **5** were hardly visible in the EI mass spectrum, but high resolution measurements of m/z 209 (M-17 and M-31, respectively) suggest that it arises after loss of a hydroxy group from $C_{11}H_{14}O_5$ in **4**, and a methoxy group from $C_{12}H_{16}O_5$ in **5**. For the ester **6**, HREI measurements with the molecular ion were in agreement with the suggested elemental composition. No molecular ion could, as expected, be observed in the EI and CI mass spectra of the two azo derivatives **7** and **8**, however, by decreasing the temperature of the ion source from 250 °C to 110 °C the ion $M+NH_4^+$ (*m/z* 300) was approximately as abundant as the $M-N_2+NH_4^+$ (*m/z* 272) in the CI (NH_3) mass spectra of both compounds. In addition, the ions for $M_2+NH_4^+$ (*m/z* 582), $M_2-N_2+NH_4^+$ (*m/z* 554) and $M_2-N_4+NH_4^+$ (*m/z* 526) became stronger (3-5 % of the base peak).

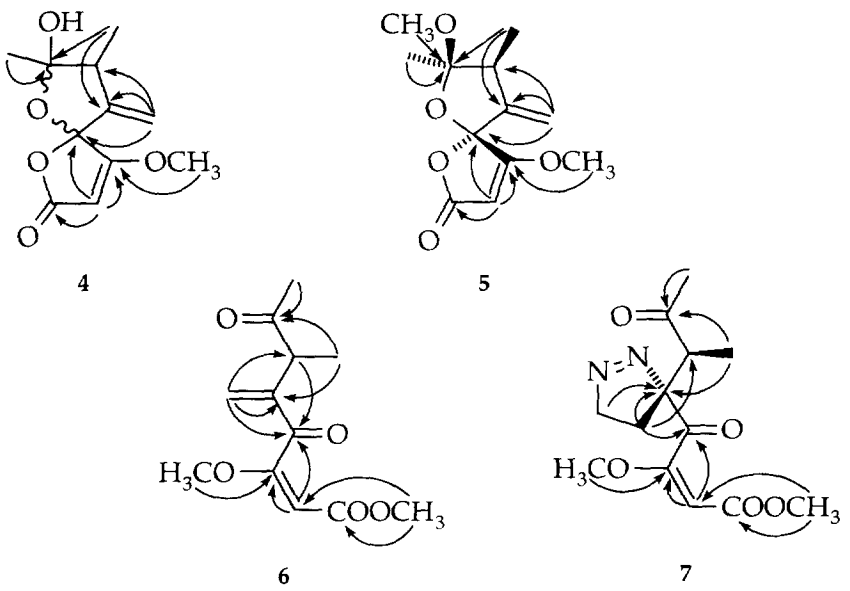

Figure 2. Pertinent HMBC correlations observed for the papyracillic acid (**4**) and its derivatives **5**, **6** and **7**.

The structure determination of papyracillic acid (**4**) and its derivatives is based on 2D NMR experiments, and pertinent HMBC correlations for compounds **4**, **5**, **6** and **7** are shown in Figure 2. The relative stereochemistry of **7** and **8** was suggested by the NOESY correlations observed and the chemical shifts of 10-H_3. The ^1H NMR spectra of the two compounds are very similar, except for the signal for 10-H_3 which is upshifted to 0.79 ppm in **7** compared to 1.17 ppm in **8**. In both compounds, 10-H_3, but not 8-H_3, give a NOESY correlation to 9-Ha, suggesting that the C-6 acetyl group is turned away from the 5-membered ring as would be expected for sterical reasons. This would bring the C-6 methyl group (C-10) closer to the azo group in **7** compared to **8**, and thereby explain the difference in chemical shift observed.

Papyracillic acid (**4**) is an analogue of penicillic acid (**12**), a classical mycotoxin produced by various fungi including strains of the genera *Penicillium* and *Aspergillus*. Together with patulin, isopatulin and

ascladiol, penicillic acid (**12**) constitutes a class of chemically relatively simple 5-membered cyclic lactones, which due to their toxicity and carcinogenicity are considered to be a potential health hazard to animals and man.[8] The toxic effects of penicillic acid (**12**) have been considered to be caused by its reaction with, for example, enzymes, and it has been shown to react with several amino acids to form less toxic products.[9] Although the chemical structure of such products has not been disclosed, it has been suggested that nucleophiles bind either to C-3 in the lactone ring or to the α,β-unsaturated keto function in the open-chain form.[9,10] Penicillic acid (**12**) is not mutagenic in the Ames' Salmonella assay,[11] and does not elicit DNA repair synthesis in primary hepatocytes,[12] although it induces single- and double-stranded DNA breaks in mammalian cells.[13] The molecular mechanism by which penicillic acid (**12**), and presumably also papyracillic acid (**4**), gives rise to toxic effects therefore remains to be clarified.

The biosynthesis of penicillic acid (**12**) has been shown to proceed from orsellinic acid (**9**), which is methylated, decarboxylated and oxidated via compound **10** to the benzoquinone **11**,[14] which is cleaved oxidatively to form **12**.[15]

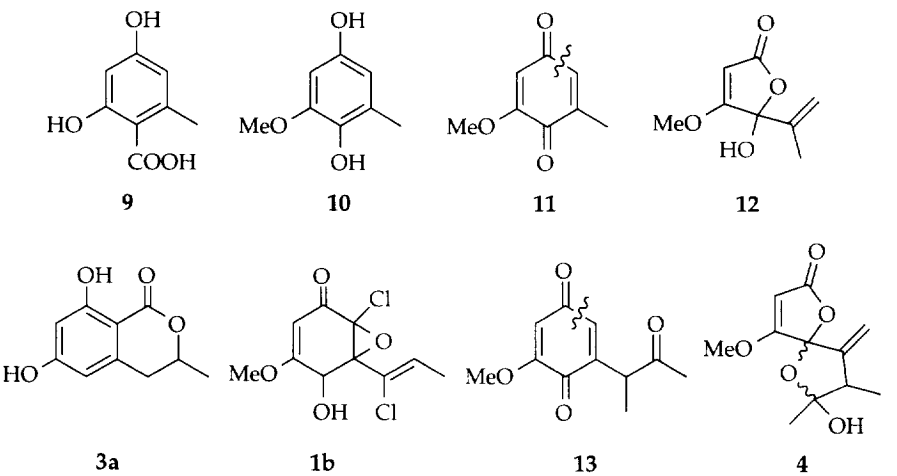

Figure 3. Compound **13** is hypothetical.

In *L. papyraceum*, the secondary metabolites originate from 3-methyl-3,4-dihydroisocoumarins [e.g. 6-hydroxymellein (**3a**)],[5] which is hydroxylated at C-5, decarboxylated and further converted to form for example lachnumon (**1b**). Although no metabolites derived from the dihydroisocoumarins that are methylated at C-4 have been isolated from *L. papyraceum* so far, compound **13** is still plausiblebiosynthetically, and, if formed, oxidative cleavage in the same way as takes place with the precursor **11** of penicillic acid (**12**) would yield papyracillic acid (**4**). However, this remains to be demonstrated by feeding experiments.

EXPERIMENTAL

The fermentation of the producing organism (*Lachnum papyraceum* (Karst.) Karst.) and the biological activities of papyracillic acid (**4**) and its derivatives will be reported separately. Papyracillic acid (**4**) (100 mg) was isolated from an ethyl acetate extract of the culture fluids (10 l), by chromatography on silica gel columns eluted by cyclohexane:ethyl acetate 1:1. The NMR spectra were recorded with a Bruker ARX500 spectrometer, the UV spectra with a Perkin Elmer λ16, the IR spectra with a Bruker IFS48, and the mass spectra with a Jeol SX102 spectrometer.

Papyracillic acid (**4**) was obtained as white crystals, m.p. 97-99 °C. $[\alpha]_D$ 0 ° (c 1.0 in methanol). UV (methanol) λ_{max} (ε): 226 nm (6,200). IR (KBr): 3450, 2920, 1770, 1640, 1360, 1210, 940 and 860 cm^{-1}. ^1H NMR (500 MHz in $CDCl_3$), δ, mult. *J* (Hz): 5.23-5.05, 2-H and 9-H_2; 3.89, 3.85, 3.84 and 3.83, 4s, 3-OCH_3; 2.89, dm, $J_{6\text{-}10}$=7.2; 2.84, 2.75 and 2.66, ddq, $J_{6\text{-}9a}$=3, $J_{6\text{-}9b}$=3, $J_{6\text{-}10}$=7, 6-H; 1.57, 1.54, 1.38 and 1.36, 4s, 8-H_3; 1.14, 1.13, 1.13 and 1.10, 4d, $J_{6\text{-}10}$=7, 10-H_3. ^{13}C NMR (125 MHz in $CDCl_3$), δ: 178.2, 177.9, 176.7 and 176.2 C-3; 170.4, 170.3, 170.2 C-1; 149.4, 148.3, 148.2 and 147.8 C-5; 111.4, 111.3, 111.2 and 110.9 C-9; 109.5, 109.2, 107.3 and 107.3 C-7; 107.3, 107.3, 107.1 and 106.2 C-4; 91.1, 90.2, 88.8, 88.4 C-2; 60.1, 60.0, 60.0, 59.8 OCH3; 47.9, 47.4, 47.0 and 45.1 C-6; 25.1, 24.4, 22.6 and 22.4 C-8; 15.2, 12.7, 10.9 and 10.4 C-10. MS (EI, 70 eV), *m/z*: 209.0791 (M^+ - OH, 100 %, $C_{11}H_{13}O_4$ requires 209.0814), 184 (12 %), 166 (56 %), 139 (29 %), 123 (13 %), 69 (18 %), 43 (24 %).

The methyl acetal **5** was formed when papyracillic acid (**4**) was left in methanol containing a catalytic amount of triflouracetic acid at room temperature for several hours. A mixture of isomers was obtained, from which compound **5** could be isolated as the major isomer. White crystals, m.p. 116-118 °C. $[\alpha]_D$ -42 ° (c 1.3 in chloroform). UV (methanol) λ_{max} (ε): 224 nm (9,800). IR (KBr): 2940, 1770, 1640, 1460, 1360, 1210, 1075, 950 and 870 cm^{-1}. ^1H NMR (500 MHz in $CDCl_3$), δ, mult. *J* (Hz): 5.12, d, $J_{6\text{-}9a}$=3, 9-Ha; 5.10, d, $J_{6\text{-}9b}$=3, 9-Hb; 5.01, s, 2-H; 3.81, s, 3-OCH_3; 3.23, s, 7-OCH_3; 2.62, ddq, $J_{6\text{-}9a}$=3, $J_{6\text{-}9b}$=3, $J_{6\text{-}10}$=6.8, 6-H; 1.43, s, 8-H_3; 1.08, d, $J_{6\text{-}10}$=6.8, 10-H_3. ^{13}C NMR (125 MHz in $CDCl_3$), δ: 178.0 C-3; 170.1 C-1; 148.5 C-5; 109.6 C-9; 109.1 C-7; 106.8 C-4; 88.1 C-2; 59.6 3-OCH_3; 49.2 7-OCH_3; 48.5 C-6; 18.7 C-8; 10.3 C-10. MS (EI, 70 eV), *m/z*: 209.0788 (M^+ - OCH_3, 54 %, $C_{11}H_{13}O_4$ requires 209.0814), 166 (100 %), 151 (29 %), 123 (31 %), 69 (39 %), 43 (43 %).

Methyl papyracillate (**6**) was obtained as a colourless oil. $[\alpha]_D$ +25 ° (c 1.0 in chloroform). UV (methanol) λ_{max} (ε): 224 nm (11,700). IR (KBr): 2950, 1715, 1685, 1620, 1370, 1195, 1145 and 1020 cm^{-1}. ^1H NMR (500 MHz in $CDCl_3$), δ, mult. *J* (Hz): 6.00 and 5.95, 2s, 9-H_2; 5.23, s, 2-H; 3.82, q, $J_{6\text{-}10}$=7.2, 6-H; 3.70, s, 3-OCH_3; 3.56, s, 1-OCH_3; 2.15, s, 8-H_3; 1.19, d, $J_{6\text{-}10}$=7.2, 10-H_3. ^{13}C NMR (125 MHz in $CDCl_3$), δ: 207.8 C-7; 191.0 C-4; 167.2 C-3; 166.2 C-1; 146.1 C-5; 129.2 C-9; 93.1 C-2; 56.8 3-OCH_3; 51.1 1-OCH_3; 45.4 C-6; 28.4 C-8; 15.1 C-10. MS (EI, 70 eV), *m/z*: 240.0976 (M^+, 20 %, $C_{15}H_{22}O_2$ requires 240.0998), 208 (42 %), 198 (99 %), 166 (60 %), 139 (100 %), 123 (29 %), 69 (42 %), 43 (73 %).

Compound **7** was obtained as white crystals, m.p. 97-99 °C, in 10 % yield after methylation of papyracillic acid (**4**) with TMS-diazomethane in methanol:benzene 1:1 at room temperature for 5 h. $[\alpha]_D$ +43 ° (c 0.6 in chloroform). UV (methanol) λ_{max} (ε): 222 nm (7,800). IR (KBr): 2920, 1710, 1700, 1620, 1445, 1360, 1200, 1140, 1070 and 815 cm^{-1}. ^1H NMR (500 MHz in $CDCl_3$), δ, mult. *J* (Hz): 5.14, s, 2-H; 4.66, ddd, $J_{9a\text{-}11a}$=6.4, $J_{9b\text{-}11a}$=10.2, $J_{11a\text{-}11b}$=18.2, 11-Ha; 4.56, ddd, $J_{9a\text{-}11b}$=9.9, $J_{9b\text{-}11b}$=5.7, $J_{11a\text{-}11b}$=18.2, 11-Hb; 4.01, q, $J_{6\text{-}10}$=7.0, 6-H; 3.80, s, 3-OCH_3; 3.57, s, 1-OCH_3; 2.23, s, 8-H_3; 2.12, ddd, $J_{9a\text{-}9b}$=13.9, $J_{9a\text{-}11a}$=6.4, $J_{9a\text{-}11b}$=9.9, 9-Ha; 2.04, ddd, $J_{9a\text{-}9b}$=13.9, $J_{9b\text{-}11a}$=10.1, $J_{9b\text{-}11b}$=5.7; 0.79, d, $J_{6\text{-}10}$=7.0, 10-H_3. ^{13}C NMR (125 MHz in $CDCl_3$), δ: 208.5 C-7; 197.7 C-4; 167.5 C-3; 166.9 C-1; 105.2, C-5; 93.0 C-2; 79.1 C-

11; 57.4 3-OCH_3; 51.6 1-OCH_3; 47.3 C-6; 31.2 C-8; 20.3 C-9; 11.2 C-10. MS (EI, 70 eV), *m/z*: 254.1137 (M^+-N_2, 5 %, $C_{13}H_{18}O_5$ requires 254.1154), 222 (8 %), 212 (32 %), 211 (19 %), 179 (17 %), 153 (100 %), 137 (18 %), 111 (21 %), 69 (26 %), 43 (50 %). MS (CI, NH_3, ion source temperature 250 °C), *m/z*: 272 (M-N_2+NH_4^+, 100 %), 255 (M-N_2+H^+, 25 %), 237 (33 %). MS (CI, NH_3, ion source temperature 110 °C), *m/z*: 300 (M+NH_4^+, 100 %), 283 (M+H^+, 3 %), 272 (M-N_2+NH_4^+, 82 %), 255 (M-N_2+H^+, 13 %), 237 (32 %).

Compound **8** was obtained as a colourless oil in 10 % yield after methylation of papyracillic acid (**4**) (*vide supra*). $[\alpha]_D$ +10 ° (c 0.5 in chloroform). UV (methanol) λ_{max} (ε): 222 nm (8,400). IR (KBr): 2920, 1700, 1615, 1435, 1360, 1190 and 1140 cm^{-1}. ^1H NMR (500 MHz in $CDCl_3$), δ, mult. *J* (Hz): 5.15, s, 2-H; 4.66, ddd, $J_{9a\text{-}11a}$=6.3, $J_{9b\text{-}11a}$=8.5, $J_{11a\text{-}11b}$=18.1, 11-Ha; 4.63, ddd, $J_{9a\text{-}11b}$=7.9, $J_{9b\text{-}11b}$=7.0, $J_{11a\text{-}11b}$=18.1, 11-Hb; 4.03, q, $J_{6\text{-}10}$=7.2, 6-H; 3.84, s, 3-OCH_3; 3.55, s, 1-OCH_3; 2.13, s, 8-H_3; 2.07, ddd, $J_{9a\text{-}9b}$=12.7, $J_{9a\text{-}11a}$=6.3, $J_{9a\text{-}11b}$=7.9, 9-Ha; 2.03, ddd, $J_{9a\text{-}9b}$=12.7, $J_{9b\text{-}11a}$=8.5, $J_{9b\text{-}11b}$=7.0; 1.17, d, $J_{6\text{-}10}$=7.2, 10-H_3 ^{13}C NMR spectrum was not recorded. MS (EI, 70 eV), *m/z*: 254.1159 (M^+-N_2, 7 %, $C_{13}H_{18}O_5$ requires 254.1154), 223 (12 %), 212 (38 %), 211 (59 %), 179 (30 %), 153 (100 %), 137 (28 %), 111 (37 %), 69 (38 %), 43 (72 %). MS (CI, NH_3, ion source temperature 250 °C), *m/z*: 272 (M-N_2+NH_4^+, 100 %), 255 (M-N_2+H^+, 27 %), 237 (34 %). MS (CI, NH_3, ion source temperature 110 °C), *m/z*: 300 (M+NH_4^+, 100 %), 272 (M-N_2+NH_4^+, 85 %), 255 (M-N_2+H^+, 8 %), 237 (23 %).

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