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Note

Isolation and characterization of the trimethyl ester of 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid, a degradation product of naphthomycin A, semisynthetically obtained from *Penicillium gladioli* cultures

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High-performance liquid chromatography (HPLC) is extensively used in isolating and analyzing natural substances of biological and pharmaceutical interest. In the present work, HPLC allowed the comparison of 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid trimethyl ester (Fig. 1, compound I), already known[1], with a product, not yet unequivocally identified, obtained by transformation of naphthomycin A (Fig. 1). Naphthomycin A is an antibacterial antibiotic, an antimetabolite of vitamin K, isolated from cultures of *Streptomyces collinus*[2]: a structure has been proposed, on the basis of spectroscopic results[3,4]. On degradation of naphthomycin A, a group of homologous fragments is obtained[5], all derived from the naphthoquinonic nucleus, according to spectroscopic characterization. Since compound I is presumably identical to one of these fragments, its semisynthesis was done according to Grove[1], starting from the fermentation of *Penicillium gladioli*, and leading to the production of gladiclic acid (Fig. 1, compound II), an antibacterial and fungistatic substance, as an intermediate.

EXPERIMENTAL

Penicillium gladioli (IMI 38567), purchased from the Commonwealth Mycological Institute, was grown in a liquid medium, according to Brian *et al.*[6], but gave only a small amount of a complex mixture of metabolites, as shown by HPLC analysis (Fig. 2), which is described in detail in *Procedures*. Owing to the low yield of the reaction, we did not attempt to isolate the gladiolic acid (compound II).

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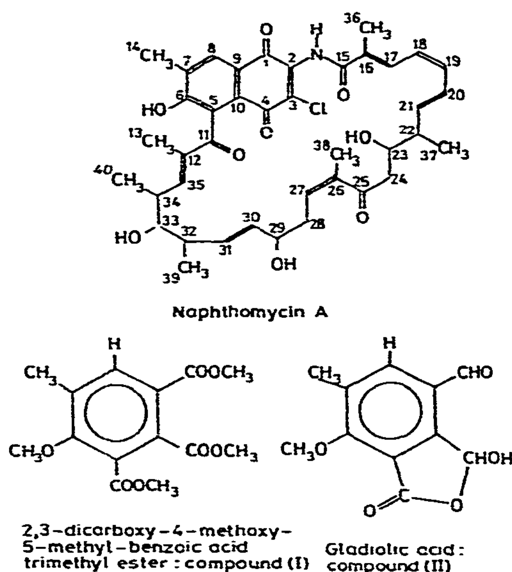


Fig. 1. Structural formulae of naphthomycin A, 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid trimethyl ester and gladiolic acid.

The extract of *Penicillium gladioli* growth medium was then directly transformed by oxidation and methylation and the new mixture was analyzed by HPLC after first checking by high-performance thin-layer chromatography (HPTLC). The peak corresponding to compound I was isolated by preparative column chromatography at medium pressure.

All the solvents utilized (unless stated otherwise) were Carlo Erba RS for HPLC; chloroform was stabilized with amylene.

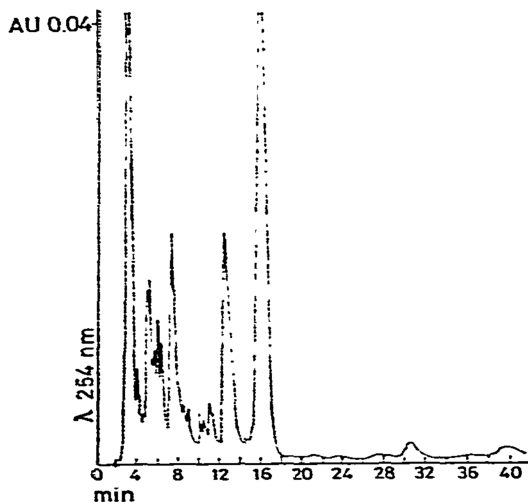


Fig. 2. HPLC of the extract of *Penicillium gladioli* growth medium before oxidation and methylation. Chromatographic conditions: LiChrosorb RP-8 (10 μm) column; eluent, acetonitrile containing 20% of 0.01 *M* H_3PO_4 at pH 3; flow-rate, 1 ml/min; pressure, 3500 p.s.i.; UV detection, λ = 254 nm.

Procedures

HPLC analyses of the extract of *Penicillium gladioli* growth medium before (a) and after (b) oxidation and methylation were performed. All separations were accomplished with an apparatus consisting of a Waters M 6000 solvent pump, a Rheodyne Model 70-10 injector valve and a Perkin-Elmer LC-75 UV–VIS detector. The columns (25 cm × 4.5 mm I.D.; Policonsult Scientifica, Rome, Italy) were packed with LiChrosorb RP-8 or Si 60 (10 μm) (E. Merck, Darmstadt, G.F.R.). The silica gel column was preceded by a pre-column (5 cm × 4.5 mm I.D.) packed with the same material.

Conditions: a, reversed-phase separation on RP-8 column, eluent acetonitrile (Merck LiChrosolv) containing 20% of 0.01 M H_3PO_4 at pH 3, UV detection at λ = 254 nm; b, normal-phase separation, after oxidation and methylation, on Si 60 column, eluent chloroform–2% ethyl acetate, UV detection at λ = 254 nm.

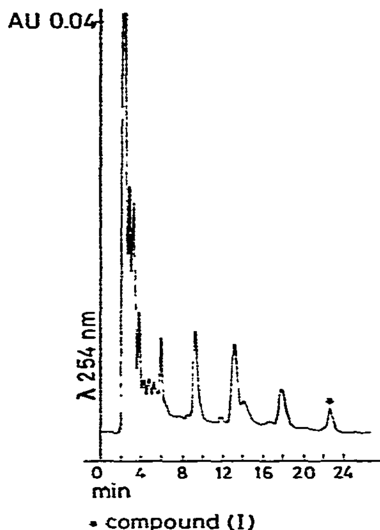
HPTLC of the extract of *Penicillium gladioli* growth medium after oxidation and methylation was carried out on Merck pre-coated plates Si 60 F_{254}, with chloroform–2% ethyl acetate as eluent. Chromatographic conditions were optimized by utilizing the degradation product of naphthomycin A as a reference.

The compound 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid trimethyl ester was isolated by medium-pressure preparative column chromatography. The apparatus consisted of a Perkin-Elmer Series 2/1 solvent pump, a Waters UK 6000 injector and a LKB Ultrorack fraction collector. For the separation, we utilized a LiChrosorb Si 60 (40–63 μm) Lobar B column (E. Merck; 310 × 25 mm I.D.) eluted with chloroform at 0.8 ml/min. The amount of mixture injected was 50 mg in 0.5 ml eluent. As a suitable detector for preparative chromatography was not available, the fractions collected were compared by TLC with the degradation product of naphthomycin A, utilizing sheets of Si 60 (Merck F_{254}) with chloroform–4% ethyl acetate as eluent. The R_F calculated for compound I under these conditions was 0.31.

RESULTS AND DISCUSSION

As Figs. 3 and 4 show, HPLC and HPTLC allowed the separation of compound I from a complex mixture of products obtained by transformation of the crude extract of cultures of *Penicillium gladioli*. In the HPTLC chromatogram the spot corresponding to compound I was preceded by one intense spot, running with the solvent front, and by several other spots. The R_F of compound I in the mixture is slightly lowered with respect to the R_F value of the degradation product of naphthomycin A utilized as a reference (R_F = 0.18 instead of 0.21), owing to a spot of lower polarity running very close to it. HPLC under the same conditions gave a better separation and the retention times for the degradation product of naphthomycin A and compound I in the mixture were the same (t_R = 22.4 min).

Preparative medium-pressure chromatography allowed the isolation of the reference product, which was characterized as compound I by elemental analysis, nuclear magnetic resonance (NMR), infrared (IR) and mass spectrometry (MS), $C_{14}H_{16}O_7$: calc. C 56.75, H 5.45; found C 57.07, H 5.47%. MS: M^+ 296, 265, 249, 233, 205, 191, 161, 146. IR (Nujol): 1760, 1450 cm^{-1}. NMR (C^2HCl_3), relative to tetramethylsilane (0 ppm): 2.34 (s, 3H), 3.84 (s, 3H), 3.87 (s, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 7.83 (s, 1H). These data correspond perfectly to those obtained for the degrada-



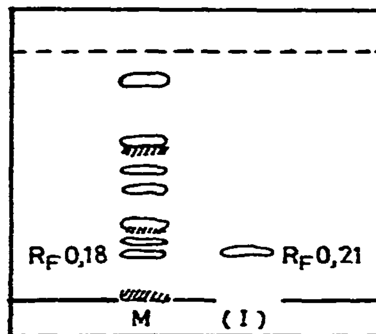


Fig. 3. HPLC of the extract of *Penicillium gladioli* growth medium after oxidation and methylation. Chromatographic conditions: LiChrosorb Si 60 (10 μm) column; pre-column packed with the same material: eluent, chloroform–2% ethyl acetate; flow-rate, 2 ml/min; pressure, 1000 p.s.i. The peak marked with an asterisk has the retention time of the degradation product of naphthomycin A and has been identified as compound I; t_R = 22.4 min.

Fig. 4. HPTLC of the extract of *Penicillium gladioli* growth medium after oxidation and methylation on Merck Si 60 F_{254} pre-coated plates. Eluent: chloroform–2% ethyl acetate. M = Extract of *Penicillium gladioli* growth medium after oxidation and methylation; (I) = degradation product of naphthomycin A.

tion product of naphthomycin A[5] whose structure (as that of one of its related compounds, previously isolated[5]) is thus unambiguously demonstrated. From these results it is therefore possible to determine the substituent pattern in the chromophore of naphthomycin A.

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