PERGAMON

Phytochemistry 54 (2000) 23–27

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Microbial hydroxylation/functionalization of terpenoid synthons derived from communic acids

Gérard Aranda[a], Robert Azerad[b,*], Michèle Maurs[b], Gildas Bertho[b], David Jiménez-González[c], Eduardo Cabrera-Torres[c], Alejandro F. Barrero[c]

[a]*Laboratoire de Synthèse Organique, UMR 3652, Ecole Polytechnique, 91128 Palaiseau Cedex, France*
[b]*Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601, Université René Descartes-Paris V, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France*
[c]*Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Granada, Spain*

Received 29 October 1999; received in revised form 29 November 1999

Abstract

Incubation of a communic acid-derived synthon with *Cunninghamella elegans* quantitatively affords 1β-, 3β- and 7β-monohydroxylated derivatives.

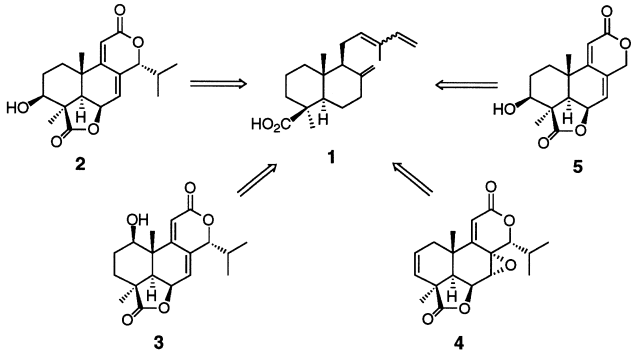
Keywords: *Cunninghamella elegans*; Biotransformation; Hydroxylation; Communic acids; Podolactones

1. Introduction

The so-called communic acids (**1**), an isomeric mixture of labdane diterpenes abundantly present in the berries of *Juniperus sabina* (Cupressaceae) (de Pascual Teresa et al., 1973) and other *Juniperus* species (Barrero et al., 1987), constitute valuable starting materials for various efficient hemisyntheses of active terpenoid molecules (Barrero et al., 1993a,b). They have been used recently in the synthesis of several members of the podolactone family (Barrero et al., 1995; Barrero et al., 1999), isolated from plants (*Podocarpus nagi*) or microorganisms (*Acrostalagmus* species), and presenting a number of interesting biological properties, including insect toxicity (Singh et al., 1973), antitumor (Hayashi et al., 1975) and antibiotic activity (Ellestad et al., 1970), plant growth inhibition (Galbraith et al., 1972), etc. Some of these podolactones, known, respectively as nagilactone F derivatives (**2**, Hayashi et al., 1978; **3**, Hayashi et al., unpublished results), podolide (**4**) (Arora et al., 1976), or wentilactone B (**5**) (Dorner et al., 1980) have been isolated as minor compounds, and are characterized by specific 1β- or 3β-hydroxylated positions, or 2–3 unsaturation, and thus, could be possibly obtained from simple microbial hydroxylation reactions of communic acid derived synthons, as previously demonstrated with a number of terpenoid compounds (Aranda et al., 1991, 1992, 1994, 1997, 1998; Azerad, 1999; Maurs et al., 1999).

* Corresponding author. Tel.: +33-1-4286-2171; fax: +33-1-4286-8387.
E-mail address: robert.azerad@biomedicale.univ-paris5.fr (R. Azerad).

0031-9422/00/$ - see front matter
PII: S0031-9422(00)00032-7



2. Results and discussion

We have thus undertaken a study of the microbial hydroxylation of communic acids **1**, or their derivatives **6**–**8** (Barrero et al., 1999), using various fungal microorganisms (*Absidia cylindrospora* LCP 57-1569, *Aspergillus niger* ATCC 9142, *Cunninghamella elegans* ATCC 36112, *Mucor plumbeus* CBS 110-16, *Rhizopus arrhizus* ATCC 11145). Communic acids **1** which, as usual for acid compounds, are poorly metabolized, and esters **6**–**8** which are rapidly hydrolyzed, were not suitable for these reactions. On the contrary, incubation of diol **9**, easily derived in 40% yield from the oxidative splitting of communic acid (Barrero et al., 1989; Barrero et al., 1993a,b, 1999), followed by LiAlH4 reduction (Barrero et al., 1993a,b), gave acceptable results, which were only hampered by the difficult separation of the variety of hydroxylated products formed. From screening experiments, *C. elegans* was selected as the best yielding microorganism and incubations of diol **9** with this microorganism (168 h, 27°C, 0.5 g l^{-1}) showed complete conversion, affording after extraction a small amount (5–10%) of 8,13-epoxides and epoxide deriving products, together with three monohydroxylated metabolites (about 80%), identified by mass spectrometry (M^+ at m/z 268), separated with difficulty by silicagel chromatography, and insoluble after purification in most usual solvents except methanol.

The major metabolic product (about 40% of the crude triol fraction, 25% yield after separation) was identified as a 3β-hydroxylated derivative **10** by ^{1}H- and ^{13}C-NMR spectroscopy (Table 1). ^{1}H-NMR showed the presence of unchanged vinylic hydrogens at 4.56 and 4.84 ppm (partly obscured by HDO resonance), primary alcohol groups as two doublets at 4.10 and 3.34 ppm (CH_2OH-16) and two multiplets at 3.62 and 3.39 ppm (CH_2OH-12), and methyl groups as singlets at 0.68 (CH_3-14) and 1.21 ppm (CH_3-15). Integration indicated the presence of an additional CHOH group (^{1}H resonance at about 3.35 ppm and ^{13}C at 81.1 ppm), but the signal was obscured by overlapping signals. ^{1}H-2D DQF COSY and ^{1}H–^{13}C heteronuclear correlations (HMQC) confirmed the presence of only eight remaining CH_2 groups and allowed to differentiate three couples of CH_2 groups, two of them involving the unchanged C6–C7 couple and C11–C12 side chain. (^3J) ^{1}H–^{13}C correlations (HMBC) showed a clear coupling of the CHOH hydrogen with CH_3-15 and CH_2OH-16, establishing the hydroxylation position at C-3 and allowing attribution of all ^{1}H and ^{13}C resonances. The assignment of a β-(equatorial) position to the 3-OH group resulted from the significant NOE enhancement of (α) H-5 and CH_3-15 upon irradiation of H-3. Moreover, the β-stereochemistry of the 3-OH group was confirmed by the observed downfield shift (0.4 ppm) of one of the CH_2OH-16 protons (at 4.10 ppm) and the absence of 1,3-diaxial interaction of the hydroxyl group with H-5 (unchanged chemical shift at 1.27 ppm).

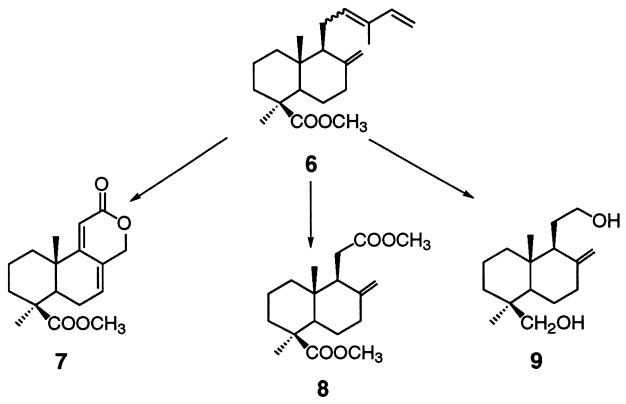
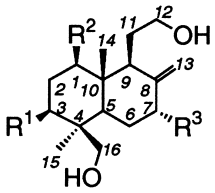
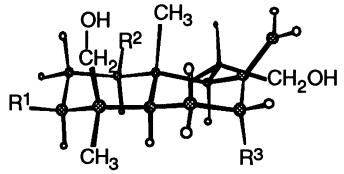


Table 1
^{13}C-NMR chemical shifts of diol **9** and metabolites **10**–**12** (125.77 MHz, solvent: MeOH-d_4). The data in parentheses indicate the number of hydrogens attached to each carbon atom, determined by distorsionless enhancement by polarization transfer (DEPT) using a flip angle of 135°

Carbon atoms numbers	9	10	11	12
C-1	40.0 (2)	38.0 (2)	78.7 (1)	40.1 (2)
C-2	19.8 (2)	29.1 (2)	30.6 (2)	20.0 (2)
C-3	36.4 (2)	81.1 (1)	34.3 (2)	36.7 (2)
C-4	40.0 (0)	43.7 (0)	39.7 (0)	39.4 (0)
C-5	57.4 (1)	56.6 (1)	56.6 (1)	49.5 (1)
C-6	25.3 (2)	25.4 (2)	25.5 (2)	32.1 (2)
C-7	39.4 (2)	39.4 (2)	39.5 (2)	74.6 (1)
C-8	149.7 (0)	149.3 (0)	149.9 (0)	150.8 (0)
C-9	53.9 (1)	53.7 (1)	54.8 (1)	48.2 (1)
C-10	40.2 (0)	39.8 (0)	46.0 (0)	40.4 (0)
C-11	27.9 (2)	28.3 (2)	30.6 (2)	27.7 (2)
C-12	62.1 (2)	62.2 (2)	62.7 (2)	62.0 (2)
C-13	106.8 (2)	107.2 (2)	107.4 (2)	109.8 (2)
C-14	15.7 (3)	15.7 (3)	11.3 (3)	14.9 (3)
C-15	27.6 (3)	23.4 (3)	27.4 (3)	27.6 (3)
C-16	64.7 (2)	65.0 (2)	64.4 (2)	65.0 (2)





10: R^1= OH; R^2 = R^3 = H

11: R^1 = R^3 = H; R^2= OH

12: R^1 = R^2 = H; R^3= OH

A second product (about 35% of the crude triol fraction, 30% after separation) was shown to be a 1β-hydroxylated derivative **11**, as demonstrated by ^{1}H- and ^{13}C-NMR (Table 1). The newly introduced CHOH group (*dd* at 3.51 ppm; ^{13}C at 78.7 ppm) was only coupled to CH_2 protons at 1.6 ppm and (^3J) ^{1}H–^{13}C correlations (HMBC) indicated a coupling with CH_3-14, C-2, C-3, C-9 and C-10. All other assignments fit correctly with the proposed structure. The β-(equatorial) position of the 1-OH group was deduced from the coupling data of H-1 with H-2 protons ($J_{ax\text{-}eq}$ = 5.0 and $J_{ax\text{-}ax}$ = 10.5 Hz), the absence of 1,3-diaxial interaction of the hydroxyl group with H-5 (unchanged chemical shift at 1.30 ppm), and the characteristic strong deshielding of one of the CH_2-11 protons (at 2.65 ppm). Moreover, significant nuclear Overhauser effects between H-1 and H-3α (at 1.08 ppm), (α)H-5, H-9, and H-11 confirmed the 1β-OH structure.

The third triol (about 25% of the crude triol fraction, 20% after separation) was a 7α-hydroxy derivative **12** as demonstrated again by ^{1}H- and ^{13}C-NMR spectroscopy (Table 1). The newly introduced CHOH group was observed at 4.29 ppm (^{13}C at 74.6 ppm), indicating an allylic position, as a broad unresolved triplet with small coupling constants resulting from its equatorial position. ^{1}H-2D DQF COSY and ^{1}H–^{13}C heteronuclear correlations (HMQC) showed the coupling of the CHOH hydrogen with only two hydrogens at 1.93 and 1.60 ppm (H-6eq and H-6ax). (^3J) ^{1}H–^{13}C correlations (HMBC) indicated a coupling of H-7 with C-5, C-8 and C-13. All other assignments fit correctly with the proposed structure. The α-(axial) position of the 7-OH group was deduced from the small coupling constants of H-7, the strong downfield shift of H-5 (at 1.80 ppm), and was confirmed by a significant nuclear Overhauser effect between H-7, both H-6, and one of the vinylic protons on C-13, together with the absence of significant nuclear Overhauser effects between H-7 and (α)H-5 or H-9.

In conclusion, the 3β- and 1β-hydroxylated derivatives of the diol **9**, obtained in fair yields by hydroxylation with *C. elegans*, constitute particularly attractive synthons for the hemisynthesis of the correspondingly hydroxylated podolactones, described as minor natural products, whose biological activities are unknown. Work is in progress to implement such hemisyntheses from the microbial hydroxylation products.

3. Experimental

3.1. General

^{1}H-NMR spectra were acquired in MeOH-d_4 solution at 200 and 500 MHz. ^{13}C-NMR spectra were determined at 125 MHz. Silicagel for column chromatography was Merck H60 (5–40 μm). Optical rotation measurements were performed on a Perkin–Elmer 241 spectropolarimeter. Incubation course was monitored by GC-MS on a Hewlett-Packard 5989 instrument, using a 25 m × 0.2 mm Ultra 2 capillary column (temperature programmed from 110 to 270°C at 8°C min^{-1}). TLC analysis was performed on precoated Merck silicagel F254 plates.

3.2. Starting materials

Diol **9** (Barrero et al., 1993a,b), m.p. 132–133.5°C, $[\alpha]_D^{22}$ + 26.3 (*c* 1.7, MeOH). CI-HRMS (NH_3): calculated for $C_{16}H_{29}O_2$ $[M+H]^+$ 253.2168, found 253.2169. ^{1}H-NMR (MeOH-d_4): δ 0.69 (3H, *s*, 14-CH_3), 0.94 (1H, *dt*, *J* = 4, 13.5 Hz, H-3β), 0.96 (3H, *s*, 15-CH_3), 1.13 (1H, *dt*, *J* = 4, 13 Hz, H-1α), 1.30 (1H, *br.d*, *J* = 13 Hz, H-5), 1.36 (1H, *dt*, *J* = 4, 13 Hz, H-6β), 1.49 (1H, *dt*, *J* = 14, 4 Hz, H-2α), 1.57 (1H, *dt*, *J* = 13.5, 3.5 Hz, H-11), 1.61 (1H, *m*, H-2β), 1.78 (2H, *m*, H-9 and H-11), 1.80 (1H, *m*, H-1), 1.83 (1H, *m*, H-6α), 1.86 (1H, *m*, H-3α), 1.97 (1H, *m*, H-7α), 2.39 (1H, *dm*, *J* = 13 Hz, H-7β), 3.28 and 3.72 (2H, 2*d*, *J* = 11 Hz, H-16), 3.39 (2H, *dt*, *J* = 10.5, 7.5 Hz, H-12), 3.62 (1H, *ddd*, *J* = 4, 8, 11 Hz, H-12), 4.55 and 4.82 (2H, 2*s*, H-13). ^{13}C-NMR, see Table 1.

3.3. Microorganisms, culture and incubation conditions

Absidia cylindrospora LCP 57-1569, *Aspergillus niger* ATCC 9142, *C. elegans* ATCC 36112, *Mucor plumbeus* CBS 110-16 and *Rhizopus arrhizus* ATCC 11145 were grown at 27°C in orbitally shaken 250 ml-conical flasks containing 100 ml of liquid medium. One litre of medium contained: corn steep liquor (Solulys L, Roquette, France) 10 g, glucose 30 g, KH_2PO_4 1 g, K_2HPO_4 2 g, $NaNO_3$ 2 g, KCl 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.02 g. After 65 h-growth, the substrate (250 mg) in EtOH (5 ml) was evenly distributed between five flasks and incubation was continued in the same conditions. Samples (1 ml) were aseptically withdrawn every day, saturated with sodium chloride

and extracted with EtOAc for TLC and GC-MS analysis. Most transformations were continued until no further increase of metabolites was observed (usually 4–7 days).

3.4. Isolation of metabolites

The crude incubation broth (relative to 100 mg of diol **9** incubated with *C. elegans* for seven days) was extracted by shaking with CH_2Cl_2 (3 × 300 ml) for two days, then continuously extracted with EtOAc (200 ml, two days). Organic layers were pooled and dried over K_2CO_3. After removal of the solvents under vacuum, the oily yellow residue was chromatographed on silicagel with increasing amounts of EtOAc (50–100%) in cyclohexane. The first eluted fraction (17 mg) corresponded to a mixture of epoxide derivatives. The second crystalline fraction (26 mg) was identified as the 3β,12,19-trihydroxy-13,14,15,16-tetranorlabd-8(17)-ene (**10**), m.p. 209–211°C (after crystallization in MeOH-EtOAc), $[\alpha]_D^{22} + 23.8$ (*c* 0.51, MeOH). CI-HRMS (NH_3): calculated for $C_{16}H_{29}O_3$ $[M+H]^+$ 269.2117, found 269.2111. ^{1}H-NMR (MeOH-d_4): δ 0.68 (3H, *s*, 14-CH_3), 1.21 (3H, *s*, 15-CH_3), 1.25 (1H, *dd*, $J = 13$, 2 Hz, H-5), 1.29 (1H, *m*, H-1), 1.35 (1H, *dt*, $J = 4.5$, 13 Hz, H-6β), 1.63 (1H, *m*, H-11), 1.70–1.85 (6H, *m*, H-1, H-2, H-6α, H-9, H-11), 1.97 (1H, *dt*, $J = 4.5$, 13 Hz, H-7α), 2.40 (1H, *dt*, $J = 13$, 3 Hz, H-7β), 3.34 and 4.10 (2H, 2*d*, $J = 11$ Hz, H-16), 3.39 (3H, *m*, H-3, H-12), 3.62 (1H, *ddd*, $J = 4.5$, 8, 11 Hz, H-12), 4.56 and 4.84 (2H, 2*s*, H-13). ^{13}C-NMR, see Table 1. The next crystalline fraction (31 mg) was identified as the 7α,12,19-trihydroxy-13,14,15,16-tetranorlabd-8(17)-ene (**12**), m.p. 200–203°C (after crystallization in MeOH-EtOAc), $[\alpha]_D^{22} - 25.7$ (*c* 1.45, MeOH). CI-HRMS (NH_3): calculated for $C_{16}H_{29}O_3$ $[M+H]^+$ 269.2117, found 269.2105. ^{1}H-NMR (MeOH-d_4): δ 0.65 (3H, *s*, 14-CH_3), 0.95 (3H, *s*, 15-CH_3), 0.98 (1H, *dt*, $J = 4$, 13.5 Hz, H-3β), 1.15 (1H, *dt*, $J = 3.5$, 13 Hz, H-1α), 1.53 (2H, *m*, H-2), 1.60 (2H, *m*, H-6β and H-11), 1.78 (2H, *m*, H-1β, H-11), 1.80 (1H, *dd*, $J = 14$, 2 Hz, H-5), 1.87 (1H, *br.d*, $J = 13.5$ Hz, H-3α), 1.93 (1H, *br.d*, $J = 14$, H-6α), 2.24 (1H, *d*, $J = 11$ Hz, H-9), 3.29 and 3.71 (2H, 2*d*, $J = 11$ Hz, H-16), 3.42 (1H, *dt*, $J = 10.5$, 7.5 Hz, H-12), 3.62 (1H, *ddd*, $J = 4$, 10, 11 Hz, H-12), 4.29 (1H, *br.t*, $w_{1/2} = 7$ Hz, H-7β), 4.68 and 5.03 (2H, 2*s*, H-13). ^{13}C-NMR, see Table 1. Further elution afforded the 1β,12,19-trihydroxy-13,14,15,16-tetranorlabd-8(17)-ene (**11**), as a colorless oil (21 mg, containing about 10% of **12**) which was chromatographed again to give an analytical sample, $[\alpha]_D^{22} + 11.2$ (*c* 0.91, MeOH). CI-HRMS (NH_3): calculated for $C_{16}H_{29}O_3$ $[M+H]^+$ 269.2117, found 269.2115. ^{1}H-NMR (MeOH-d_4): δ 0.73 (3H, *s*, 14-CH_3), 0.94 (3H, *s*, 15-CH_3), 1.08 (1H, *dt*, $J = 4$, 13.5 Hz, H-3α), 1.30 (1H, *m*, H-5), 1.44 (1H, *ddt*, $J = 4.5$, 13, 13 Hz, H-6), 1.60–1.70 (3H, *m*, H-2α, H-2β and H-11), 1.80–1.90 (2H, *m*, H-6β and H-3β), 1.92 (1H, *m*, H-7), 1.96 (1H, *br.d*, $J = 11.5$ Hz, H-9), 2.37 (1H, *ddd*, $J = 2.5$, 4, 13 Hz, H-7), 2.64 (1H, *ddt*, $J = 2.5$, 14, 9 Hz, H-11), 3.26 and 3.69 (2H, 2*d*, $J = 11$ Hz, H-16), 3.43 (1H, *dt*, $J = 14$, 8 Hz, H-12), 3.51 (1H, *dd*, $J = 5$, 10.5 Hz, H-1), 4.55 and 4.82 (2H, 2*s*, H-13). ^{13}C-NMR, see Table 1.

Acknowledgements

This research was supported by the Centre National de la Recherche Scientifique, France, and the Dirección General de Investigación Científica y Técnica, Spain (DGICYT, Project No. PB 95/1192). We thank Nicole Morin and Michel Levart for all HRMS measurements and the Spanish Ministerio de Educación y Cultura for the grant provided to D. Jiménez-González.

References

Aranda, G., Bertranne-Delahaye, M., Azerad, R., Maurs, M., Cortes, M., Ramirez, H., Vernal, G., Prangé, T., 1997. Synth. Commun. 27, 45.

Aranda, G., Bertranne-Delahaye, M., Lallemand, J.-Y., Azerad, R., Maurs, M., Cortes, M., Lopez, J., 1998. J. Mol. Catalysis: B. Enzymatic (Special issue: Biotrans'97, R. Azerad ed.) 5, 255.

Aranda, G., ElKortbi, M.S., Lallemand, J.Y., Neuman, A., Hammoumi, A., Facon, I., Azerad, R., 1991. Tetrahedron 47, 8339.

Aranda, G., Facon, I., Lallemand, J.Y., Leclaire, M., Azerad, R., Cortes, M., Lopez, J., Ramirez, H., 1992. Tetrahedron Lett. 33, 7845.

Aranda, G., Lallemand, J.-Y., Azerad, R., Maurs, M., Cortes, M., Ramirez, H., Vernal, G., 1994. Synth. Commun. 24, 2525.

Arora, S.K., Bates, R.B., Chou, P.C.C., Sanchez, L.W.E., Brown Jr., K.S., Galbraith, M.N., 1976. J. Org. Chem. 41, 2458.

Azerad, R. 2000. Microbial hydroxylation of terpenoid compounds. In: Patel, R. (Ed.), Stereoselective Biocatalysis. Marcel Dekker, New York, p. 153.

Barrero, A.F., Altarejos, J., Alvarez Manzaneda, E.J., Ramos, J.M., Salido, S., 1993a. Tetrahedron 49, 6251.

Barrero, A.F., Alvarez Manzaneda, E.J., Altarejos, J., Ramos, J.M., Salido, S., 1993b. Bull. Soc. Chim. Fr 130, 700.

Barrero, A.F., Sanchez, J.F., Altarejos, J., 1987. Ars Pharmaceutica 28, 449.

Barrero, A.F., Sanchez, J.F., Altarejos, J., 1989. Tetrahedron Lett. 30, 5515.

Barrero, A.F., Sanchez, J.F., Elmerabet, J., 1995. Tetrahedron Lett. 36, 5251.

Barrero, A.F., Sanchez, J.F., Elmerabet, J., Jimenez-Gonzales, D., Macias, F.A., Simonet, A.M., 1999. Tetrahedron 55, 7289.

de Pascual Teresa, J., San Feliciano, A., Barrero, A.F., 1973. An. Quim. 69, 1065.

Dorner, J.W., Cole, R.J., Springer, J.P., Cox, R.H., Cutler, H., Wicklow, D.T., 1980. Phytochemistry 19, 1157.

Ellestad, G.A., Evans, R.H., Kunstman, M.P., Lancaster, J.E., Morton, G.O., 1970. J. Am. Chem. Soc. 92, 5483.

Galbraith, M.N., Horn, D.H.S., Ito, S., Kodama, M., Sasse, J.M., 1972. Agr. Biol. Chem. 36, 2393.

Hayashi, Y., Matsumoto, T., Sakan, T., 1978. Heterocycles 10, 123.
Hayashi, Y., Sakan, T., Sakurai, Y., Tashiro, T., 1975. Gann 66, 587.
Maurs, M., Azerad, R., Cortes, M., Aranda, G., Bertranne-Delahaye, M., Ricard, L., 1999. Phytochemistry 52, 291.
Singh, P., Fenemore, P.G., Russell, G.B., 1973. Aust. J. Biol. Sci. 26, 911.