Monatshefte für Chemie 130, 589–595 (1999)

Monatshefte für Chemie
Chemical Monthly

Printed in Austria

Synthesis of Some New Benzylic Ethers from 1,8-Cineole with Antimicrobial Activity

Armando J. D. Silvestre[1], **José A. S. Cavaleiro**[1,*], **Sónia S. Feio**[2], **José C. Roseiro**[2], **Bernard Delmond**[3], and **Claude Filliatre**[3]

[1] Department of Chemistry, University of Aveiro, P-3810 Aveiro, Portugal
[2] Instituto Nacional de Engenharia e Tecnologia Industrial, IBQTA, Laboratório de Microbiologia Industrial, P-1699 Lisboa Codex, Portugal
[3] Institut du Pin, Université de Bordeaux I, F-33405 Talence, France

Summary. The synthesis and structural characterization of several benzylic derivatives of 3-*exo*-hydroxy-1,8-cineole is described. The new compounds show antimicrobial activity against *Cladosporium cucumerinum*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, and *Candida albicans*.

Keywords. 1,8-Cineole; Chemical transformation; 3-*exo*-Benzyloxy-1,8-cineole; Antimicrobial activity.

Synthese einiger neuer Benzylether des 1,8-Cineols mit antimikrobieller Aktivität

Zusammenfassung. Die Synthese und strukturelle Charakterisierung einiger Benzylderivate des 3-*exo*-Hydroxy-1,8-cineols werden beschrieben. Die neuen Verbindungen zeigen antimikrobielle Aktivität gegen *Cladosporium cucumerinum*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa* und *Candida albicans*.

Introduction

1,8-Cineole (**1**) is an abundant monoterpene present in the essential oils of several species, *e.g. Eucalyptus globulus* Labill [1–3]. At the beginning of the present century, due to their antibacterial properties [4, 5] 1,8-cineole rich essential oils were economically important products because of their pharmacological uses in the treatment of respiratory problems; however, the development of new and more effective drugs quickly replaced the 1,8-cineole containing mixtures.

Apart from the pharmacological applications cited above, 1,8-cineole finds little use as a raw material, mainly because it is a chemically inert compound [6]. Despite its chemical stability, some studies have been carried out on the oxygenation of 1,8-cineole by chemical [7, 8] and biological [9–16] processes. However, none of these transformations represents a significant breakthrough on

* Corresponding author

the transformation of this abundant and cheap substance into more valuable products. Studies on the transformation of 1,8-cineole into new and more valuable products [17–19] can be of great significance.

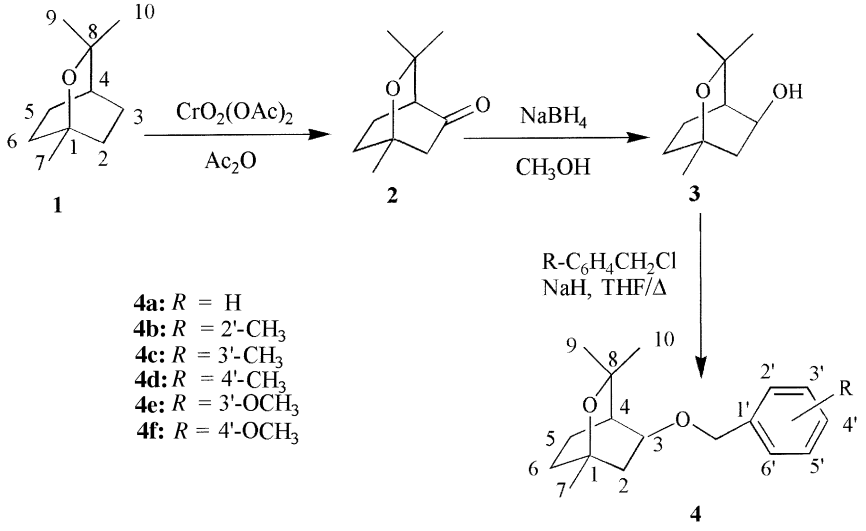
Benzylic ether derivatives of 1,8-cineole might become useful derivatives taking into account that similar compounds are being marketed as agrochemicals [20]. The synthesis and antimicrobial evaluation of such cineole-derived ethers is described in the present work.

Results and Discussion

Syntheses

3-Keto-1,8-cineole (**2**) was obtained by oxidation of **1** with chromyl acetate in acetic anhydride; the ketone **2** was then selectively reduced to 3-*exo*-hydroxy-1,8-cineole (**3**) [7]. Attempts to benzylate the alcohol **3** using benzyl chloride in the presence of Na_2CO_3/KI in acetone [21] were unsuccessful; however, such benzylations were achieved in good yields (85–95%) by refluxing **3** with benzyl chloride or derivatives thereof in the presence of sodium hydride in dry tetrahydrofuran. Pure benzyl ethers **4a–f** were obtained as pale yellow liquids after chromatographic purification.

Compounds **4a–f** were characterized using ^1H and ^{13}C NMR and mass spectrometry. Definitive assignments of aliphatic carbon and proton resonances were achieved using HETCOR experiments; the carbon and proton resonances of the aromatic part of the molecule were assigned considering the shifts induced by methyl and methoxyl groups on the aryl moiety. From ^1H NMR data it can be seen that the equatorial protons of the aliphatic CH_2 groups always appear at higher chemical shifts than the corresponding axial protons. This can be explained by the deshielding effect exerted by the 1,8-cineole oxygen atom upon the equatorial



Scheme 1

protons. The values of the coupling constants found for H-3 confirm its axial position, thus establishing the *exo*-position for the benzyloxy substituent. In all cases, the benzylic protons appear at $\delta = 4.48$–4.52 ppm as an AB system with coupling values characteristic of geminal protons.

The mass spectra of compounds **4a–f** are characterized by the presence of the molecular ion and two characteristic fragmentations. One fragment corresponds to the loss of a methyl group ($M^{+\cdot} - 15$); the formation of this species in the case of compound **4a** indicates that the methyl group must be lost from the aliphatic part of the molecule, as no methyl group exists in the aromatic moiety of **4a**. A second fragment, which gives rise to the base peak in each spectrum, is assigned to the benzylic substructure.

Antimicrobial activity essays

The multiwell plates assays used in the present work are based on qualitative techniques as it is currently used in the assessment of hydrophobic compounds. The amounts of the test compounds in these techniques are usually higher than those applied in the assessment of the activity of hydrophilic compounds using serial dilutions with nutrient broth. This is due to the fact that the contact of the hydrophobic compounds with the microorganism is restricted by transport hindrances and biodisponibility. Therefore, the high concentration is apparent and only indicates the potential antimicrobial activity of the test compounds. The concentration of the test compound is also related to the density of the microbial population used in the test. We used 108 cfu ml^{-1} rather than the much lower suspension density usually described in literature. The motivation behind this procedure is based on the fact that a higher density reduces the occurrence of false positive results. The concentration of the compounds used in the present work was selected from *Franich et al.* [22] (50 mg test compound per ml acetone) and corresponds to a dosage of 1 mg which is in contact with 0.02 ml of the solidified culture medium containing the microorganism after evaporation of acetone [23].

Compounds **4a–f** inhibited the growth of *C. cucumerinum* in the antifungal bioassay, compound **4f** being the most active and compound **4a** the least active substance. **4b–e** have shown similar fungicidal behaviour although to a smaller extent than **4f**.

Compounds **4b–d** and **4f** also show fungistatic activity against *C. albicans*. Compound **4a** partially inhibited yeast development, whereas compound **4e** did not show antimicrobial activity against *C. albicans*.

The results of antimicrobial tests are summarized in Table 1. It could be demonstrated that the growth of *S. aureus* is partially inhibited by compounds **4e** and **4f** and completely inhibited by compounds **4a–d**. When samples **4a–d** were inoculated into fresh culture medium, no development of the microorganism was observed, indicating bactericidal activity. *M. smegmatis* development was also inhibited by compounds **4a–f**; however, only bacteriostatic activity was observed.

The growth of *P. aeruginosa* was not inhibited by any of the test compounds. This might be associated with the transport of the compounds through the membrane. In fact, *gram*-negative bacteria contain an outer lipopolysaccharide wall layer [24] where lipophilic compounds are easily trapped producing an

Table 1. Antimicrobial activity of compounds **4a–f** by multiwell plate method (50 mg · ml^{-1}); +: absence of growth, ±:presence of rare colonies, −: normal growth

	4a	**4b**	**4c**	**4d**	**4e**	**4f**
C. albicans	±	+	+	+	−	+
M. smegmatis	+	+	+	+	+	+
P. aeruginosa	−	−	−	−	−	−
S. aureus	+	+	+	+	±	±

effective transport hindrance [25]. The mechanism behind this observation may result from the solubility behaviour of the compounds which do not pass easily through the *gram*-negative membrane structure with a lipid content of up to 25% of dry weight. Conversely, lipophilic compounds could pass through the *gram*-positive cell walls which contain 0–2.5% of lipids. Therefore, antimicrobial activity can be designed in such a way that lipophilic compounds can be used against *gram*-positive bacteria, whereas the less lipophilic compounds can be used effectively against *gram*-negative bacteria.

It can be concluded that all tested compounds show antimicrobial activity, suggesting that the active function is constitutive of this kind of molecule and not strictly related with the presence of substituents in the aromatic ring. However, the results observed also suggest some degree of association between the antimicrobial activity and the aromatic substituents. Compounds containing a methyl group in the aromatic positions 2, 3, and 4 (**4b**–**d**) show a wider activity profile than compounds **4a**, **4e**, and **4f**. The position of the methyl group in the aromatic ring does not seem to interfere with the activity of the test compounds; however, due to its lipophylic character the methyl moiety could contribute to enhance the antimicrobial activity against the microorganisms tested.

In compounds **4e** and **4f**, the position of the methoxyl group in the aromatic ring seems to influence the activity: **4f** inhibited the growth of *C. albicans*, whereas **4e** did not show any activity towards this microorganism.

Experimental

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AMX 300 spectrometer at 300.13 and 75.47 MHz (*TMS* as an internal reference). In the 2D heteronuclear shift correlation (^{1}H, ^{13}C), the initial matrix of 2048×512 data points was zero-filled to 2048×1024 points; the relaxation delay was 2 s. Mass spectra were measured in the electron impact mode at 70 eV on a VG Autospec Q instrument. Column chromatography was performed on silica gel (Merck silica gel 60, 70–230 mesh). All chemicals and solvents were obtained from commercial sources and used as received or dried using standard procedures.

Organisms

The antimicrobial activity of the test compounds was tested against fungus *Cladosporium cucumerinum* CCMI 206, *gram*-positive bacteria *Staphylococcus aureus* CCMI 335, acid fast bacteria *Mycobacterium smegmatis* CCMI 690, *gram*-negative bacteria *Pseudomonas aeruginosa*

CCMI 352, and yeast *Candida albicans* CCMI 209. All microorganisms were obtained from the Culture Collection of Industrial Microorganisms at the Laboratório de Microbiologia Industrial, Instituto Nacional de Engenharia e Tecnologia Industrial, Lisbon, Portugal.

Antifungal bioassay using the bioautographic method

The antifungal activity of compounds **4a–f** was tested using the bioautographic method on thin layer chromatography (TLC) against *Cladosporium cucumerinum* according to the procedure described by *Homans* and *Fuchs* [26]. Compounds **4a–f** were dissolved in acetone at a concentration of $10\,mg \cdot ml^{-1}$. A volume of 0.02 ml of each suspension was then applied on silica gel F_{254} 0.2 mm Merck plates. The TLC plates were developed in standard tanks lined with filter paper eluting with light petroleum:CH_2Cl_2 (7:3). The dried plates were sprayed with a $10^8\,cfu \cdot ml^{-1}$ suspension of *Cladosporium cucumerinum* and incubated at 25°C in a moist chamber for 72 h. Zones of inhibition were observed as clear spots against the colored background of the fungus developed in the plates.

Antimicrobial bioassay using the multiwell plates method

The antibacterial activity of compounds **4a–f** was tested against *S. aureus*, *M. smegmatis*, and *P. aeruginosa* in multiwell plates according to the procedure described by *Feio et al.* [23]. The same method was used to assess the activity against *Candida albicans*. The test compounds, dissolved in acetone at a concentration of $50\,mg \cdot ml^{-1}$, were applied to the multiwell plates containing the microorganism incorporated in the culture medium. Brain Heart Infusion (Difco) with 2% agar was used for *S. aureus* and *M. smegmatis* and Nutrient Agar (Difco) for *P. aeruginosa. C. albicans* was grown in Malt Extract Agar. The plates were incubated at 37°C in a moist chamber for 24 h. The Difco plates were observed by light microscopy, and the presence of antibacterial or antifungal activity was defined as absence of colony development. The bactericidal/fungicidal or bacteriostatic/fungistatic activity was determined by the observation of non-growth or growth in the appropriate fresh culture medium after contact with the test samples.

Synthesis of 3-exo-benzyloxy-1,8-cineole derivatives (**4a–f**)

300 mg 3-*exo*-hydroxy-1,8-cineole (**3**, 1.76 mmol) and 2.12 mmol of the required benzyl chloride (1.2 eqs) were dissolved in dry 20 ml *THF*; the resulting solution was then treated with 63.50 mg sodium hydride (2.65 mmol). The mixture was refluxed under a N_2 atmosphere for 12 h, poured into an ice/H_2O mixture, and extracted with dichloromethane. The organic solution was dried over anhydrous Na_2SO_4 and concentrated by vacuum distillation. The reaction mixture was purified by column chromatography on silica gel, eluting first with petroleum ether to remove the excess of the benzyl chloride and then with petroleum ether:CH_2Cl_2 (7:3) to obtain the benzylic derivatives **4a–f**.

3-exo-Benzyloxy-1,8-cineole (**4a**; $C_{17}H_{24}O$)

Yield: 435 mg (95%); HR-MS: found 260.1776, $C_{17}H_{24}O$ requires 260.1776; ^1H NMR (300 MHz, δ, $CDCl_3$): 1.11 (3H, s, H-7), 1.25 (3H, s, H-9), 1.31–1.39 (2H, m, H-5_{ax},6_{ax}), 1.46 (3H, s, H-10), 1.53–1.64 (1H, m, H-5_{eq}), 1.78–1.85 (2H, m, H-2_{ax},4_{eq}), 1.97–2.10 (2H, m, H-2_{eq},6_{eq}), 3.81 (1H, ddd, $J = 10.3$, 6.2, and 1.9 Hz, H-3_{ax}), 4.52 (2H, AB system, $J_{AB} = 11.8$ Hz, Ar-CH_2), 7.12–7.25 (5H, m, H 2′–6′) ppm; ^{13}C NMR (75 MHz, δ, $CDCl_3$): 21.4 (C-5), 24.8 (C-7), 27.0 (C-6), 30.1 (C-9), 30.3 (C-10), 36.3 (C-4), 41.1 (C-2), 69.4 (C-1), 70.1 (C-3), 73.4 (C-8), 77.7 (Ar-CH_2), 128.5 (C 2′,6′), 127.2 (C 4′), 127.3 (C3′,5′) ppm; MS: $m/z = 260$ (1%), 245(2), 91(100), 43(35).

3-exo-(2-Methylbenzyloxy)-1,8-cineole (**4b**, $C_{18}H_{26}O_2$)

Yield: 410 mg (85%); HR-MS: found 274.1929, $C_{18}H_{26}O_2$ requires 274.1932; ^{1}H NMR (300 MHz, δ, $CDCl_3$): 1.12 (3H, s, H-7), 1.25 (3H, s, H-9), 1.28–1.34 (2H, m, H-5$_{ax}$,6$_{ax}$), 1.46 (3H, s, H-10), 1.53–1.64 (1H, m, H-5$_{eq}$), 1.78–1.85 (2H, m, H-2$_{ax}$,4$_{eq}$), 1.98–2.09 (2H, m, H-2$_{eq}$,6$_{eq}$), 2.35 (3H, s, 2′-CH_3), 3.82 (1H, ddd, $J = 10.2$, 6.3, and 2.0 Hz, H-3$_{ax}$), 4.52 (2H, AB system, $J_{AB} = 11.8$ Hz, Ar-CH_2), 7.22–7.28 (4H, m, H-2′,3′,5′,6′) ppm; ^{13}C NMR (75 MHz, δ, $CDCl_3$): 18.8 (2′-CH_3), 21.4 (C-5), 26.9 (C-7), 30.0 (C-6), 30.4 (C-9,10), 36.3 (C-4), 41.1 (C-2), 69.4 (C-1), 70.3 (C-3), 73.6 (C-8), 78.0 (Ar-CH_2), 125.8 (C-5′), 127.4 (C-3′), 130.0 (C-4′), 136.0 (C-2′), 136.7 (C-1′) ppm; MS: $m/z = 274$ (3%), 259(2), 105(100), 43(20).

3-exo-(3-Methylbenzyloxy)-1,8-cineole (**4c**; $C_{18}H_{26}O_2$)

Yield: 429 mg (89%); HR-MS: found 274.1931, $C_{18}H_{26}O_2$ requires 274.1932; ^{1}H NMR (300 MHz, δ, $CDCl_3$): 1.12 (3H, s, H-7), 1.25 (3H, s, H-9), 1.29–1.33 (2H, m, H-5$_{ax}$,6$_{ax}$), 1.47 (3H, s, H-10), 1.51–1.63 (1H, m, H-5$_{eq}$), 1.79–1.84 (2H, m, H-2$_{ax}$,4$_{eq}$), 1.98–2.09 (2H, m, H-2$_{eq}$,6$_{eq}$), 2.34 (3H, s, 3′-CH_3), 3.81 (1H, ddd, $J = 10.1$, 6.4 and 2.1 Hz, H-3$_{ax}$), 4.52 (2H, AB system, $J_{AB} = 11.8$ Hz, Ar-CH_2), 7.22–7.28 (4H, m, H-2′,3′,5′,6′) ppm; ^{13}C NMR (75 MHz, δ, $CDCl_3$): 20.0 (3′-CH_3), 21.3 (C-5), 26.8 (C-7), 30.1 (C-6), 30.4 (C-9,10), 36.3 (C-4), 41.1 (C-2), 69.3 (C-1), 70.3 (C-3), 73.5 (C-8), 78.0 (Ar-CH_2), 125.6 (C-6′), 127.8 (C-5′), 128.6 (C-4′), 129.2 (C-2′), 136.8 (C-3′), 138.7 (C-1′) ppm; MS: $m/z = 274$ (5%), 259(36), 105(100), 43(25).

3-exo-(4-Mehtylbenzyloxy)-1,8-cineole (**4d**; $C_{18}H_{26}O_2$)

Yield: 458 mg (94%); HR-MS: found 274.1931, $C_{18}H_{26}O_2$ requires 274.1931; ^{1}H NMR (300 MHz, δ, $CDCl_3$): 1.11 (3H, s, H-7), 1.24 (3H, s, H-9), 1.29–1.38 (2H, m, H-5$_{ax}$,6$_{ax}$), 1.46 (3H, s, H-10), 1.55–1.64 (1H, m, H-5$_{eq}$), 1.79–1.86 (2H, m, H-2$_{ax}$,4$_{eq}$), 1.98–2.06 (2H, m, H-2$_{eq}$,6$_{eq}$), 2.34 (3H, s, 4′-CH_3), 3.79 (1H, ddd, $J = 9.9$, 6.3, and 2.1 Hz, H-3$_{ax}$), 4.52 (2H, AB system, $J_{AB} = 11.7$ Hz, Ar-CH_2), 7.13 (2H, d, $J = 7.9$ Hz, H-3′,5′), 7.23 (2H, d, $J = 7.9$ Hz, H-2′,6′) ppm; ^{13}C NMR (75 MHz, δ, $CDCl_3$): 21.0 (4′-CH_3), 21.3 (C-5) 26.9 (C-7), 30.0 (C-6), 30.4 (C-9,10), 36.3 (C-4), 41.1 (C-2), 69.7 (C-1), 70.1 (C-3), 73.4 (C-8), 77.4 (Ar-CH_2), 127.5 (C-3′,5′), 128.9 (C-2′,6′), 135.6 (C-1′), 136.9 (C-4′) ppm; MS: $m/z = 274$ (7%), 259(64), 105(100), 43(12).

3-exo-(3-Methoxybenzyloxy)-1,8-cineole (**4e**; $C_{18}H_{26}O_3$)

Yield: 470 mg (92%); HR-MS: found 290.1884, $C_{18}H_{26}O_3$ requires 290.1881; ^{1}H NMR (300 MHz, δ, $CDCl_3$): 1.12 (3H, s, H-7), 1.25 (3H, s, H-9), 1.28–1.38 (2H, m, H-5$_{ax}$,6$_{ax}$), 1.47 (3H, s, H-10), 1.56–1.65 (1H, m, H-5$_{eq}$), 1.79–1.88 (2H, m, H-2$_{ax}$,4$_{eq}$), 1.98–2.07 (2H, m, H-2$_{eq}$,6$_{eq}$), 3.80 (1H, m, H-3$_{ax}$), 3.80 (3H, s, 3′-OCH_3), 4.48 (2H, AB system, $J_{AB} = 11.9$ Hz, Ar-CH_2), 6.8 (1H, d, $J = 8.3$ Hz, H-4′), 6.89–6.92 (2H, m, H-2′,6′), 7.26 (1H, d, $J = 8.3$ Hz, H-5′) ppm; ^{13}C NMR (75 MHz, δ, $CDCl_3$): 21.3 (C-5), 26.7 (C-7), 30.1 (C-6), 30.4 (C-9,10), 36.4 (C-4), 41.1 (C-2), 55.1 (3′-OCH_3), 69.7 (C-1), 70.1 (C-3), 73.4 (C-8), 77.6 (Ar-CH_2), 112.6 (C-4′), 112.8 (C-2′), 129.3 (C-5′), 140.4 (C-1′), 159.6 (C-3′) ppm; MS: $m/z = 290$ (5%), 275(30), 121(100), 43(17).

3-exo-(4-Methoxybenzyloxy)-1,8-cineole (**4f**; $C_{18}H_{26}O_3$)

Yield: 480 mg (94%); HR-MS: found 290.1880, $C_{18}H_{26}O_3$ requires 290.1881; ^{1}H NMR (300 MHz, δ, $CDCl_3$): 1.11 (3H, s, H-7), 1.24 (3H, s, H-9), 1.29–1.36 (2H, m, H-5$_{ax}$,6$_{ax}$), 1.45 (3H, s, H-10), 1.56–1.65 (1H, m, H-5$_{eq}$), 1.78–1.87 (2H, m, H-2$_{ax}$,4$_{eq}$), 1.97–2.09 (2H, m, H-2$_{eq}$,6$_{eq}$), 3.80 (3H, s, 4′-OCH_3), 3.81 (1H, m, H-3$_{ax}$), 4.48 (2H, AB system, $J_{AB} = 11.9$ Hz, Ar-CH_2), 6.8 (2H, d,

$J = 8.4$ Hz, H-3′,5′), 7.23 (2H, d, $J = 8.4$ Hz, H-2′,6′) ppm; ^{13}C NMR (75 MHz, δ, $CDCl_3$): 21.4 (C-5), 27.0 (C-7), 30.0 (C-6), 30.4 (C-9,10), 36.3 (C-4), 41.1 (C-2), 55.2 (4′-OCH_3), 69.5 (C-1), 70.1 (C-3), 73.4 (C-8), 77.5 (Ar-CH_2) 113.7 (C-3′,5′), 128.8 (C-2′,6′), 129.4 (C-1′), 158.9 (C-4′) ppm; MS: $m/z = 290$ (6%), 275(11), 121(100), 43(7).

References

[1] Simonsen JL (1947) The Terpenes. University Press, vol 1. Cambridge, p 422
[2] Silvestre AJD, Cavaleiro JAS, Delmond B, Filliatre C, Bourgeois G (1994) Flavour Fragr J **9**: 51
[3] Silvestre AJD, Cavaleiro JAS, Delmond B, Filliatre C, Bourgeois G (1997) Ind Crops Products **6**: 27
[4] Hassan M, Benouda M, Benjilali B (1988) Fitoterapia **59**: 115
[5] Kumar A, Sharma VD, Sing AK, Singh K (1988) Fitoterapia **59**: 141
[6] Carman RM, Fletcher MT (1983) Aust J Chem **36**: 1483
[7] Boggiatto MV, Heluani CS, Fenik IJS, Catalán CAN (1987) J Org Chem **52**: 1505
[8] Asakawa Y, Matsuda R, Tori M, Hashimoto T (1988) Phytochemistry **27**: 3861
[9] Flynn TM, Southwell IA (1979) Aust J Chem **32**: 2093
[10] MacRae IC, Alberts V, Carman RM, Shaw IM (1979) Aust J Chem **32**: 917
[11] MacRae IC, Carman RM, Perkins MV (1986) Aust J Chem **39**: 1739
[12] Liu WG, Rosazza JPN (1990) Tetrahedron Lett **31**: 2833
[13] Carman RM, Klika KD (1992) Aust J Chem **45**: 651
[14] Bull SD, Carman RM, Carrick FN, Klika KD (1993) Aust J Chem **46**: 441
[15] Carman RM, Garner AC, Klika KD (1994) Aust J Chem **47**: 1509
[16] Carman RM, Rayner AC (1994) Aust J Chem **47**: 2087
[17] Cavaleiro JAS, Nascimento GMS, Neves MGPMS, Silvestre AJD, Vicente MGH (1996) Tetrahedron Lett **37**: 1983
[18] Silvestre AJD, Cavaleiro JAS, Silva AMS, Delmond B, Filliatre C (1996) Heterocyclic Commun **2**: 371
[19] Silvestre AJD, Cavaleiro JAS, Silva AMS, Delmond B, Filliatre C (1997) J Chem Res (S) 228; J Chem Res (M) 1516
[20] Cremlyn RJ (1991) Agrochemicals, Preparation and Mode of Action. Wiley, Chichester, p 249
[21] Jain AC, Gupta RC, Khazanchi R (1979) Tetrahedron **35**: 413
[22] Franich RA, Gadgil PD, Shain L (1983) Physiol Plant Pathol **23**: 183
[23] Feio SS, Roseiro JC, Gigante B, Marcelo-Curto MJ (1997) J Microbiol Meth **28**: 201
[24] Brock DT, Madigan TM, Martinko MJ, Parker J (1994) Biology of Microorganisms. Prentice Hall, New Jersey, p 61
[25] Kobayashi K, Nishino C, Fukushima M, Shiobara Y, Kodama M (1988) Agric Biol Chem **52**: 77
[26] Homans LA, Fuchs A (1970) J Chromatogr **51**: 327

Received July 20, 1998. Accepted (revised) September 28, 1998