Pyrrolizidine Alkaloids of Cynoglossum officinale and Cynoglossum amabile (Family Boraginaceae)

A. EL-SHAZLY,† T. SARG, A. ATEYA, E. ABDEL AZIZ, L. WITTE‡ and M. WINK§

*Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt;
†Institut für Pharmazie, Technische Universität Braunschweig, Mendelianstr. 1, D-38106 Braunschweig, Germany;
‡Institut für Pharmazie, Universität Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany

Key Word Index—Cynoglossum officinale; Cynoglossum amabile; Boraginaceae; pyrrolizidine alkaloids; capillary GC; GLC-MS; 1H; 13C NMR.

Abstract—Heliosupine, heliosupine N-oxide, 3'-acetylheliosupine, and viridiflorine were isolated and identified on the base of MS, 1H and 13C NMR from Cynoglossum officinale. Altogether 14 pyrrolizidine alkaloids were separated and identified by GLC and GLC-MS in the alkaloid extracts from different parts of Cynoglossum officinale. From Cynoglossum amabile five pyrrolizidine alkaloids were recorded: supinine, amabiline, rinderine, echinatine, and 3'-O-acetyllchinine. Copyright © 1996. Published by Elsevier Science Ltd

Introduction
Species of the genus Cynoglossum (family Boraginaceae) naturally occur mainly in the Old World, although a few taxa (such as C. grande) also inhabit North America. Cynoglossum officinale is widespread in the U.S. as an introduced weed (Knight et al., 1984). Similar to the situation in many other Boraginaceae, Cynoglossum spp. are also known to accumulate pyrrolizidine alkaloids as a major means of chemical defence (Knight et al., 1984; Roeder, 1995; Hartmann and Witte, 1995; Van Dam et al., 1995b).

Heliosupine, 3'-acetylheliosupine, echinatine and 7-angeloylheliotridine have been reported as the main pyrrolizidine alkaloids of Cynoglossum officinale (inhabiting south-west Europe and north Asia) (Bull et al., 1968; Pedersen, 1970; Robins, 1982; Knight et al., 1984). Recently, rinderine, viridiflorine, 3'-O-acetyllchinine and traces of amabiline were additionally found (Van Dam et al., 1995a,b). For Cynoglossum amabile (occurring in west China and Tibet) amabiline and echinatine have been reported (Culvenor and Smith, 1967). Since capillary GLC-MS is a method of choice for analysis and sensitive identification of pyrrolizidine alkaloids in complex mixtures (Culvenor et al., 1981; Bicchi et al., 1989; Borstel et al., 1989; Stelljes et al., 1991; Witte et al., 1992, 1993) extracts of these plants were subjected to GLC and GLC-MS analysis.

In this communication, we report on the occurrence of 14 pyrrolizidine alkaloids in C. officinale and of five pyrrolizidine alkaloids in C. amabile. In addition, the organ-specific alkaloid variation is documented.

§Corresponding author.

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Materials and Methods

Plant material and alkaloid extraction. Flowering and fruiting plants of Cynoglossum officinale L. were collected on sand dunes near Schwetzingen (Germany) in May 1993. C. amabile Stapf et J.R. Drum was grown in a garden at Heidelberg and harvested in August 1994. Voucher specimens were deposited at the Institut für Pharmazeutische Biologie, University of Heidelberg. The fresh plant materials were homogenized with an Ultra turrax in 0.5 N HCl and left to stand for 1 h at least. The acidic aqueous extracts of C. amabile and half of that of C. officinale were adjusted to 2N HCl and the N-oxides were reduced with Zn dust with stirring overnight. Excess Zn was removed by filtration. Basification of the aqueous acid solutions with NaOH was followed by extraction with methylene chloride. Evaporation of the solvent. The residue represents total pyrrolizidine alkaloids as tertiary bases. The remaining half of the extract of C. officinale was treated in the same way, but without Zn/H⁺ reduction. The crude PA-bases of C. officinale were chromatographed on a silica gel column (1.5×50 cm, 30 g) with CH₂Cl₂-MeOH gradients, to give four fractions. Fractions I–IV were subjected to prep. TLC [silica gel F₂54, CH₂Cl₂-MeOH-NH₄OH (25%), 85:15:2] and yielded alkaloids 1–3 with RI 0.7, 0.64 and 0.14, respectively. The unresorbed alkaloid extract of C. officinale was subjected to prep. TLC [silica gel F₂54, CH₂Cl₂-MeOH-NH₄OH (25%), 75:23:2] and yielded heliosupine N-oxide 4, RI 0.30.

GLC-MS Analysis. A Carlo Erba Mega 5160 gas chromatograph equipped with a fused silica column (DB1, J and W Scientific) was employed. The capillary column was directly coupled to a quadrupole mass spectrometer (Finnigan MAT 4515). EI-mass spectra were recorded at 40 eV. Conditions: injection 250°C; temperature program 150–300°C, 6°C min; split ratio 1:20; carrier gas He 0.5 bar. GLC conditions. A Carlo Erba IU 600 gas chromatograph equipped with FID and Spectra Physics integrator and a DB1-30W (J and W Scientific) fused silica capillary column 30 m × 0.17 mm film thickness; carrier gas He; detector temp. 300°C; injector temp. 250°C; oven temp. program: initial temp. 170°C, 5 min isothermal, 170–300°C, 10°C/min, 300°C, 15 min isothermal). Retention Indices (RI): Kovats indices (Kovats, 1958) were calculated with respect to a set of co-injected even numbered hydrocarbons (C14–C28). Each RI was subjected to a library search by comparison with the reference RI stored in a data base of the Institute.

NMR. ¹H- and ¹³C NMR spectra were recorded with a AC 300 Bruker instrument in CDCl₃, at 300 and 75 MHz, respectively, or with a AC 400 Bruker, at 400 and 100 MHz, respectively. Spectroscopic data of alkaloids: 3'-Acetylheliosupine (1). Oily; [α]₂₀ = -2° (EtOH, c0.1), RI 2636, GLC-EIMS m/z (rel. int.): C₂₂H₂₀NO₅ [M⁺] 439(0), 424(1), 322(1.2), 321(2.2), 221(26), 220(91), 138(4), 137(5), 136(80), 121(15), 120(100), 119(97), 94(25), 93(60), 83(16), 80(7), 59(11), 55(13), 43(28). ¹H NMR (300 MHz, CDCl₃) spectra were identical with those of Resch and Meinwald (1982); ¹³C NMR (75 MHz, CDCl₃), 813.56 (C-1), 128.81 (C-2), 64.24 (C-3), 54.26 (C-5), 30.33 (C-6), 78.75 (C-7), 78.79 (C-8), 62.64 (C-9), 173.23 (C-11), 82.79 (C-2'), 72.60 (C-3'), 20.43 (C-4'), 73.05 (C-5'), 26.51 (C-6'), 24.51 (C-7'), 167.89 (C-1'), 127.66 (C-2''), 138.46 (C-3''), 15.78 (C-4''), 20.97 (C-5''), 169 (C-3'OAc: C = O), 15.23 (C-3'OAc: Me).

Heliosupine (2). Oily; [α]₂₀ = -9° (EtOH, c0.1), RI 2550, GLC-EIMS m/z (rel. int.): C₂₀H₂₁NO₅ [M⁺] 397(1.1), 382(0.5), 362(0.1), 338(0.1), 321 (0.1), 297(1.5), 238(5), 221 (26), 220(90), 138(8), 137(5), 136(50), 121(50), 120 (100), 119(78), 106(10), 94 (30), 93(52), 83(18), 59 (10), 55(30), 43(14). ¹H and ¹³C NMR were identical with those published (Asial et al., 1989; Roeder, 1990; Logie et al., 1994). Viridiflorine (or its stereoisomer)(3). Oily, RI 1983, GLC-EIMS m/z (rel. int.): C₁₅H₁₄NO₅ [M⁺] 285(0.1), 284(0.3), 267(6), 252(6), 240(9), 142(70), 126(25), 124(100), 117(9), 110(8), 83(35), 82(22), 70(15), 65(25), 43(21). ¹H and ¹³C NMR were identical with those reported by Kelley and Seiber (1992).

Heliosupine N-oxide (4). Gummy; [α]₂₀ = -4° (EtOH, c 0.5), FAB [M + H⁺], 414 (100); C₂₀H₂₀NO₅. ¹H and ¹³C NMR were identical with those published by Asial et al. (1989).

The minor alkaloids were identified by capillary GLC-MS; [M⁺], retention index and characteristic mass fragments are given.

7-Angeloyllithraimidine (5). RI 1820, C₁₅H₁₄NO₅ [M⁺] 237(1), 219(2), 138(5), 137(34), 136 (17), 124(22), 120(5), 111(32), 106(95), 94(21), 108(100), 68(10), 56(22) (Witte et al., 1993; Roeder et al., 1987).

7-Tigloyllithraimidine (6). RI 1873, C₁₅H₁₄NO₅ [M⁺] 237(0.5), 137(55), 136(15), 124(18), 120(5), 111(41), 107(10), 106(20), 85(6), 83(12), 80(95), 68(5), 55(19) (Witte et al., 1993).

7-Angeloyl-1-formyl-6,7-dihydro-5H-pyrrolizine (7). RI 1920, [M⁺] 233(2), 215(3), 150(100), 134(92), 133(35), 122(4), 106(15), 105(38), 104(15), 83(10), 79(16), 55(20).

Supinine (8). RI 1978, C₁₅H₁₄NO₅ [M⁺] 283(0.2), 140(8), 123(20), 122 (100), 121 (37), 120 (40), 108 (9), 93 (6), 80 (10), 45 (8), 43 (28) (Witte et al., 1993).

Amabiline (9). RI 1985, C₁₅H₁₄NO₅ [M⁺] 283 (1), 140 (8), 123 (30), 122 (100), 121 (46), 120 (51), 108 (17), 93 (25), 80 (13), 70 (17), 53 (7), 45 (6), 43 (19) (Witte et al., 1993; Roeder and Bouruelle, 1992).
7-Angeloylinderine (10), RI 2485, [M]+ 381 (0.1), 336 (2), 281 (1), 238 (10), 221 (34), 220 (80), 141 (11), 136 (10), 137 (9), 136 (53), 121 (70), 120 (100), 119 (69), 117 (4), 106 (10), 94 (28), 93 (50), 83 (15), 80 (10), 55 (17), 45 (8), 43 (25).

Rinderine (11), RI 2153, C_{13}H_{26}NO_6: [M]+ 299 (0.5), 266 (0.3) 254 (0.6), 240 (0.1), 156 (9), 139 (38), 138 (100), 137 (10), 136 (9), 120 (5), 95 (13), 94 (25), 93 (78), 80 (10), 43 (17) (Witte et al., 1993).

Echinatine (12), RI 2172, C_{13}H_{26}NO_6: [M]+ 298 (0.2), 284 (0.1), 256 (0.4), 254 (1), 236 (0.2), 156 (8), 139 (33), 138 (100), 137 (9), 136 (8), 120 (5), 95 (13), 94 (25), 93 (76), 80 (11), 43 (17) (Witte et al., 70; Pedersen and Larsen, 1970).

7-O-Acetylchinatine (13), RI 2235, C_{17}H_{32}NO_6: [M]+ 341 (0.1), 281 (2), 198 (6), 181 (39), 180 (100), 136 (18), 121 (35), 120 (70), 119 (28), 101 (9), 94 (18), 93 (55), 80 (7), 43 (33).

7-Angeloyl-9-(2,3-dihydroxybutyl)heliotrione (14), RI 2183, [M]+ 321 (0.5), 221 (43), 220 (70), 195 (5), 141 (28), 138 (4), 137 (9), 136 (85), 121 (13), 120 (100), 119 (66), 106 (13), 94 (59), 93 (71), 83 (23), 80 (15), 67 (4), 57 (27), 55 (30); this PA could be a decomposition product of 7-O-acetylchinatine produced by GC conditions.

7-Angeloyl-9-(2,3-dihydroxybutyl)heliotrione (15), RI 2333, [M]+ 339 (1), 324 (1), 294 (1), 239 (6), 222 (25), 221 (25), 220 (65), 219 (8), 138 (20), 137 (10), 136 (81), 121 (24), 120 (100), 119 (85), 106 (15), 94 (50), 93 (85), 83 (24), 80 (18), 75 (2), 57 (10), 55 (25), 45 (10).

Isomer of 16 (16), RI 2348, [M]+ 339 (1), 324 (1), 294 (1), 239 (6), 222 (19), 221 (25), 220 (65), 219 (8), 138 (20), 137 (10), 136 (81), 121 (24), 120 (100), 119 (85), 106 (15), 94 (50), 93 (85), 83 (24), 80 (18), 75 (2), 57 (10), 55 (25), 45 (10).

7-Angeloylchinatine (17), RI 2487, [M]+ 381 (n.d.), 336 (2), 281 (1), 238 (11), 221 (42), 220 (86), 141 (11), 136 (11), 137 (9), 136 (55), 121 (78), 120 (100), 119 (60), 117 (4), 106 (9), 94 (30), 93 (52), 83 (17), 80 (10), 55 (17), 45 (8), 43 (21).

Results and Discussion

Identification of pyrolyzidine alkaloids

Four pyrolyzidine alkaloids (1–4) were isolated from the alkaloidal extract of C. officinale and their structures were determined by MS, 1H- and 13C NMR: Compounds 1 and 2 were identified as 3′-acetylheliosupine (Resch and Meinwald, 1982) and heliosupine (Pedersen and Larsen, 1970; Asibal et al., 1989), respectively, which had been previously isolated from this plant (Mattocks, 1986). GLC-EIMS of compound 3 shows a M+ at m/z 285, which corresponds to the molecular formula C_{15}H_{22}NO_4. The MS of this compound exhibited a base peak at m/z 124 and the typical fragmentation pattern of a saturated neine of the trachelanthamidine type (Kelley and Seiber, 1992). With the aid of 1H and 13C NMR, the structure of 3 was identified as viridiflorine. Heliosupine N-oxide 4 was isolated from the unreduced alkaloidal extract and the identification was based on 1H- and 13C NMR data which were essentially identical with that reported (Asibal et al., 1989). The isolated PAs have been characterized biochemically in another study (Schmeller et al., 1996).

In addition, the alkaloid extracts of different organs of C. officinale were analysed by capillary GLC (Table 1) and GLC-MS. Altogether, 14 alkaloids were detected in C. officinale of which alkaloids 1–3, 7-angeloylheliosupidine 5, 7-tigloylheliosupine 6, rinderine 11 and echinatine 12 were unequivocally identified by their MS fragmentation patterns and retention indices as compared to authentic samples (Witte et al., 1993). Compounds 7, 14 to 17 were tentatively identified: The molecular ion at m/z 233 (C_{13}H_{15}NO_3) as well as the base peak at m/z 150 and fragment ions at m/z 134 suggest that compound 7 is 7-angeloyl-1-formyl-6, 7-dihydro-5H-pyrrolizine (Segall et al., 1984) confirming that pyrrole (dihydropyrrrolizine) are genuine plant constituents (Huizing et al., 1986). Compound 10 shows the same fragmentation pattern as compound 17 with small differences in the relative intensity of some fragments indicating that 10 is a diastereoisomer of 17. Thus, based upon mass fragmentation and biogenic consideration, 7-angeloylchinatine is proposed as the structure for 17 and 7-angeloylinderine for 10. Compound 13 exhibited a base peak at m/z 180 providing strong evidence for the presence of an 7-acegony group. Based upon biogenic considerations and mass fragmentation
(Pedersen, 1975a), compound 13 was tentatively identified as 7-acetylechinatine (or its stereoisomer). The mass spectrum of compound 14 showed a molecular ion at m/z 321 which is consistent with the molecular formula C_{19}H_{27}NO_{4}. The fragment m/z 220 possibly refers to 7-angeloyldehydroxyheliotridine (Mₚ minus C9 ester) and m/z 221 (Mₚ minus angelic acid). A peak at m/z 57 (C₄H₉) was probably derived from the side-chain at C9 after decarboxylation. Based upon biogenic considerations and mass fragmentation, compound 14 was tentatively identified as 7-angeloyl-9-(2-methylbutyryl)heliotridine (or its isomer). The mass
TABLE 1. ALKALOIDAL PROFILES AND CONTENTS (TOTAL ALKALOID =100%) IN DIFFERENT ORGANS OF C. OFFICINALÉ

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Stems</th>
<th>Leaves</th>
<th>Flowers</th>
<th>Fruits</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Acetylheliospine*</td>
<td>6.6</td>
<td>2.7</td>
<td>4.6</td>
<td>3.9</td>
<td>5.0</td>
</tr>
<tr>
<td>2 Heliospine*</td>
<td>19.6</td>
<td>6.9</td>
<td>11.0</td>
<td>11.3</td>
<td>16.4</td>
</tr>
<tr>
<td>3 Viridiflorine*</td>
<td>2.7</td>
<td>1.8</td>
<td>4.8</td>
<td>tr</td>
<td>6.8</td>
</tr>
<tr>
<td>5 7-Angeloylheliotridine*</td>
<td>20.3</td>
<td>11.8</td>
<td>14.1</td>
<td>11.1</td>
<td>16.8</td>
</tr>
<tr>
<td>6 7-Tigiloylheliotridine</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>1.4</td>
</tr>
<tr>
<td>7 7-Angeloyl-1-foxyly-6,7-dihydro-5H-pyrolizine§</td>
<td>3.1</td>
<td>3.7</td>
<td>2.4</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>10 7-Angeloylerinine§</td>
<td>tr</td>
<td>2.1</td>
<td>1.1</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>11 Rinderine*</td>
<td>tr</td>
<td>tr</td>
<td>0.94</td>
<td>0.99</td>
<td>tr</td>
</tr>
<tr>
<td>12 Echinatine*</td>
<td>19.5</td>
<td>63.5†</td>
<td>28.4</td>
<td>31.0</td>
<td>29.9</td>
</tr>
<tr>
<td>14 7-Angeloyl-9-(2-methylbutyryl) heliotridina§</td>
<td>19.0</td>
<td>63.5†</td>
<td>24.8</td>
<td>32.1</td>
<td>16.2</td>
</tr>
<tr>
<td>15 7-Angeloyl-9-(2,3-dihydroxybutyryl) heliotridina§</td>
<td>1.1</td>
<td>0.7</td>
<td>3.1</td>
<td>2.3</td>
<td>3.6</td>
</tr>
<tr>
<td>16 Isomer of (16)§</td>
<td>6.8</td>
<td>6.8</td>
<td>4.4</td>
<td>4.5</td>
<td>0.7</td>
</tr>
<tr>
<td>17 7-Angeloylheliotridine§</td>
<td>1.4</td>
<td>tr</td>
<td>0.4</td>
<td>0.5</td>
<td>0.8</td>
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<tr>
<td>Alkaloid content</td>
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<td>0.20</td>
<td>0.98</td>
<td>0.71</td>
<td>0.05</td>
</tr>
</tbody>
</table>

tr = Traces; --- = Not detected; * = Known alkaloid for C. officinalé; † = Per cent by fresh weight; § = Compound co-eluted; this value refers to the sum of compound 14 and 15; †† = Tentatively identified by GLC-MS

spectra of compounds 15 and 16 showed a molecular ion at m/z 339 corresponding to C\(_{17}\)H\(_{28}\)N\(_{6}\). Compound 15 gives m/z 239 (M\(^+\) minus angelic acid) and m/z 220 (M\(^+\) minus C\(_9\) ester). The fragment at m/z 57 could be derived from m/z 75 through a rapid loss of one molecule of water. A small signal at m/z 294 indicated the loss of 45 mass units (C\(_2\)H\(_3\)O) from the parent ion. The same fragmentation pattern as in compound 15 was present in the mass spectrum of compound 16. The only difference is that the latter shows delayed retention time in GLC-MS indicating that 16 is a diastereomer of 15. Based upon the mass fragmentation and biogenetic considerations alkaloid 15 was tentatively identified as 7-angeloyl-9-(2,3-dihydroxybutyryl)heliotridine (or a closely related isomer). The molecular ion could not be defined in the mass spectrum of compound 17, but an ion at m/z 281 (M\(^+\) minus angelic acid) and m/z 220 (M\(^+\) minus an ester at C-9) was recorded. The ion at m/z 336 is probably due to M\(^+\) minus C\(_2\)H\(_3\)O and the ion at m/z 43 represents C\(_3\)H\(_7\). The fragment at m/z 99 was probably derived from m/z 117 through the loss of H\(_2\)O (—18).

GLC-MS analysis of C. amabile revealed supinine 8, amabiline 9, rinderine 11, and echinatine 12. Compound 13 was tentatively identified as 7-acetylenchlinatine (see above) (Table 2).

TABLE 2. ALKALOID PROFILES AND CONTENTS (TOTAL ALKALOID 100%) IN DIFFERENT ORGANS OF FRUITING C. AMABILE PLANTS

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Stems</th>
<th>Leaves</th>
<th>Fruits</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Supinine</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>9 Amabiline*</td>
<td>87.6</td>
<td>95.2</td>
<td>94.79</td>
</tr>
<tr>
<td>11 Rinderine</td>
<td>2.8</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>12 Echinatine*</td>
<td>6.9</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>13 7-Acetylenchlinatine§</td>
<td>tr</td>
<td>---</td>
<td>tr</td>
</tr>
<tr>
<td>Unknown</td>
<td>2.7</td>
<td>2.3</td>
<td>2.9</td>
</tr>
<tr>
<td>Total alkaloid</td>
<td>0.01</td>
<td>0.009</td>
<td>0.074</td>
</tr>
</tbody>
</table>

tr = Traces; --- = Not detected; * = Known alkaloid for C. amabile; † = Per cent fresh weight; § = Tentatively identified by GLC-MS
Organ-specific variation of pyrrolizidine alkaloids

Pyrrolizidine alkaloids mainly occur as their N-oxides in Cynoglossum (Hartmann and Witte, 1995; Van Dam et al., 1995a,b). This feature has to be kept in mind, when only the free PAs, which were freed from their N-oxides by reduction are discussed above and in the following.

For C. officinale (Table 1) the alkaloids viridiflorine (3), 7-angeloylheliotridine (5), echinatine (12), 7-angeloyl-9-(2-methylbutyryl)heliotridine (14) and heliosupine (2) were recorded as the major components in all plant organs, thus confirming previous studies (Bull et al., 1968; Robins, 1982; Mattocks, 1986; Van Dam et al., 1995a). A number of the minor compounds have been recorded for the first time in this species (Table 1); Van Dam et al. (1995b) reported 3'-acetylechinatine and traces of amabiline for C. officinale which were not encountered in our study. The difference might be attributed to geographical as well as ecological variations or the presence of different chemotypes.

Cynoglossum is a biennial plant, showing a rosette stage in the first year and developing stems and flowers in its second year. Young leaves and rosettes (which were not analyzed in our study) contain substantial amounts of PA (Knight et al., 1984; Van Dam et al., 1995b). Flowers and fruits (which are concomitantly present in second year plants) appear especially rich in PAs (Table 1). PA accumulation in these organs supports the view that PAs are predominantly stored in reproductive organs (plants in their second year) or organs important for growth and survival (especially in their first year) which are thus effectively defended against herbivores (Knight et al., 1984; Hartmann and Witte, 1995; Van Dam et al., 1995a,b; Wink, 1993).

In agreement with literature data (Culvenor and Smith, 1967). C. amabile (Table 2) accumulates amabiline (9) as the dominant alkaloid (87–95%) in stems, leaves and fruits. Also the minor alkaloids are almost equally present in all organs, including rinderine (11), supinine (8), 7-acetylechinatine (13) but also the known echinatine (12) (Culvenor and Smith, 1967).

References