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New Pyrrole Acids by Oxidative Degradation of Eumelanins with Hydrogen Peroxide. Further Hints to the Mechanism of Pigment Breakdown.

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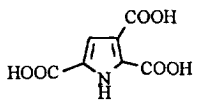
Abstract. Oxidative degradation of natural and synthetic eumelanins with alkaline H_2O_2 afforded a complex mixture of low molecular weight products which comprised, besides **1** and **2**, three novel pyrrole acids. These were isolated and identified as 2-carboxyhydroxymethylpyrrole-3,5-dicarboxylic acid (**3**), 2-carboxymethylpyrrole-3,5-dicarboxylic acid (**4**) and 2-hydroxymethylpyrrole-3,5-dicarboxylic acid (**5**). Investigation of the oxidative degradation of the eumelanin precursors 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, as well as of the oligomers **6-10** showed that pyrrole acids **3-5** originate by peroxidative disruption of both carboxylated and non-carboxylated indole units, and that pyrrole **4** arises mainly from indole units not substituted at the 7-position. None of the new pyrroles was converted to **1** by treatment with alkaline H_2O_2, suggesting that they are formed by different degradation routes. These results can be accommodated into an improved mechanistic scheme for rationalising the origin of pyrrole acids by H_2O_2 degradation of eumelanins.

Recent advances in the chemistry of eumelanins, the black-to-brown pigments of mammalian skin, hair and eyes, have allowed unprecedented insight into the complex mechanisms underlying pigment formation by oxidative polymerisation of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).[1,2] The emerging perception of eumelanin structure and role in skin (photo)protection is however still biased by certain misconceptions and gaps in current knowledge of the oxidation behaviour and mode of degradation of the pigment backbone. In particular, the mechanism of disruption of 5,6-indolequinone units by hydrogen peroxide, leading to gradual solubilisation and bleaching of eumelanin granules, has remained uncharted. This is yet a reaction of the greatest importance both from the investigative and practical viewpoints, being widely exploited in cosmetics,[3] and in histochemistry.[4] Moreover, the peroxidative breakdown of indolequinone units is now definitely recognised as a major post-synthetic modification suffered by natural and synthetic eumelanins, on account of an irreversible loss of integrity of the indole units.[5,6]

A considerable hurdle in studies of the reaction of eumelanins with hydrogen peroxide is the tendency of the pigment to undergo extensive degradation, with formation of a collection of small fragments all in very low amounts.[7-11] A similar pattern of products is obtained by degradation of synthetic melanins prepared by oxidation of dopa, DHI and DHICA.[8,11] Of these products, only a few have been identified and include, notably, pyrrole-2,3,5-tricarboxylic acid (**1**) and pyrrole-2,3-dicarboxylic acid (**2**) as well as some related pyrrole acids. The origin of **1**, especially, has been the subject of much speculation and has recently been shown to involve degradation of units derived from both DHICA and, to a lesser extent, DHI,[12] in contrast to a previous suggestion.[13] Nevertheless, the significance of this pyrrole remains at present limited, as its structural anatomy conveys little information about the detailed mode of cleavage of the precursor units.



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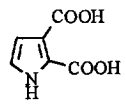

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New clues to the mode of degradation of melanins came from a re-investigation of the mixture obtained by treatment of DHICA melanin with hydrogen peroxide in 1 M K_2CO_3. HPLC analysis revealed the formation, besides **1**, of a complex pattern of products whose properties were suggestive of low molecular weight pyrrole acids. Attempts at isolating the most abundant of such products by preparative HPLC were discouraging, as the yields were too small to permit adequate spectral characterisation. The problem was however circumvented by oxidation of DHICA, which gave a similar pattern of products in higher yields.

One of these products (λ_{max}= 260 nm), which eluted faster than **1**, could be obtained by preparative HPLC. The ^{1}H-NMR spectrum consisted of two singlets (1H each) at δ 5.61 and 7.00, whereas the ^{13}C-NMR spectrum displayed signals for four sp^2 carbons, three carboxy groups and a deshielded aliphatic CH carbon at δ 65.91. Overall, these data suggested a pyrroledicarboxylic acid bearing a CH(OH)COOH grouping. Of the possible isomers that could be considered, keeping into account the constraints posed by the structure of the indole precursor, 2-carboxyhydroxymethylpyrrole-3,5-dicarboxylic acid (**3**) was favoured on the basis of the ^{13}C-NMR resonance pattern.[14] The above structural assignment was substantiated by spectral analysis of the trimethyl ester of **3**, exhibiting a molecular ion peak in the EI-MS spectrum at m/z 271.

Another, relatively more abundant product eluted after **3** and **1**, and exhibited an absorption spectrum similar to that of **3**. Considerable analogies were also apparent from the NMR spectra, save for the lack of the aliphatic CH group, which was replaced by a methylene group, appearing as a 2H singlet at δ 3.81 in the ^{1}H-NMR spectrum and as a signal at δ 32.49 in the ^{13}C-NMR spectrum (DEPT evidence). By straightforward analysis of the corresponding trimethyl ester (molecular ion peak at m/z 255), it was possible to unambiguously formulate the compound as 2-carboxymethylpyrrole-3,5-dicarboxylic acid (**4**).[15]

Further inspection of the degradation mixtures revealed the presence of a product which eluted close to **1**. This product was eventually isolated and was found to possess only two carboxy groups, linked to the α- and β-positions of the pyrrole ring, along with a deshielded methylene group (δ 54.93) accounting for a 2H singlet at δ 4.68 in the proton spectrum. Combined spectral analyses of the acid and the corresponding dimethyl ester allowed formulation of the product as 2-hydroxymethylpyrrole-3,5-dicarboxylic acid (**5**).

In subsequent experiments we investigated the formation of pyrroles **3-5** under a variety of oxidation conditions using DHICA melanin as a reference pigment. With hydrogen peroxide, the product yields in 1 M K_2CO_3 compared favourably with those obtained in neutral or strongly alkaline media.[12] Strong oxidants, like $KMnO_4$, gave only **1**, whereas $K_3Fe(CN)_6$ afforded traces of **3-5**. This would suggest either that the new pyrroles arise specifically by attack of hydrogen peroxide anion to indolequinone units, and/or that, though formed by breakdown of the pigment backbone, they do not survive under strongly oxidising conditions. Control experiments also showed that pyrroles **3-5** are not formed by treatment of melanins with alkali under an oxygen-free atmosphere, thus ruling out the possibility that they are produced by a simple hydrolytic process. Since the focus of this work was primarily on the hydrogen peroxide-induced degradation of eumelanins, we next examined formation of pyrroles **1-5** by hydrogen peroxide/K_2CO_3 oxidation of a set of synthetic eumelanins prepared from dopa, DHI and DHICA. Sepia ink and black human and mouse hair were also included as representative sources of natural eumelanins. The data in Table 1 clearly show that the new

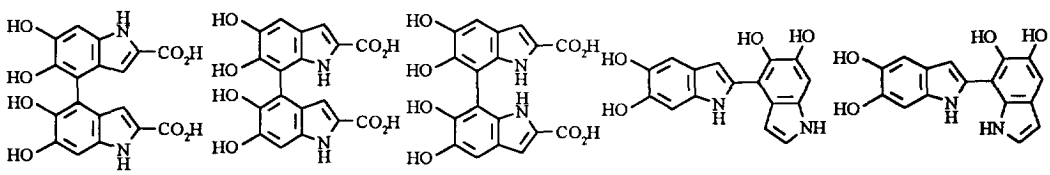
pyrroles, though usually less abundant than **1**, are formed by all types of eumelanins, including natural pigments. Pyrroles **3** and **4** usually predominate in oxidation mixtures from DHICA melanins and are formed in much lower yields from dopa and DHI melanins, consistent with the lower proportion or the lack of carboxylated units in the latter polymers.

Table 1. Yields of Pyrroles by Oxidative Degradation of Natural and Synthetic Melanins

Oxidant or Source	**Yield (nmol/mg sample)** [a]				
	1	**2**	**3**	**4**	**5**
Synthetic Melanins					
DHICA-Melanin	280	--	78.7	78.2	18.0
DHI-Melanin	44.7	15.7	5.6	2.2	5.2
Dopa-Melanin	22.4	10.8	0.7	0.7	1.9
Natural Melanins					
Sepia ink	118	--	16.9	3.3	19.0
Black human hair[b]	0.46	--	0.7	--	--
Black mouse hair[b]	6.3	--	0.7	1.6	0.5

[a]Determined by HPLC, average of three experiments, S.D. $\leq$ 5%. [b]Intact hair.

With a view to investigating in more detail the origin of the new pyrrole acids and their structural significance, we assessed their formation by hydrogen peroxide oxidation of some representative oligomers of DHICA and DHI, namely **6-10**, which could serve as models of the pigment polymer. In these experiments, air was rigorously excluded from the degradation mixture to minimise concurrent alkali-catalysed oxidative polymerisation of the compounds to melanin pigments. The results, summarised in Table 2, indicate that pyrroles **3-5** arise preferentially from oxidative fission of DHICA units not substituted at the 7-position. This holds especially for **4**, which is a significant product from oxidation of the 4,4'- and 4,7'-biindolyls **6** and **7**, but is absent in the mixture from the 7,7'-biindolyl **8**, and may therefore be regarded as a marker of terminal units of DHICA polymers possessing a free 7-position. Formation of **1** and **3-5** from DHI oligomers, e.g. **9** and **10**, is a relatively more complex process which involves exclusively 2-substituted units and requires at some stage a carboxyl-forming fission of the attached indolyl moiety on the 2-position. Another issue that was addressed concerns the mechanistic relationship between **1** and **3-5**. Oxidation of DHICA dimers, e.g. **6**, with a larger excess of hydrogen peroxide than that used in the above experiments was found to give much higher yields of **1** at the expenses of **3-5**. This raised the possibility that **1** arises at least in part from further oxidation of **3-5** by alkaline hydrogen peroxide.



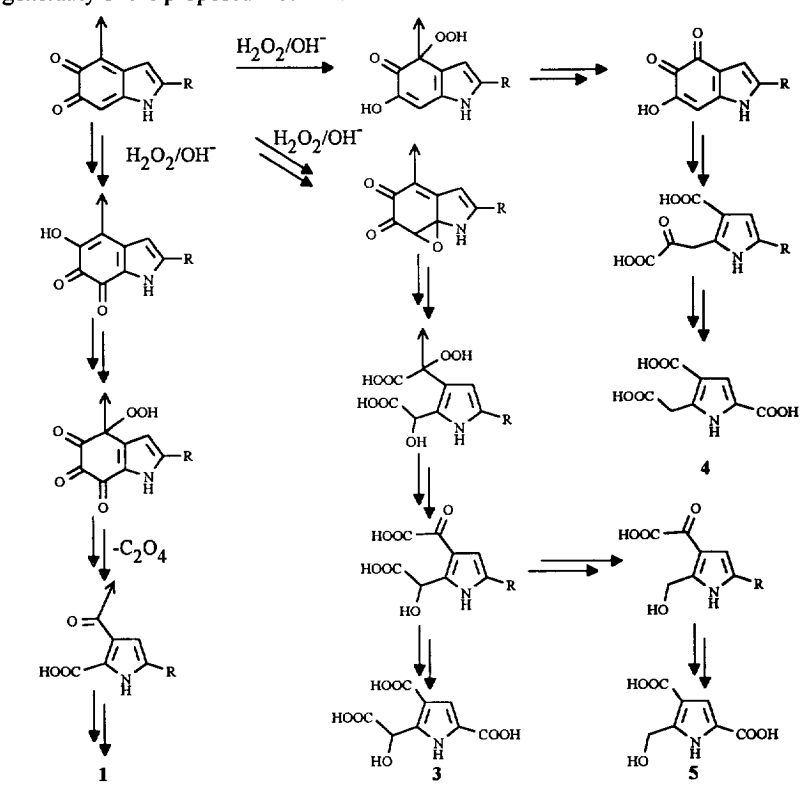
6 **7** **8** **9** **10**

Table 2. Yields of Pyrrole Acids by Oxidative Degradation of DHICA and DHI Oligomers

Compound	Yield (%) [a]				
	1	**2**	**3**	**4**	**5**
6	3.9	--	1.8	1.7	1.7
7	4.3	--	1.6	1.8	1.3
8	4.0	--	1.0	--	0.6
9	0.13	0.58	0.1	0.05	0.04
10	0.18	0.37	0.1	0.08	0.05

[a] Determined by HPLC, average of three separate experiments, S.D. ≤ 5%.

To assess this point, **3-5** were subjected to oxidation with hydrogen peroxide. No significant formationof **1** was detected, thus ruling out the intermediacy of **3-5** in the degradative route leading to **1**. Attempts to convert **3** into **5** by treatment with acids or alkali also met with failure, suggesting that **5** does not arise from decarboxylation of **3**, but is produced by a related, yet distinct degradative route. A plausible mechanism for the peroxidative degradation of eumelanins is outlined in the Scheme and develops from 4-linked DHICA (R=COOH) or 2-substituted DHI (R= 5,6-dihydroxybiindolyl) quinones as model pigment units. The same scheme can be extended to other possible units, e.g. a 4,7-disubstituted DHICA quinone, in accord with the generality of the proposed mechanisms.



Scheme

One main route, leading to **4**, is envisaged to involve nucleophilic attack of the hydrogen peroxide anion to the electrophilic 4-position of the indolequinone system, followed by muconic-type cleavage[16] of the ring and subsequent oxidative loss of carbon dioxide. The addition of hydrogen peroxide to the 7-position of the indolequinone system, possibly to give an epoxide intermediate in the early stages,[17] can reasonably be invoked for the alternative paths leading to pyrroles **3** and **5**. Fragmentation to **1**, on the other hand, is likely to involve a different type of fission of the indole units, which would result from attack of the oxidant at both the 4- and 7-positions of the indole ring with possible production of oxalic acid.[7,8]

EXPERIMENTAL

M.ps. were determined with a Kofler hot-stage apparatus. UV spectra were performed with a Perkin-Elmer Lambda 7 spectrophotometer. EI and HR-EI (70 eV) mass spectra were determined with a Kratos MS 50 spectrometer. ^{1}H-NMR (270 MHz) and ^{13}C-NMR (67.9 MHz) spectra were recorded on a Bruker AC 270 spectrometer. Analytical and preparative HPLC was carried out on a Gilson apparatus using a Spherisorb S5 ODS2 column (4.6 x 250 mm) or an Econosil C_{18} 10 μ (22 x 250 mm) column, respectively. The flow rate was maintained at 1 ml/min or at 10 ml/min. Detection was set at 270 nm.. The yields of pyrroles **1-5** in the reaction mixtures were determined by measurement of peak areas and comparison with external calibration curves. Mushroom tyrosinase (E.C. 1.14.18.1, 4300 units/mg) and horseradish peroxidase (EC 1.11.1.7) type II (220 units/mg, RZ E_{430}/E_{275}=2.0) were from Sigma. DHI and DHICA were synthesised according to a standard procedure.[18] Dimers **6-8** were obtained as reported.[19] Dimers **9-10** were prepared by hydrolysis of the corresponding *O*-acetyl derivatives[20] as reported.[12] Melanins by tyrosinase-catalysed oxidation of dopa (2.5 mM), DHI (12.5 mM) and DHICA (12.5 mM) were prepared as described.[5,12] Melanins were dried over silica gel and NaOH overnight and then equilibrated with saturated $CaCl_2$. Sepia melanin was purified as previously reported.[21] Mouse hair was from C57 BL/6J-a/a mice.

Oxidative degradation of melanins and dimers **6-10**.

A suspension of the appropriate melanin (10 mg) in 1 M K_2CO_3 (3 ml) was treated with 30% H_2O_2 (20 μl) and vigorously stirred at 25°C for 3 h. The reaction was stopped by addition of 5% $NaHSO_3$ (500 μl), the mixture was acidified to around pH 2 with 1 M HCl and extracted with ethyl acetate (4x1 ml). The combined organic layers were dried over sodium sulphate and evaporated to dryness. The residue was taken up in water (1 ml) and analysed by HPLC using as the mobile phase a mixture of 0.05 M citric acid and 0.3 M formic acid containing 3% acetonitrile brought to pH 2.5 with conc. ammonium hydroxide. Degradation of dimers **6-10** was carried out as described.[12]

Isolation of pyrroles **3-5**.

DHICA (1 g) in 1 M potassium carbonate (500 ml) was reacted with 30% hydrogen peroxide (10 ml) under stirring. After 3 h, the mixture was treated with 5% sodium bisulphite (10 ml), acidified to around pH 2 with 1 M HCl and extracted with ethyl acetate (4x200 ml). After evaporation to dryness, the oily residue (600 mg) was fractionated by preparative HPLC using 1% acetic acid containing 4% acetonitrile. Peaks eluting at 11, 27 and 33 min were collected and taken to dryness to afford pure **3** (18 mg), **5** (15 mg) and **4** (25 mg), in that order. Under such conditions, **1** eluted at 26 min.

*2-Carboxyhydroxymethylpyrrole-3,5-dicarboxylic acid (**3**).* M.p. 230-233°C (dec.); UV λ_{max} (EtOH) 260 nm (log ε 3.76); ^{1}H-NMR (DMSO-d_6) δ: 5.61 (1H, s, -CHOH), 7.00 (1H, s, H-4), 10.43 (1Hx3, bs, COOH), 12.00 (1H, bs, NH); ^{13}C-NMR (DMSO-d_6), δ: 65.91 (-CHOH), 113.92 (C-3), 116.38 (C-4), 122.52 (C-5), 140.31 (C-2), 161.58 (C-5-COOH), 165.95 (C-3-COOH), 172.15 (-CH(OH)COOH).

*2-Carboxymethylpyrrole-3,5-dicarboxylic acid (**4**).* M.p. 211-214 °C (dec.); UV λ_{max} (EtOH) 265 nm (log ε 3.53); ^{1}H-NMR (DMSO-d_6) δ: 3.81 (2H, s, -CH_2), 6.96 (1H, bs, H-4), 8.0-9.0 (1Hx3, s, COOH), 12.12 (1H, bs, NH); ^{13}C-NMR (DMSO-d_6), δ: 32.49 (CH_2-COOH), 114.51 (C-3), 116.16 (C-4), 121.85 (C-5), 136.12 (C-2), 161.62 (C-5-COOH), 165.44 (C-3-COOH), 170.93 (CH_2COOH).

2-Hydroxymethylpyrrole-3,5-dicarboxylic acid (5). M.p. 220-222 °C (dec.); UV λ_{max} (EtOH) 264 nm (log ε 3.67); ^{1}H-NMR (DMSO-d_6) δ: 4.68 (2H, s, -$\underline{CH_2}$OH), 6.95 (1H, s, H-4), 11.75 (1H, bs, NH); ^{13}C-NMR (DMSO-d_6) δ: 54.93 ($\underline{CH_2}$OH), 113.12 (C-3), 116.17 (C-4), 121.83 (C-5), 142.40 (C-2), 161.34 (C-5-$\underline{C}$OOH), 165.46 (C-3-$\underline{C}$OOH).

Pyrroles **3-5** were esterified by treatment with HCl-saturated anhydrous methanol (100 µl/mg).

Trimethylester of **3**. UV λ_{max} (EtOH) 262 nm; HR EIMS, *m/z* 271.0698 (M^+) (calc. for $C_{11}H_{13}NO_7$: 271.0692); EIMS m/z 271 (61), 240 (100); ^{1}H-NMR (DMSO-d_6) δ: 3.60 (3H, s, -OCH_3), 3.69 (3H, s, -OCH_3), 3.78 (3H, s,-OCH_3), 5.40 (1H, bs, OH), 5.65 (1H, s, -CHOH), 7.04 (1H, s, H-4).

Trimethyl ester of **4**. UV λ_{max} (EtOH) 264 nm; HR EIMS, *m/z* 255.0753 (M^+) (calc. for $C_{11}H_{13}NO_6$: 255.0743); EIMS m/z 255 (16), 224 (28), 223 (100), 196 (31), 191 (64), 164 (85), 149 (19); ^{1}H-NMR (DMSO-d_6) δ: 3.60 (3H, s, -OCH_3), 3.67 (3H, s, -OCH_3), 3.77 (3H, s, -OCH_3), 3.97 (2H, s, CH_2) 7.01 (1H, s, H-4), 12.56 (1H, bs, NH).

Dimethyl ester of **5**. UV λ_{max} (EtOH) 264 nm; HR EIMS *m/z* 213.0631 (M^+) (calc. for $C_9H_{11}NO_5$: 213.0637); EIMS m/z 213 (10), 212 (50), 180 (52), 149 (42), 148 (100); ^{1}H-NMR (DMSO-d_6) δ: 3.71 (3H, s, -OCH_3), 3.77 (3H, s, -OCH_3), 4.68 (2H, s, $\underline{CH_2}$OH), 7.06 (1H, s, H-4).

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REFERENCES AND NOTES.

1. Prota, G. *Melanins and Melanogenesis*, Academic Press: San Diego, 1992.
2. Prota, G. *Fortschr.Chem. Org. Naturst.* **1995**, *64*, 93-148.
3. Zviak,C.;Dawber,P.In *The Science of Hair Care;*Zviak,C. Ed.;Marcel Dekker:New York **1986**;pp.1-45.
4. Barden, H.; Brizzee, K.R. *Adv Biosci.* **1987**, *64*, 339-392
5. Crescenzi, O.; d'Ischia, M.; Napolitano, A.; Prota, G. *Gazz. Chim. Ital.* **1993**, *123*, 241-242
6. Napolitano,A.;Pezzella,A.;Prota,G.;Seraglia,R.;Traldi,P.*Rapid Commun. Mass Spectrom.***1996**, in press.
7. Panizzi, L.; Nicolaus, R.A. *Gazz. Chim. Ital.* **1952**, *82*, 435-460.
8. Piattelli, M; Fattorusso, E.; Magno, S.; Nicolaus, R.A. *Tetrahedron* **1962**, *18*, 941-949
9. Swan, G.A. *Fortschr. Chem. Org. Naturst.* **1974**, **31**, 521-582.
10. Swan, G.A.; Waggott., A. *J. Chem. Soc. (C)*, **1970**, 1409-1418.
11. Binns, F.; Chapman,R.F.; Robson, N.C.; Swan,G.A.; Waggott.,A. *J. Chem. Soc. (C)*, **1970**,1128-1134.
12. Napolitano, A.; Pezzella, A.; Vincensi, M.R.; Prota, G. *Tetrahedron*, **1995**, *51*, 5913-5920.
13. Ito, S. *Biochim. Biophys. Acta* **1986**,*883*,155-161. Ito,S.; Fujita,K. *Anal. Biochem.* **1993**,*215*, 273-277.
14. Carbon resonances of pyrrole acids, e.g. **1** and **2**, fall within the following ranges: α-COOH: δ 160-162; COOH-bearing α-carbons: δ 120-122; β-COOH: δ 165-167; COOH-bearing β-carbons: δ 113-116.
15. Compound **4** was first synthesised by Swan and Waggot.[22] However, ^{1}H NMR data do not match at all with those of the product isolated in the present study. We suspect that the product synthesised by the previous authors and claimed to be **4** was in fact an isomer, possibly 2-carboxymethylpyrrole-3,4-dicarboxylic acid. This would also provide a plausible explanation of why the product could not be detected by the previous workers[11] in the oxidation of eumelanins, in spite of very careful experiments.
16. Grinstead, R.R. *Biochemistry* **1964**, *3*, 1308-1314. Pandell, A.J. *J. Org. Chem.* **1976**, *41*, 3992-3996. Sawaki, Y.; Foote, C.S. *J. Am. Chem. Soc.* **1983**, *105*, 5035-5040.
17. Matsuura, T. *Tetrahedron*, **1977**, *33*, 2869-2905. Gellerstedt, G.; Hardell, H-L.; Lindfors, E-L- *Acta Chem. Scand* **1980**, *34(B)*, 669-673.
18. Benigni, J.D.; Minnis, R.L. *J. Heterocycl. Chem.* **1965**, *2*, 387-392
19. Pezzella, A.; Napolitano, A.; d'Ischia, M.; Prota, G. *Tetrahedron*, **1996**, in press.
20. d'Ischia, M.; Napolitano, A.; Tsiakas, K.; Prota, G. *Tetrahedron*, **1990**, *46*, 5789-5796.
21. Palumbo,A.;d'Ischia,M.;Misuraca, G.;Prota, G.;Schultz,T.M.*Biochim. Biophys. Acta*,**1988**,*964*,193-199
22. Swan, G.A.; Waggott, A. *J. Chem. Soc. (C)* **1970**, 285-290.

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