Tetrahedron. Vol. 27, pp. 1831 to 1837 Pergamon Press 1971. Printed in Great Britain

HUMIC ACID—IV

THE REACTION OF α-AMINO ACID ESTERS WITH QUINONES

P. A. CRANWELL and R. D. HAWORTH
University of Sheffield

(*Received in the UK* 24 *October* 1970; *Accepted for publication* 4 *November* 1970)

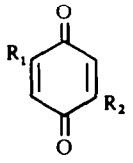
Abstract—The synthesis is described of quinones containing α-amino acid ester substituents and the structure of the products is established by NMR and mass spectroscopic techniques. Hydrolysis of the products with 6N HCl has been studied; the products behave as vinylogous amides but the release of nitrogen in the form of amino acid is low, particularly from the amino acid group directly attached to the quinone nucleus, and suggests that compounds of this type may contribute to the stability of soil organic nitrogen.

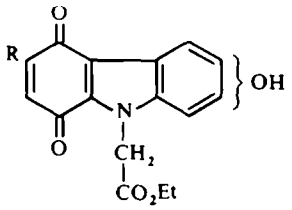
THE biological stability of organically bound nitrogen in soils has been attributed to combination of amino acids, peptides and proteins with quinones during the early stages of decomposition of organic matter.[1,2] Soil humic acids, containing 1·5–6·0% nitrogen, give, on hydrolysis, 20–50% of their nitrogen as α-amino acid nitrogen, up to 10% as amino-sugar nitrogen and a trace of heterocyclic nitrogen, but approximately 50% is unidentified.[3] Failure to obtain evidence for the presence of peptide bonds led Swaby and Ladd[1] to propose initially that amino acids were incorporated as single units during the oxidative polymerisation of phenols but we have shown that the water extract of a humic acid contained amino acids only after hydrolysis with 6N HCl.[4] In addition Ladd and Brisbane[5] have recently shown that peptides are present by the release of amino acids on treatment of soil humic acids with the proteolytic enzyme pronase and the isolation of a humoprotein component of soil humic acid has also been reported.[6]

Previous studies of the amorphous products arising from catechol or hydroquinone derivatives and amino acids, peptides or proteins in presence of silver oxide[7] or phenoloxidase[8] have indicated (a) that the proportion of α-amino acid nitrogen liberated by hydrolysis with 6N HCl increased as the number of peptide bonds increased and (b) that the amino terminal amino acid was particularly resistant to hydrolysis. But no studies have been made previously on the pure compounds which may be prepared by the reaction of quinones with amino acids or peptides.

Fischer and Schrader[9] reported that *p*-benzoquinone condensed with glycine ethyl ester to give I and also obtained the corresponding product with alanine ethyl ester and a similar disubstituted glycine ester of *p*-toluquinone. In a previous paper[4] we reported the synthesis of compounds II and III from glycine ethyl ester with *p*-toluquinone and methoxy-*p*-benzoquinone respectively and also reported the liberation of glycine on hydrolysis of compounds I–III with 6N HCl. Structure I suggested by Fischer and Schrader[9] and based upon analogy with the structure of the quinone–aniline products, is consistent with the additive properties of *p*-benzoquinones discussed by Erdtman[10] and with the preferred formation of *para*

diglycine ester derivatives from chloro- *p*-benzoquinones and glycine ethyl ester.[11] The structures of compounds I–III have now been confirmed by examination of IR, UV, NMR and mass spectra and the glycyl-L-leucine ethyl ester derivative IV has also been prepared.





I: $R_1 = R_2 = -NH.CH_2.CO_2Et$
II: $R_1 = Me, R_2 = -NH.CH_2.CO_2Et$
III: $R_1 = OMe, R_2 = -NH.CH_2.CO_2Et$
IV: $R_1 = R_2 = -NH.CH_2.CO.NH.CH(CH_2(Me)_2CH).CO_2Et$

V: $R = -NH.CH_2CO_2Et$

The major product obtained from the condensation of *p*-benzoquinone with glycine ethyl ester had m.p. 200–202° (dec). A minor product, obtained by TLC separation of the mother liquors, showed the same melting point, 214–215°, as that reported by Fischer and Schrader for the product to which they assigned structure I. We assign structure I to the major product m.p. 200–202° (dec) from the NMR spectrum showing absorption at 8·68 τ (triplet, J = 7·5 Hz; 6 protons) and 5·7 τ (quartet, J = 7·5 Hz; 4 protons) typical of an ethyl ester, a doublet at 6·14 τ (J = 5·5 Hz; 4 protons) assigned to the glycine methylene groups, a broad peak at 8·13 τ (2 protons) assigned to the —NH— groups and a singlet at 4·73 τ (2 protons) assigned to the quinone ring protons. The mass spectrum (Fig 1) showed M^+ at *m/e* 310 and strong fragment ions at *m/e* 237 (M-73) and 163 (M-147) due to successive loss of the carbethoxyl groups and reductive acetylation gave a tetra-acetate showing M^+ 480 and sequential loss of four ketene units supported by metastable ions.

The minor product, which had the same m.p. as Fischer and Schrader reported for I, analysed as $C_{20}H_{20}O_7N_2$ in agreement with the M^+ at *m/e* 400 in the mass spectrum (Fig 1). Fragment ions occurred at *m/e* 327 and 253 (successive loss of 73 and 74 as in compound I) and the corresponding doubly charged ions were also observed at *m/e* 163·5 and 126·5. The solubility in alkali, the IR spectrum, showing absorption at 3450 cm^{-1} and the UV spectrum, showing a bathochromic shift on addition of alkali suggested the presence of a phenolic hydroxyl group. The NMR spectrum in $(CD_3)_2SO$ solution showed the presence of two non-equivalent ethyl ester groups absorbing at 8·75, 8·78 (triplets, J = 7 Hz) and 5·82, 5·84 τ (quartets, J = 7 Hz). A doublet at 6·02 τ (J = 5·5 Hz; 2 protons) showed the presence of a —NH—CH_2— group, while a singlet at 4·65 τ (2 protons) was assigned to the other methylene group derived from a glycine unit, now attached to a fully substituted N-atom. One quinone ring proton was observed at 4·9 τ and the aromatic region, after shaking with D_2O

to remove a broad N–H absorption, showed an ABX system with proton resonances at 2·52, 2·63 and 3·11 τ respectively (J_{AB} = 0Hz, J_{AX} = 2 Hz and J_{BX} = 9 Hz) while a further singlet at 0·62 τ (1 proton), removed by D_2O was assigned to the phenolic —OH group. The mechanism of formation of this minor product is unknown and we have not been able to prepare it by reaction of I with *p*-benzoquinone, but structure V with a hydroxyl group in either position shown is consistent with the chemical and spectral evidence and with the known additive behaviour of quinones.

Chromatographic separation of the product from *p*-toluquinone and glycine ethyl ester gave a red crystalline compound, $C_{11}H_{13}O_4N$, which was supported by the mass spectrum (Fig 1) showing M^+ at *m/e* 223 and a strong fragment ion at *m/e* 150 (M-73). The NMR spectrum showed, in addition to protons of the glycyl ester side chain, a doublet at 7·93 τ (J = 2 Hz; quinone Me group), quinone ring protons at 4·58 τ (singlet) and 3·52 τ (multiplet) assigned to protons ortho to the amino side chain and methyl group respectively by comparison of the chemical shift and multiplicity with the corresponding protons in I and *p*-toluquinone[12] respectively. This evidence eliminates a possible structure in which the Me and glycine ester substituents are ortho related and the *para* relation of these groups as shown in the assigned structure II is preferred by analogy with other 1,4-addition reactions of *p*-toluquinone.[10]

The product formed from methoxy-*p*-benzoquinone and glycine ethyl ester was assigned structure III by analogy with the toluquinone product. The NMR spectrum

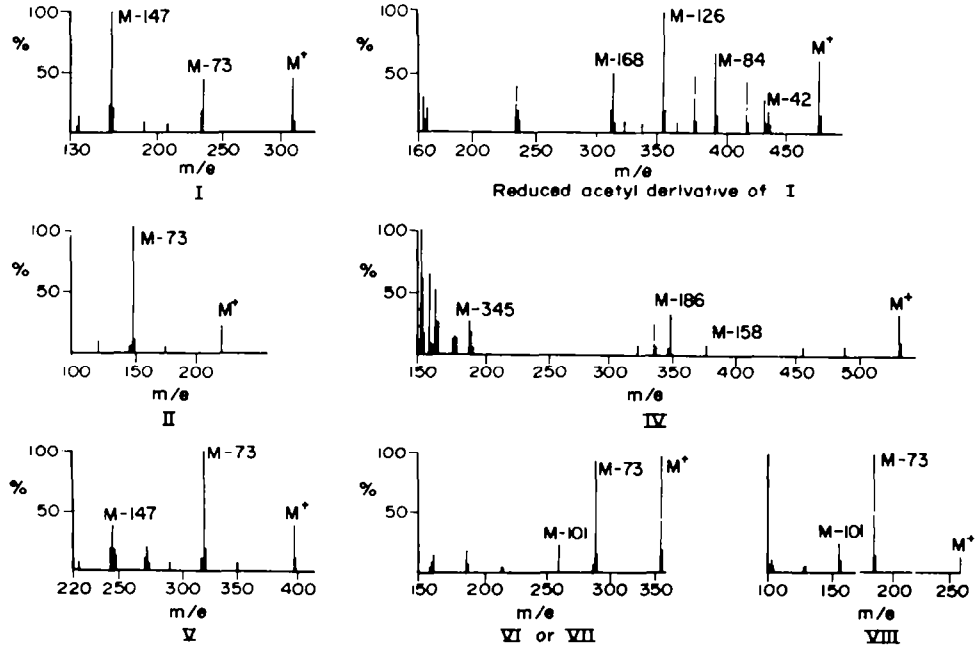


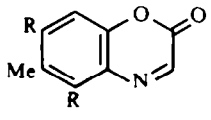
FIG 1. Mass spectra of amino ester substituted quinones (ions with an abundance of more than 5% of the base peak are shown).

showed one ring proton at 4·68 τ, typical of protons *ortho* to the glycine ester side chain, and another at 4·21 τ assigned to a proton *ortho* to the OMe group by comparison with the shift of a similar proton in 4-methoxydalbergione.[13]

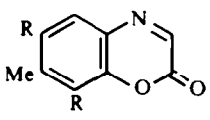
p-Benzoquinone condensed with glycyl-L-leucine ethyl ester to give a crimson product IV which was shown to be disubstituted by its analysis which indicated the formula $C_{26}H_{40}O_8N_4$. The mass spectrum (Fig 1) showed M^+ at *m/e* 536, in agreement with the formula, and fragment ions at *m/e* 378, 350 and 191 corresponding to side chain cleavage either side of the peptide carbonyl group in each chain. Metastable ions supported the transitions 536 → 350 → 191 and 536 → 378.

Condensation of *o*-benzoquinone with glycine ethyl ester gave a resinous product from which no pure compound could be obtained. 4-Methyl-*o*-benzoquinone gave, in very poor yield, a yellow basic product on reaction with glycine ethyl ester. Analysis indicated the formula $C_{17}H_{21}O_6N_3$ and the mass spectrum (Fig 1) showed M^+ at *m/e* 363 in agreement with the empirical formula. Fragment ions occurred at *m/e* 290 (M-73), 262 (M-101) and 188 (262–274) with metastable ions supporting the transitions 363 → 290 → 262 → 188.

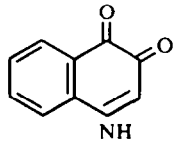
The analytical and mass spectral data suggest that while three nitrogen atoms are present, only two Et groups derived from glycine ethyl ester have been retained, and the basic properties indicate the removal of quinone groupings. Two of several possible structures are the lactones VI and VII which could arise by cyclisation of the intermediate anils, but the UV, IR, and NMR spectrum (100 MHz) do not enable an unequivocal assignment to be made. The IR spectrum indicated the presence of —NH, ester carbonyl, C=N and aromatic groups. The NMR spectra showed signals at 8·73, 8·70, 5·76 and 5·74 τ assigned to the protons of two ethyl ester groups. A singlet at 7·81 τ (3 protons) was assigned to an aromatic Me group, while that at 6·12 τ (2 protons) was attributed to a glycine methylene group. The quartets (4 protons) centred at 5·75 τ obscure two singlets at 5·85 τ (1 proton) and 5·79 τ (2 protons) which may be assigned to an —NH group and to the second glycine methylene group respectively. A signal at 3·76 τ consists of a broad peak (1 proton) and a sharp signal (1 proton) due to an —NH group and an aromatic proton respectively and a sharp singlet at 2·88 τ is assigned to the olefinic proton of the HC=N group.



VI: R = $-NH.CH_2.CO_2Et$



VII: R = $-NH.CH_2.CO_2Et$



VIII

1,2-Naphthoquinone condensed with glycine ethyl ester to give an orange-brown product obtained previously[14] but not fully characterised. Analytical and mass spectral data suggested structure VIII of molecular weight 259. Fragment ions (Fig 1) were obtained at *m/e* 186 (M-73) and 158 (186–28) together with metastable ions corresponding with the transitions 259 → 186 → 158. Comparison of the UV spectrum of the product in ethanol (page 1837) with the reported UV spectra [15] of

tautomers of 4-amino-1,2-naphthoquinones supports structure VIII. The NMR spectrum, measured in trifluoroacetic acid because of low solubility in other solvents, showed, in addition to the protons of the aromatic ring and ester group, a singlet at 3·07 τ (one quinone ring proton) and a singlet at 5·03 τ (2 protons) due to the methylene group, showing that no proton was attached to the adjacent nitrogen atom. This evidence supports the tautomeric 2-hydroxy-1,4-quinone-4-imine structure; it has been shown[15] that 4-amino-1,2-naphthoquinones exist as 1,4-quinone imines below pH 4.

Glycine was slowly liberated from compounds I, II and V by hydrolysis with boiling water and detected by paper chromatography of the hydrolysate using ninhydrin spray. Hydrolysis was more rapid with boiling 6N HCl and the liberated amino acids were estimated by two methods which gave results in good agreement. In the case of compounds I, II and VIII direct measurements of the intensity of the colouration with ninhydrin were used[16] and the hydrolysates from I, II, IV, V and VIII were kindly examined for us by Professor R. L. M. Synge, F.R.S., at the Food Research Institute, Norwich using an automatic amino acid analyser.[17] The results (Table 1) show that glycine was obtained from compounds I, IV and VIII in 19–27%

TABLE 1. RECOVERY OF ACIDS AND AMMONIA ON HYDROLYSIS

	%N recovered in hydrolysis			
		Autoanalyser		
Compound	Direct ninhydrin	Glycine	Leucine	Ammonia
I	28–35	23·4	—	2·0
II	79–85	86·0	—	very small
IV	—	19·6	89·0	3·8
V	—	62·0	—	8·5
VIII	38–44	27·0	—	*

* Considerable ammonia is produced but the estimation is complicated by at least two unidentified nitrogenous compounds.

yield, but the toluquinone product II gave a high recovery (86%) while compound V gave 62% yield, indicating partial release of both potential glycine groups. The high recovery (89%) of leucine from compound IV showed that only the amino terminal amino acid was recovered in poor yield.

Some nitrogen is liberated as ammonia (Table 1) which may be formed by the reaction: quinone + glycine → hydroquinone + $HN{=}CHCO_2H \rightarrow NH_3$ + glyoxylic acid. The glyoxylic acid content of the acid hydrolysate of compound I was determined spectrophotometrically by conversion to 1,5-diphenylformazancarboxylic acid.[18] The yield obtained (12%) must be less than the true quantity since authentic glyoxylic acid, when subjected to the hydrolysis procedure, gave only a 42% recovery.

During hydrolysis of compound I with water or 6N HCl, 5% yields are obtained of an insoluble black substance containing 3·5% nitrogen, but in view of the small

yields the black product cannot be responsible for the retention of significant amounts of nitrogen. However, the reactions between quinones and glycine esters are complicated; the quinonoid nature of the products and the isolation of hydroquinone derivates suggest that the redox potentials of reactants and products are important factors. As expected, the quinonoid products behave as vinylogous amides on hydrolysis although the recovery of amino acids is usually low and the liberation of ammonia and glyoxylic acid suggests that secondary reactions between the resultant quinones and glycine may be partly responsible. There is evidence from the hydrolysis of IV that amino acids attached to the quinone ring are not recovered as fully as more remote amino acids but there is no support for the suggestion[8] that the N-terminal amino acids are not hydrolysed by 6N HCl.

EXPERIMENTAL

UV and IR spectra were determined using Perkin–Elmer Model 137 and Unicam SP200 spectrometers respectively. NMR spectra were determined at 60 MHz in $CDCl_3$ unless otherwise stated and mass spectra in an AEI MS9 using 70 eV ionising electrons. Amino acids were analysed on a Beckman–Spinco Model 120C, using norleucine as internal standard. Preparative TLC was accomplished on plates coated with Kieselgel G.

2,5-*Di*-(N-*carbethoxymethylamino*)1,4-*benzoquinone*(1) and 3-(N-*carbethoxymethylamino*)6(or 7)-*hydroxy*-N-*carbethoxymethylcarbazole*-1,4-*quinone* (V). Glycine ethyl ester (2.06 g) was treated with *p*-benzoquinone (3·24 g) as described.[9] The ppt was recrystallised from Chf-EtOH to give **1** (1·0 g) as crimson prisms m.p. 200–202° (dec). (Found: C, 53·9; H, 6·1; N, 9·0. $C_{14}H_{18}O_6N_2$ requires: C, 54·2; H, 5·8; N, 9·0%); λ_{max} (EtOH): 337, 460–480 nm; ν_{max} ($CHCl_3$): 3380, 3300, 1740, 1650, 1595, 1510 cm^{-1}.

Reductive acetylation of **1** with Zn and Ac_2O gave, in good yield, a *tetra-acetyl* derivative as colourless prisms from benzene–ether, m.p. 134–136°. (Found: C, 55·2; H, 6·0; N, 5·6; $C_{22}H_{28}O_{10}N_2$ requires: C, 55·0; H, 5·8; N, 5·8%); ν_{max} ($CHCl_3$): 1765, 1740, 1665, 1515 cm^{-1}.

The combined filtrate and crystallisation mother liquors from **1** were concentrated and separated by preparative TLC using Chf-MeOH (95:5) for development. The red band of higher R_f gave **1**, while the lower R_f band gave, on extraction with and crystallisation from EtOH, compound V (0·15 g) as red-brown needles, m.p. 214–215°. (Found: C, 60·2; H, 5·4; N, 6·9. $C_{20}H_{20}O_7N_2$ requires: C, 60·0; H, 5·0; N, 7·0%); λ_{max} (EtOH): 268, 275, 347, 452 nm; λ_{max} (EtOH–NaOH): 268, 277, 355, 580 nm; λ_{max} (nujol): 3450, 3400, 1740, 1705, 1660, 1620, 1602, 1532, 1512, 872, 800 cm^{-1}.

2-(N-*Carbethoxymethylamino*)5-*methyl*-1,4-*benzoquinone* (II). Ice cold solns of glycine ethyl ester (1·3 g) in EtOH (5 ml) and *p*-toluquinone (3·05 g) in EtOH (60 ml) were mixed and the soln kept at 0° for 2 days. The residue obtained by evaporation was chromatographed on silica gel (50 g) and the first Chf-benzene (3:1) eluate gave the product II (0·4 g), obtained as orange plates from benzene-light petroleum (b.p. 40–60°) (1:2), m.p. 102–103°. (Found: C, 59·1; H, 6·0; N, 6·0. Calc for $C_{11}H_{13}O_4N$: C, 59·2; H, 5·8; N, 6·3%); λ_{max} (EtOH) 275, 470 nm; ν_{max} ($CHCl_3$): 3400, 1740, 1665, 1640, 1603, 1525 cm^{-1}. 2,5-Dihydroxytoluene was recovered from later eluates.

2-(N-*Carbethoxymethylamino*)5-*methoxy*-1,4-*benzoquinone* (III) was prepared as described previously.[4]

2,5-*Di*-(N-*glycl*-L-*leucine ethyl ester*)1,4-*benzoquinone* (IV). A soln containing glycyl-L-leucine ethyl ester hydrochloride (0·7 g), *p*-benzoquinone (0·4 g), EtOH (20 ml) and pyridine (1 ml) was kept at 0° for 24 hr. The product, obtained by evaporation, was chromatographed on silica gel (30 g), eluted with Chf-MeOH (97:3) and purified by TLC using the same solvent system. The product IV crystallised from benzene as crimson prisms (0·08 g) m.p. 154–156° (dec). (Found: C, 58·0; H, 7·5; N, 10·3. $C_{26}H_{40}O_8N_4$ requires: C, 58·2; H, 7·5; N, 10·5%); λ_{max} (MeOH): 337, 470–490 nm.

Reaction of 4-*methyl*-1,2-*benzoquinone with glycine ethyl ester.* Ice cold solns of 4-methyl-1,2-benzoquinone (3·0 g) in EtOH (90 ml) and glycine ethyl ester (2·05 g) in EtOH (10 ml) were mixed and kept at 0° for 24 hr. The soln was concentrated and the crystalline material was collected and recrystallised from EtOH giving yellow needles (0·02 g) m.p. 187–189°. (Found: C, 55·7; H, 5·4. $C_{17}H_{21}O_6N_3$ requires: C, 56·2; H, 5·8%); λ_{max} (EtOH): 254, 380 nm; λ_{max} (EtOH–HCl): 249, 358 nm; λ_{max} (EtOH–NaOH); 238 sh, 307 nm; ν_{max} ($CHCl_3$): 3380, 2985, 2895, 1733, 1625, 1590, 1515, 1384 and 1346 cm^{-1}. The residual soln was

evaporated to dryness and the residue was chromatographed on silica gel but no crystalline material could be obtained.

4-(N-*Carbethoxymethylamino*)1,2-*naphthoquinone* (VIII) was obtained from 1,2-naphthoquinone (3·1 g) and glycine ethyl ester (1·0 g) as for compound I. The product was chromatographed on silica gel (100 g) and the red band eluted with Chf-MeOH (95:5) was crystallised from MeOH to give VIII (0·8 g) as red-brown needles, m.p. 182–184° (dec). (Found: C, 64·8; H, 5·2; N, 5·2. $C_{14}H_{13}O_4N$ requires: C, 64·9; H, 5·0; N, 5·4%); λ_{max} (EtOH): 238, 268, 295 sh, 335 sh, 440–450 nm; ν_{max} (Nujol): 3300, 1740, 1692, 1645, 1620, 1600, 1550, 845, 785, 750, 730 cm^{-1}.

Hydrolysis of compounds I, II, V, VIII *with water*. The product (50 mg) was boiled with water (20 ml) for 20 hr, the soln was filtered, concentrated and examined by two dimensional paper chromatography using n-BuOH: HOAc: H_2O (12:3:5) and EtOH:H_2O:35%NH_4OH (18:1:1) for development and ninhydrin spray to locate any glycine liberated. Glycine was detected in the hydrolysate of compounds I, II and V but not from VIII.

Acid hydrolysis. The compound (20 mg) was boiled with 6N HCl (200 ml) for 24 hr. The soln was filtered, evaporated and the residue dissolved in citrate buffer, pH 5 or pH 2·2 for the direct or automatic analyser methods respectively and in water (250 ml) for estimation of glyoxylic acid. The glyoxylic acid concentration was determined by reference to a calibration curve. The results are given in Table 1.

Acknowledgements—We wish to thank the Agricultural Research Council, who supported the work described in this paper, Professor R. L. M. Synge, F.R.S. for the determination of amino acids on the Beckman-Spinco apparatus, and Dr. I. O. Sutherland for discussion on the N.M.R. spectra.

REFERENCES

[1] R. J. Swaby and J. N. Ladd, *Trans. Int. Soc. Soil Sci.* Commissions IV and V p 197. New Zealand (1962)
[2] W. R. C. Handley, *Plant and Soil* **15**, 37 (1961)
[3] J. M. Bremner, *Soil Biochemistry* (Edited by A. D. McLaren and G. H. Peterson) Chap. 2. Arnold, London (1967)
[4] M. V. Cheshire, P. A. Cranwell, C. P. Falshaw, A. J. Floyd and R. D. Haworth, *Tetrahedron* **23**, 1669 (1967)
[5] J. N. Ladd and P. G. Brisbane, *Aust. J. Soil Res.* **5**, 161 (1967)
[6] P. Simonart, L. Batistic and J. Mayaudon, *Plant and Soil* **27**, 153 (1967)
[7] J. N. Ladd and J. H. A. Butler, *Aust. J. Soil Res.* **4**, 41 (1966)
[8] K. Haider, L. R. Frederick and W. Flaig, *Plant and Soil* **22**, 49 (1965)
[9] E. Fischer and H. Schrader, *Ber. Dtsch. Chem. Soc.* **43**, 525 (1910)
[10] H. G. H. Erdtman, *Proc. Roy. Soc.* A, **143**, 183 (1933)
[11] I. S. Ioffe and Z. Ya. Khavin, *J. Gen. Chem. USSR* (English trans) **24**, 539 (1954)
[12] R. K. Norris and S. Sternhell, *Aust. J. Chem.* **19**, 617 (1966)
[13] W. B. Eyton, W. D. Ollis, I. O. Sutherland, O. R. Gottlieb, M. Taveira Magalhaes and L. M. Jackman, *Tetrahedron* **21**, 2683 (1965)
[14] H. Cassebaum, *Chem. Ber.* **90**, 2876 (1957)
[15] R. E. Harmon, L. M. Phipps, J. A. Howell and S. K. Gupta, *Tetrahedron* **25**, 5807 (1969)
[16] S. Moore and W. H. Stein, *J. Boil. Chem.* **176**, 367 (1948)
[17] D. H. Spackman, W. H. Stein and S. Moore, *Analyt. Chem.* **30**, 1190 (1958)
[18] D. N. Kramer, N. Klein and R. A. Baselice, *Ibid.* **31**, 250 (1959)