ACTA CHEMICA SCANDINAVICA 23 (1969) 371–378

The Structure Elucidation of A Fluorescent Substance from Bovine Liver Hydrolysates

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A crystalline blue fluorescent solid isolated from bovine liver has been found by chemical degradation and spectroscopic methods to be L-(−)-8-hydroxy-5-methyldihydrothiazolo(3,2-a)pyridinium-3-carboxylate.

Acid hydrolysis of bovine liver has recently been found by Laland *et al.*[1] to furnish a white crystalline solid, m.p. 152–53°, which exhibited an intense blue fluorescence in ultraviolet light. The empirical formula was reported [1] as $C_9H_9NO_3S$ while a preliminary X-ray crystallographic investigation gave a weight of 439 for the asymmetric unit in the crystal [2] and from alkali titration the equivalent weight was 212.[1] We now report the structure of this material.

High resolution mass spectrometry gives an apparent molecular ion C_8H_9NOS (*m/e* 167). But there is also a prominent peak at *m/e* 44 due to carbon dioxide which at first decreases with time. The IR spectrum as discussed below confirms the presence of a carboxyl group and would suggest the molecule to be $C_9H_9NO_3S$ which is being decarboxylated by pyrolysis in the mass spectrometer. The asymmetric unit in the crystalline state should then be due to the monohydrated dimer of the acid, the theoretical unit being 440. Drying experiments also confirmed this crystal composition.

The substance is water soluble and insoluble in most of the usual organic solvents. It is very acid resistant and can be recovered after refluxing in 6 N HCl over longer periods. It is gradually attacked by aqueous alkali, but was recovered unchanged after heating in aqueous or ethanolic ammonia. The material is ninhydrin negative and is not acetylated under normal conditions. Therefore the nitrogen must be tertiary or quaternary. With ferric chloride a pink coloration is observed, indicating a phenol or enolic structure. The substance is not attacked by HBr in acetic acid, thus excluding a thio-

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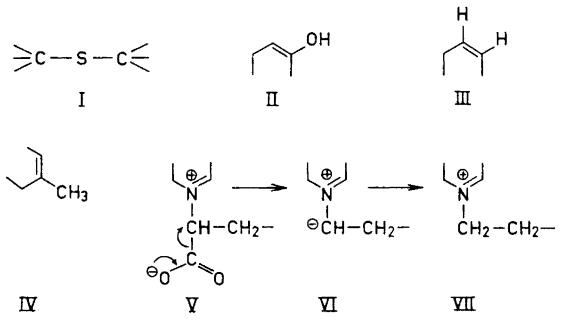
lactam structure.[3] The tests for thiol were negative and with the oxygen atoms otherwise bonded the sulphur must be present as a thioether.

The substance absorbs strongly in UV with λ_{max} in 0.1 N HCl at 340 mμ (log ε 4.01) and 240 mμ (log ε 3.85); in 0.1 N NaOH at 360 mμ (log ε 4.05) and 245 mμ (log ε 4.01). This suggestion of an aromatic structure would seem to be confirmed by IR absorption bands in nujol at 1575, 1525, 1470, and 1440 cm^{-1}, which could well be interpreted as due to aromatic ring vibrations. The observed red shift of 10 and 20 units (see above) for the respective UV absorption bands with increase in pH would support a phenolic structure since the phenolate anion absorbs 15—17 mμ higher than phenol itself [4] and the carboxyl group as shown below is isolated from the chromophore.

Additional prominent features in the IR spectrum are a broad H-bonded OH-band at about 2500 cm^{-1} and a broad and strong band at 1640 cm^{-1}, indicating a zwitterionic carboxylate structure. When the ionisation of the carboxyl group is suppressed as in the acid hydrochloride, the strong absorption in the carbonyl region is shifted to 1740 cm^{-1} as in α-amino acids. Common to all IR spectra besides aromatic absorption bands in the 1400—1600 cm^{-1} region is a sharp band at 850 cm^{-1}, indicating a 1,2,3,4-tetrasubstituted phenyl ring system.[5] This conclusion is supported by the presence in the NMR spectrum of 2 proton signals in the aromatic region making up an AB system (2.13—2.67 τ in TFA, J=8.5 cps). A singlet at 7.23 τ is due to the 3 protons in an "isolated" methyl group. The remaining 3 protons resonate as a single proton quartet at 3.67—3.84 τ and as a two-proton triplet at 5.72—5.80 τ. The latter would suggest two protons of a methylene carbon adjacent to an asymmetrically substituted methine carbon atom, a collapsed ABX system. The ready decarboxylation of the material was confirmed by heating in powdered glass either under nitrogen or *in vacuo*, in aqueous DMSO or in quinoline. The UV spectra were the same after heat treatment, but loss of absorption in the carbonyl region in IR confirms decarboxylation. The molecular peak in the mass spectrum was found at *m/e* 167 further confirming decarboxylation. The NMR spectrum shows the loss of the methylene-methine multiplets with the appearence of two new triplets centered at 6.17 and 4.90 τ (J=8.0 cps, in TFA), which must be ascribed to two adjacent methylene groups. The original material is optically active, $[\alpha]_D = -130°$ (c 1.2 in 0.1 N NaOH), but the optical activity is completely lost on decarboxylation. Since the rest of the molecule is intact on decarboxylation the carboxyl group must be attached to the asymmetric methine carbon.

The methine proton resonates at unusually low field in the NMR spectrum (3.67—3.84 τ in TFA) as do the protons in the new methylene group (4.90 τ in TFA). The methine group besides having attached a methylene and a carboxyl group must be attached to a carbon, which carries no protons which can couple vicinally or to the nitrogen or the sulphur. But since the absorption in NMR lies at an unusually low field [6] an ammonium or sulphonium structure would suggest itself. Attempts to make a methiodide under the usual conditions did not lead to any stable derivative, the starting material being recovered. This might suggest a quaternary nitrogen. The ready decarboxylation could then be explained by the reaction sequence V→VI→VII the intermediate anion (VI) being highly stabilized by the positive charge on

the neighbouring annular nitrogen atom. Furthermore, the acid was only very slowly esterified with diazomethane in methanol. The reaction could be catalyzed with perchloric acid, but the esterification was best carried out by heating in methanolic HCl. The resultant ester was unstable and was readily hydrolyzed. These results could well support the idea that the carboxyl group and a quaternary nitrogen are attached to the same carbon (V). The following structural units are thus indicated:

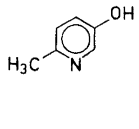


To verify that the sulphur is present as a thioether, oxidations were carried out. Thus hydrogen peroxide in formic acid furnished a product showing the characteristic sulphoxide absorption at 1050 cm^{-1}. In no case was the corresponding sulphone obtained even with excess hydrogen peroxide. More vigorous conditions such as the use of potassium permanganate or chromic oxide lead to extensive decomposition. The product had molecular composition $C_9H_9NO_4S$ in agreement with sulphoxide formation. In the NMR spectrum the vicinal aromatic protons have been shifted about 0.7 ppm towards lower field, 1.54—2.00 τ (J=9.0 cps). The methyl group has suffered a downfield shift from 7.23 to 7.02 τ. With sulphur directly attached to the aromatic ring a lone pair of electrons on the sulphur atom would be conjugated with the π-electron system while in the sulphoxide such an electron pair would no longer be available to the same extent for conjugation because of the electronegative oxygen. This should result in a more electron deficient ring system and therefore a chemical shift towards lower field. The loss of such an auxochrome is also reflected in a blue shift of about 10 mμ in the UV spectrum. In the infrared the absorption in the carbonyl region is shifted to a more usual value of 1700 cm^{-1} showing that the carboxyl group is not simply involved in a betaine structure.

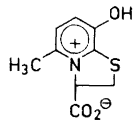
In the absence of steric hindrance about equal amounts of the diastereomeric sulphoxides would be expected to have been formed. However, chromatography clearly showed the ratio to be more like 9:1, which means that we here have a stereospecific oxidation of sulphur. This interesting observation is the basis for a later paper. The major optical isomer was readily obtained by crystallization from water, $[\alpha]_D = +169$ (c=0.2 in water).

Raney nickel desulphurisation in 15 % NaOH gave rise to two major isolated products. Repeated ether extractions of the neutralized reaction

mixture furnished a solid, m.p. 169—170°. The base peak in the mass spectrum is the molecular ion C_6H_7NO (*m/e* 109) in accordance with a simple aromatic structure. The NMR spectrum shows an isolated methyl group (7.60 τ in DMSO-d_6). The molecule, $C_5H_4NO(CH_3)$, must be a pyridine to accommodate four double bond equivalents. This is also verified by the presence of three aromatic protons. The IR spectrum is characterised by a broad absorption band at about 2500 cm^{-1} with no absorption in the range 1600—1700 cm^{-1}, the range in which 2- and 4-pyridones absorb.[7] The strongly hydrogen bonded hydroxy group in 3-hydroxypyridine has a broad absorption band around 2500 cm^{-1}.[8] The hydroxy group can therefore be assigned to the β-position. In NMR the aromatic protons give rise to an ABX spectrum, 2.77—3.03 τ (2H, multiplet, J=8.6, 2.2, 1.2 cps) and a quartet centered at 1.87 τ. The low chemical shift of the new proton shows that this must have been introduced into the α-position in the pyridine and the values for the coupling constants are only consistent with a 2,5-disubstituted pyridine.[9] As the IR data show the hydroxy group to be in the β-position the substance must be 2-methyl-5-pyridinol. This conclusion was confirmed by the UV absorption data which were as recorded in the literature [10] and finally by comparison with an authentic specimen prepared synthetically from α-picoline by sulphonation and potassium hydroxide fusion.[11]



VIII

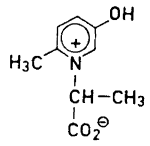


IX

From the evidence cited above the original material should have structure IX. On the other hand, quaternary pyridinium salts are fairly easily reduced in contrast to the parent base although some failures have been recorded.[12] Attempts to prepare a dihydro derivative with dithionite or sodium borohydride as in the case of quaternary nicotinamides [13] or to achieve reduction over platinum oxide, palladium on charcoal, or Raney nickel failed, the original material being recovered.

The final proof for structure IX comes from the isolation of the second degradation product from the desulphurization. Its molecular formula was found to be $C_9H_{11}NO_3$.

The UV absorption, in 0.1 N HCl at 295 mμ (log ε=3.80) and shoulder at 220 mμ (log ε 3.72), in 0.1 N NaOH at 325 (log ε=3.75) and 246 mμ (log ε=3.91) is very similar to that recorded for *N*-methylpyridinium-3-oxide.[10] The IR spectrum (KBr) is characterized by a carboxyl group present in a zwitterion, broad band at 2300—2600 cm^{-1} and 1630 cm^{-1} as in the original material. In the NMR spectrum (TFA) the aromatic protons constitute an ABX system, 2 H's in an apparent sextet at 1.85—2.30 (J=9.0, 2.5 and <1) and 1H in doublet centered at 1.15 τ and the singlet of a methyl group at 7.13 τ in agreement with the previously obtained 2-methyl-5-pyridinol. The protons in the *N*-alkyl side-chain resonate at 4.13 (1H, quartet) and at 7.88 τ (3H,

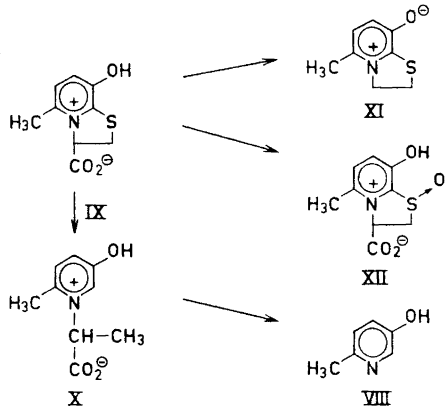


X

doublet) with vicinal coupling, $J=7.5$ cps. These results are only consistent with structure X.

The correctness of this structure was proved by synthesis from 2-methyl-5-pyridinol and α-bromopropionate ester. Acid hydrolysis of the resultant ester furnished a substance with the same chromatographic and absorption data as the degradation product. The latter, as first obtained, was optically inactive. When the reaction time was shortened to reduce the degree of racemisation the material isolated was weakly dextrorotatory, $[\alpha]_D^{25}=+4°$ (NaOH). The same compound has been synthesized from L-alanine without affecting the configuration at the asymmetric carbon atom in alanine [14] and was found to be dextrorotatory, $[\alpha]_D^{25}=+14°$ (H_2O). Therefore the degradation product has the L-configuration and the original substance (IX) must also have the L-configuration. This has been further proved by syntheses from L-cysteine.[15,16]

The structure of the liver factor has thus been shown to be L-(−)-8-hydroxy-5-methyldihydrothiazolo(3,2-a)pyridinium-3-carboxylate. The reactions discussed in this paper are shown schematically below:



EXPERIMENTAL

Hydrochloride of crystalline substance from liver hydrolysates.[1] Prepared by concentrating a solution of the substance in dilute HCl; white needles. IR spectrum in KBr: λ_{max} at 3340, 3300 (broad band), 1740, 1580, 1520, 1450, 1380, 1320, 1270, 1220, and 840 cm^{-1}.

NMR spectrum in deuterium oxide: Peaks at 7.36 (3H, singlet, aryl−CH_3); 5.95–5.70 (2H, octet, $J=12$, 6.5, 3.5 cps) and 3.87 (1H, quartet, $-CH_2-CH-$); and 2.62–2.32 (2H, AB, $J=8.5$ cps, $-CH=CH-$).

Decarboxylation. a) *Action of heat.* 500 mg of the material was ground together with finely crushed pyrex glass (3 ml) and the mixture heated at 120° for 10 h at 0.5 mm Hg. Alternatively the heating was carried out in an atmosphere of nitrogen by allowing the nitrogen to pass slowly through the apparatus and into a barium hydroxide solution when barium carbonate was precipitated and could be used to follow the progress of the decarboxylation.

The reaction mixture was extracted with hot ethanol (3×20 ml), the extracts concentrated to about 15 ml and applied to 2 Whatman No. 17 papers (45×45 cm) and developed overnight with BuOH:EtOH:H_2O (4:1:5). Water elution of the faster moving fluorescent band and freeze-drying, yielded the decarboxylated material (250 mg). Recrystallization from acetone-MeOH gave white prismatic needles, m.p. 218–220°. This substance contained water of crystallization and was dried at 100° *in vacuo* for elementary analysis. (Found: C 57.20; H 5.94; N 8.12. C_8H_9NOS requires: C 57.45; H 5.43; N 8.38). An aqueous solution was optically inactive.

UV spectrum: λ_{max} in 0.1 N HCl at 340 mμ (log ε=3.98) and 230 (log ε=3.70) with shoulder at 245 mμ (log ε=3.69); in 0.1 N NaOH at 360 mμ (log ε=4.02) and 245 (log ε=3.89) with shoulder at 260 (log ε=3.81). The IR spectrum in nujol showed no strong band in the 1550–1750 cm^{-1} region. NMR spectrum in TFA: Peaks at 7.28 (3H, singlet, aryl-CH_3), centered at 6.17 and 4.90 (2×2H, 2×triplets, J=8.0 cps, $-CH_2-CH_2-$), and 2.29–2.80 τ (2H, AB, J=8.0 cps, aryl-2H).

b) *Heating in DMSO.* 50 mg of the material was added to a solution of DMSO (2 ml) and water (3 drops) and the mixture heated to 120° for 6 h. The solid material gradually dissolved during this period. The solvent was removed under reduced pressure, the residual material dissolved in acetone-methanol (3 ml) and decolourized with charcoal. Addition of ether precipitated the decarboxylated material (22 mg), m.p. 204–214° (decomp.) which was further purified by chromatography as above.

A similar result was obtained on heating in quinoline.

Sulphoxide formation. To 220 mg of the substance dissolved in formic acid (20 ml) was added 30 % hydrogen peroxide (0.2 ml) and the clear solution left at room temperature. Chromatography showed that the reaction was essentially complete after 2 h. After evaporation under reduced pressure below 40° to prevent decomposition, the residual white solid was dissolved in water (5 ml) without warming. After standing in the cold overnight, a white crystalline solid (50 mg) had separated, m.p. 170° (decomp.).

The filtrate was freezed-dried and the solid residue triturated with acetone several times. The residual white solid (150 mg) had m.p. 170° (decomp.).

Chromatography of the material which crystallized out, showed this to be homogeneous while the solid obtained by evaporation contained a second component with about the same R_F value, ratio roughly 5:1. Elementary analyses gave the same results for both fractions and therefore these contain only sulphoxides. The diastereomeric sulphoxides can be separated by crystallization from water.

The white crystalline materials were analysed without any further purification. (Found: C 47.5; H 4.0; N 6.04. $C_9H_9NO_4S$ requires: C 47.56; H 3.99; N 6.17). The diastereomer isolated had $[\alpha]_D^{20}=+169$ (*c*, 0.2 in water). The IR spectrum in KBr showed the sulphoxide band at 1050 cm^{-1} and carboxyl at 1700 cm^{-1}.

The NMR spectrum in TFA has peaks at 7.02 (3H, singlet, aryl-CH_3), 5.32–6.13 (2H, multiplet, J=15, 7.5 and 0.8 cps, $-CH_2-$), 3.27–3.39 (1H, doublet) and 1.54–2.00 (2H, AB, J=9.0 cps).

UV absorption: λ_{max} in 0.1 N HCl at 312 (log ε 3.87), and at 235 mμ (log ε=3.82); in 0.1 N NaOH at 350 (log ε=3.83), and 230 mμ (log ε=4.17) with shoulder at 250 mμ (log ε=3.91).

Desulphurisation with Raney-nickel. 500 mg of the substance were dissolved in aqueous 15 % NaOH (20 ml) and 4 teaspoons full of freshly prepared Raney-nickel added. The catalyst used was obtained by heating nickel-aluminium alloy in NaOH as recently described.[17]

The suspension was heated at 100° with vigorous stirring overnight, the metal removed by centrifuging, washed twice with aqueous ethanol and the washings combined with the original solution. TLC on silica gel in BuOH:HOAc:H_2O (100:22:50) showed 2 fluorescent spots R_F=0.17 (90 %) and R_F=0.40 (10 %). The solution was brought to pH 7 with HCl, some white amorphous solid (100 mg) filtered off, the filtrate concentrated *in vacuo* to 20 ml and extracted with ether (4×10 ml). Evaporation of the dried ether

extracts furnished a whitish solid (33 mg), m.p. 169—170°. The m.p. was unchanged after recrystallization from benzene. TLC as above showed this to be the minor component in the reaction mixture. High resolution mass spectrometry gave *m/e* 109.0529, C_6H_7NO, for the molecular ion in agreement with the elementary analysis. (Found: C 66.1; H 6.4; N 12.7. Calc. for C_6H_7NO: C 66.04; H 6.46; N 12.83). NMR spectrum in DMSO-d_6. Peaks at 7.60 (3H, singlet, aryl-CH_3), 2.77—3.03 (2H, multiplet, $J=8.6$, 2.2, 1.2) and a quartet centered at 1.87. This ABX system is due to a 2.5-disubstituted pyridine. IR spectrum in KBr: Broad strong band at 2500 cm^{-1} (strongly bonded OH), 1580, 1510, 1280, 1230, 1140, 830, and 750 cm^{-1}. UV spectrum: λ_{max} in 0.1 N HCl at 297 (log $\varepsilon=3.79$) and 220 mμ (log $\varepsilon=3.63$); λ_{max} in 0.1 N NaOH at 306 (log $\varepsilon=3.63$) and 240 mμ (log $\varepsilon=4.02$).

All these data including R_F values were the same as those found for synthetic 3-hydroxy-6-methyl pyridine prepared by sulphonation of α-picoline followed by alkali fusion.[11]

The aqueous layer after the ether extractions was concentrated to 2—3 ml and ethanol (20 ml) added. The precipitated salt was removed by filtration and the filtrate evaporated to dryness. The residue was triturated with hot ethanol, insoluble material filtered off, the filtrate evaporated and this extraction procedure repeated once more. Then the extraction procedure was repeated with isopropanol containing a little acid and finally extracted with boiling isopropanol. The hot solution on cooling precipitated a solid (250 mg), m.p. 130—150° which after 2 recrystallizations from a small volume of water had m.p. 145—147°. A further recrystallization from wet isopropanol gave m.p. 146—148°. TLC as above. R_F 0.20, the spot being blue fluorescent under the UV lamp. This material was optically inactive, but when the temperature of the reaction was reduced to 80° and the heating time to 1½ h the product was weakly dextrorotatory. $[\alpha]_D^{25}=+4.0$ ($c=1.0$ in 0.1 N NaOH). (Found: C 59.68; H 6.08; N 8.03. $C_9H_{11}NO_3$ requires: C 59.68; H 6.12; N 7.73). NMR spectrum in TFA: peaks at 7.13 (3H, singlet, aryl-CH_3), 7.88 (3H, doublet, $J=7.5$; $-CH_3$) and 4.13 (1H, quartet, $J=7.5$, $-CH-$); 1.85—2.20 (2H, sextet, $J=9.0$, 2.5 and <1) and 1.15 τ (doublet), both groups part of reduced ABX system.

The IR spectrum in KBr showed strong carboxylate absorption at 1630 cm^{-1}. UV spectra: λ_{max} in 0.1 N HCl at 295 mμ (log $\varepsilon=3.80$), shoulder at 220 mμ (log $\varepsilon=3.72$). In 0.1 N NaOH at 325 (log $\varepsilon=3.75$) and 246 mμ (log $\varepsilon=3.91$).

All these data are the same as those obtained for synthetic α-(3-hydroxy-6-methyl-pyridinium)propionic acid, and the sign of the optical rotation is the same as that of the L-enantiomer otherwise obtained.[14]

α-(3-Hydroxy-6-methylpyridinium)propionate (X). A solution of 3-hydroxy-6-methyl-pyridine[11] (0.33 g, 0.003 mole) and methyl α-bromopropionate (0.50 g, 0.003 mole) in anhydrous toluene (45 ml) was refluxed for 20 h. Evaporation of the toluene left the ester as a greyish solid which was hydrolysed to the acid in N NaOH (10 ml) at room temperature overnight. After adjustment of the pH to 7.6 with HCl the solution was repeatedly extracted with ether to remove any 3-hydroxy-6-methylpyridine present and the concentrated solution (to about 4 ml) preparatively chromatographed on Whatman No. 17 paper, using the developer BuOH:EtOH:NH_3:H_2O (4:1:2:1). Eluting the fluorescent band, $R_F=0.2-0.25$, with water furnished the desired pyridinium salt after evaporation. To remove inorganic impurities the solid was extracted with isopropanol (2×20 ml) at 60° and the filtrate evaporated. The extraction procedure was repeated twice, finally leaving 30 mg of a whitish solid, m.p. 145—147° after recrystallization from isopropanol.

The identity of the synthetic and degradative products was established by them both having the same physical chemical properties.

Acknowledgements. The authors are indebted to Dr. B. A. Hems and his staff at the Glaxo Research Laboratories, London, England, for the use of Glaxo research facilities during the summer of 1965, to Dr. E. Page of the Glaxo Research Laboratories for NMR spectra, and to Dr. E. S. Waight of Imperial College, London, for mass spectra.

Mass spectra were recorded on an A.E.I. MS 9 double focussing mass spectrometer, the NMR spectra on a Varian A-60 spectrometer, the IR spectra on a Perkin-Elmer 237 infrared spectrophotometer, and the UV spectra on a Beckmann DB-G ultraviolet spectrophotometer.

REFERENCES

1. Laland, P., Alvsaker, J. O., Haugli, F., Dedichen, J., Laland, S. and Thorsdalen, N. *Nature* **210** (1966) 917.
2. Furberg, S. *Nature* **210** (1966) 919.
3. Renault, J. *Compt. Rend.* **232** (1951) 77.
4. Jaffe, H. H. and Orchin, M. *Theory and Application of Ultraviolet Spectroscopy*, Wiley, New York 1962, p. 257.
5. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules*, Methuen, London 1958, p. 78.
6. Nakanishi, K. *Infrared Absorption Spectroscopy*, Holden-Day, San Francisco and Nankado & Co. Ltd., Tokyo 1962, Appendix I, p. 233.
7. Katritzky, A. R. and Ambler, A. P. In Katritzky, A. R. *Physical Methods in Heterocyclic Chemistry*, Academic, London 1963, Vol. II, p. 266.
8. Heinert, D. and Martell, A. F. *J. Am. Chem. Soc.* **81** (1959) 3933.
9. White, R. F. M. In Katritzky, A. R. *Physical Methods in Heterocyclic Chemistry*, Academic, London 1963, Vol. II, p. 142.
10. Metzler, D. E. and Snell, E. E. *J. Am. Chem. Soc.* **77** (1955) 2431.
11. Marion, L. and Cockburn, W. F. *J. Am. Chem. Soc.* **71** (1949) 3402.
12. Baker, B. R. and McEvoy, F. J. *J. Org. Chem.* **20** (1950) 118.
13. Shaw, E. N. In Klingsberg, E. *Heterocyclic Compounds, Pyridine and Derivatives*, Part II, Interscience, London 1961, p. 47.
14. Undheim, K. and Greibrokk, T. *Unpublished data.*
15. Holtermann, H. *Unpublished data.*
16. Undheim, K., Røe, J. and Greibrokk, T. *Unpublished data.*
17. Dominguez, X. A., Lopez, I. C. and Franco, R. *J. Org. Chem.* **26** (1961) 1625.

Received June 19, 1968.