The results of pharmacological tests, including the evaluation of the relationship between chemical structure and analgesic activity (1–6), became the basis for kinetic and chromatographic studies of the stability of new N-substituted derivatives of 4-alkoxy-6-methyl-1H-pyrrolo[3,4-c]pyridine-1,3(2H)-dione. As a consequence of our observations, the studies on the mechanism of the reactions observed can also be interesting. The analgesic activity of these newly synthesized compounds (Fig. 1) was superior to that of acetylsalicylic acid in two different tests (“writhing syndrome” and “hot plate”). What is more, the results of the writhing syndrome test indicated that compounds I–IV were more potent than morphine (5, 6).

Based on the studies of the stability of other imides, such as thalidomide (2-phthalimidoglutaramide), a variety of possible degradation compounds can be formed during hydrolysis. Thalidomide is known to degrade rapidly in solutions at both physiological pH and in alkaline conditions. The main hydrolytic product is N-(2,6-dioxo-3-piperidin-3-yl)-phthalamic acid with a cleaved glutarimide ring, whereas the glutarimide ring is more stable and, therefore, the hydrolytic products (4-carbamoyl-4- and 4-carbamoyl-2-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-butyric acid) occur only as minor fractions. These three products are subjected to further hydrolysis (7, 8). Thalidomide is hydrolyzed in vivo and in vitro to 12 different products (8), 11 of which retain the chiral centre. Hydroxylation is possible at five different carbon atoms and with two of these the new chiral centres would be created (9).

The results of our previous kinetic and HPLC (HPLC-UV, HPLC-MS) studies suggest that the decomposition of the imide bond is followed by the

STUDIES OF THE DEGRADATION MECHANISM OF PYRROLO[3,4-C]PYRIDINE-1,3(2H)-DIONE DERIVATIVES WITH ANALGESIC ACTIVITY: ISOLATION AND IDENTIFICATION OF PRODUCTS AND SUMMARY

IZABELA MUSZALSKA

Department of Pharmaceutical Chemistry, Poznań University of Medical Sciences, Grunwaldzka 6, 60-780 Poznań, Poland

Abstract: Within the framework of the studies concerning the decomposition of N-substituted derivatives of 4-alkoxy-6-methyl-1H-pyrrolo[3,4-c]pyridine-1,3(2H)-dione, the isolation of the alkaline hydrolysis product was performed (pH 10.5, room temperature). Subsequently, based on NMR spectra and two-dimensional spectra, the chemical structure of the isolated compounds was established. The interpretation of COSY, HSQC and HMBC spectra proved that the C1-N2 bond of the pyrrolopyridinedione ring undergoes cleavage under the influence of OH ions and generates a product which is an isonicotinic acid derivative. Owing to the analysis of previous studies, including results presented in this paper, the decomposition mechanism of the compounds studied could be determined.

Keywords: pyrrolo[3,4-c]pyridine-1,3(2H)-dione derivatives, NMR spectra, COSY, HSQC, HMBC

* Corresponding author: e-mail: imuszals@ump.edu.pl

Figure 1. Chemical structures of pyrrolo[3,4-c]pyridine-1,3(2H)-dione derivatives
formation of two structural isomers (A and B; Scheme 1). Either can appear as the main product, depending on the concentration of hydrogen or hydroxyl ions. Furthermore, the successful staining using ninhydrine as a TLC visualizing reagent indicates that there is one amino derivative of product A or/and B after the hydrolysis of the amine group (C; Scheme 1) among the degradation products (10–12). The aim of this study was to prove and define the chemical structure of one of the two structural isomers by its isolation from the alkaline medium and NMR spectral identification (product A). On the other hand, our purpose was to determine the degradation mechanism of the newly synthesized pyrrolo[3,4-c]pyridine-1,3(2H)-dione derivatives (with proven analgesic activity, I – IV) in the aqueous environment.

EXPERIMENTAL

Materials and methods
All of the derivatives of 4-alkoxy-6-methyl-1H-pyrrolo[3,4-c]pyridine-1,3(2H)-dione (I – IV) were synthesized by means of published methods (5, 6) and supplied from the Department of Chemistry of Drugs at the Wroclaw University of Medicine.
All the solvents or chemicals (POCh, Gliwice, Poland) were of analytical reagent grade.

The spectrophotometric analysis of the isolated products of alkaline hydrolysis (1H-NMR; 13C-NMR; 2D: 1H-1H, 1H-13C short-range and long-range: COSY, HSQC, HMBC) was performed at the Advanced Chemical Equipment and Instrumentation Facility, Faculty of Chemistry, Adam Mickiewicz University in Poznań using: Bruker Avance 600 MHz NMR spectrometer, internal standard – tetramethylsilane (TMS), solvent – dimethyl sulfoxide (DMSO).

Isolation of the products of alkaline hydrolysis

Degradation products of derivatives containing 3-(4-phenylpiperazin-1-yl)propan-1-yl group (I – IV) formed at room temperature in the sodium hydroxide solution (0.01 mol L⁻¹, pH 10.5) were isolated. In order to do so, approximately 50 mg of the substrate was dissolved in a mixture of 3 mL of ethanol (760 g L⁻¹) and 0.5 mL of hydrochloric acid (1 mol L⁻¹). Subsequently, 1.4 mL of deionized water with approximately 1.2 mL of the sodium hydroxide solution (0.5 mol L⁻¹) was added and the pH of the mixture was brought to approximately 10.5. The mixture was left to evaporate to dryness at room temperature. Subsequently, the precipitate was dissolved in 5 mL of ethanol (760 g L⁻¹) and 0.5 mL of hydrochloric acid (0.01 mol L⁻¹), and filtered through a hard paper filter. The filtrate was evaporated and dried at room temperature. Finally, the precipitate was subjected to spectrophotometric analysis.

RESULTS

NMR spectra interpretation

**Product I A:** 3-[2-hydroxy-3-(4-phenylpiperazin-1-yl)propyl]carbamoyl]-2-methoxy-6-methylisonicotinic acid

1H-NMR (δ, ppm): 2.37 (s, 3H, CH3); 3.78 (s, 3H, OCH3); 2.61, 3.37 (t, 2 × 4H, 2 × CH2, piperazine); 3.26, 3.00 (d, 2 × 2H, 2 × CH2, propyl); 3.96 (m, 1H, CH, propyl); 5.37 (s, 1H, OH); 6.76 (t 1H, C6, phenyl); 6.92 (d, 2H, C2,6, phenyl); 7.20 (t, 2H, C3,5, phenyl); 7.05 (s, 1H, CH, pyridine); 7.83 (s, 1H, NH, amide).

13C-NMR (δ, ppm): 23.7 (CH3); 53.0 (OCH3); 44.5, 53.4 (2 × CH2, piperazine); 48.2, 61.8 (2 × CH2, propyl); 66.4 (CH, propyl); 115.3 (C2,5, phenyl); 118.7 (C6, phenyl); 128.9 (C3,5, phenyl); 151.1 (C1, phenyl); 115.8 (C2, pyridine); 117.2 (C5, pyridine); 149.6 (C4, pyridine); 153.9 (C6, pyridine); 159.5 (C2, pyridine); 167.8 (COOH); 167.4 (CO-NH).

**Product IIA:** 2-methoxy-6-methyl-3-[3-(4-phenyl-piperazin-1-yl)propyl]carbamoyl]-isonicotinic acid (Fig. 2)

1H-NMR (δ, ppm): 2.35 (s, 3H, CH3); 3.79 (s, 3H, OCH3); 3.09, 3.41 (t, 2 × 4H, 2 × CH2, piperazine); 1.65, 2.40, 3.18 (m, t, 3 × 2H, 3 × CH2, propyl); 6.77 (t, 1H, C6, phenyl); 6.91 (d, 2H, C2,6, phenyl); 7.20 (t, 2H, C3,5, phenyl); 6.98 (s, 1H, CH, pyridine); 7.92 (s, 1H, NH, amide).

13C-NMR (δ, ppm): 23.7 (CH3); 53.9 (OCH3); 48.2, 52.8 ppm (2 × CH2, piperazine); 25.9, 37.4, 55.6 (3 × CH2, propyl); 115.3 (C2,6, phenyl); 118.9 (C4, phenyl); 128.9 (C3,5, phenyl), 151.1 (C1, phenyl); 115.4 (C6, pyridine); 116.6 (C5, pyridine); 150.2 (C4, pyridine); 154.1 (C6, pyridine); 159.6 (C2, pyridine); 168.6 (COOH); 167.0 (CO-NH).

**Product IIIA:** 2-ethoxy-3-[2-hydroxy-3-(4-phenyl-piperazin-1-yl)propyl]carbamoyl]-6-methylisonicotinic acid

1H-NMR (δ, ppm): 2.37 (s, 3H, CH3); 1.24 (t, 3H, CH2, ethoxy); 4.25 (q, 2H, OCH2); 2.61, 3.37 (t, 2 × 4H, 2 × CH2, piperazine); 3.10, 3.03 (d, 2 × 2H, 2 × CH2, propyl); 3.95 (m, 1H, CH, propyl); 5.34 (s, 1H, OH); 6.76 (t, 1H, C6, phenyl); 6.92 (d, 2H, C2,6, phenyl); 7.18 (t, 2H, C3,5, phenyl); 7.01 (s, 1H, CH, pyridine); 7.78 (s, 1H, NH, amide).

13C-NMR (δ, ppm): 23.7 (CH3); 14.6 (CH2, ethoxy); 61.0 (OCH3); 44.5, 53.4 (2 × CH2, piperazine); 48.2, 61.9 (2 × CH2, propyl); 66.5 (CH2, propyl); 115.3 (C2,6, phenyl); 118.7 (C5, phenyl); 128.9 (C3,5, phenyl); 151.1 (C1, phenyl); 115.5 (C2, pyridine); 117.1 (C5, pyridine); 150.0 (C6, pyridine); 154.0 (C6, pyridine); 159.2 (C2, pyridine); 168.0 (COOH); 167.4 (CO-NH).

**Product IV A:** 2-methoxy-6-methyl-3-[3-(4-phenyl-piperazin-1-yl)propyl]carbamoyl]-isonicotinic acid

1H-NMR (δ, ppm): 2.34 (s, 3H, CH3); 3.76, 3.78 (2 × s, 3H, 2 × OCH3); 2.90, 3.38 (t, 2 × 4H, 2 × CH2, piperazine); 1.64, 2.40, 3.18 (m, t, 3 × 2H, 3 × CH2, propyl); 6.84 – 6.92 (m, 4H, 4 × CH, phenyl); 6.96 (s, 1H, CH, pyridine); 7.90 (s, 1H, NH, amide).

13C-NMR (δ, ppm): 23.7 (CH3); 53.1, 55.3 (2 × OCH3); 50.1, 53.0 (2 × CH2, piperazine); 25.9, 37.6, 55.8 (3 × CH2, propyl); 111.9 (C1, phenyl); 117.8 (C6, phenyl); 120.8 (C2, phenyl); 122.3 (C5, phenyl); 141.3 (C1, phenyl); 154.0 (C2, phenyl); 115.0 (C3, pyridine); 115.4 (C5, pyridine); 116.5 (C2, pyridine); 150.6 (C6, pyridine); 159.6 (C2, pyridine); 168.5 (COOH); 166.9 (CO-NH).

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DISCUSSION AND CONCLUSIONS

The previously published results of studies on the stability of the pyrrolo[3,4-c]pyridine-1,3(2H)-dione derivatives concerned the kinetic interpretation of the hydrolysis reaction of these derivatives with regard to pH values ranging from 0.4 to 7.0 and the estimation of the kinetic parameters for the formation and decomposition of the observed products (13–16). The analysis of results, including the observation of HPLC-UV and HPLC-MS chromatograms, did not solve the problem concerning the chemical structure of the intermediate product, being a derivative of nicotinic or isonicotinic acid (10–13). Considering the hydrolysis reaction mechanism of the compounds within the group of derivatives containing the 3-(4-phenylpiperazin-1-yl)propyl substituent (I – IV), it was shown that during the first phase of the reaction, the cleavage of the C1-N2 or N2-C3 bond within the pyrrole ring and the formation of the product (A or B) containing carboxyl and amide groups in the pyridine ring occur (Scheme 1) (10–13). As a consequence of this cleavage, peaks with the m/z values equal to the sum of the molecular weights of the substrate and water (M + 18) are present in HPLC-MS chromatograms (10–13). The differences in retention times observed in these chromatograms as well as in the HPLC-UV chromatograms suggest different susceptibility of the bonds to the action of hydrogen and hydroxyl ions being dependent on the electronic and steric properties of the neighboring substituents. Similarly, the effect of structure stiffening in the 3-(4-phenylpiperazin-1-yl)propyl derivatives due to the formation of hydrogen bond between the OH group of the propylene chain and the CO group of the pyrrolopyridinedione ring may be a possible explanation of the observed stability increase of these derivatives (I, III) in the alkaline environment, compared to the stability of derivatives lacking the alcohol group (II, IV). The observed hydrolysis reaction is reversible, although only within a narrow

Figure 2. Chemical structure and NMR spectra (1H-NMR, 13C-NMR, COSY) of isolated product IIA
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range of pH values from 3 to 6 (10–16) [similarly as in the case of noraminophenazone (17–19)]. The reversible character of hydrolysis of imides with a cyclic structure was described both with respect to the alkaline hydrolysis of barbiturates (leading to the formation of a malonuric acid compound (18, 26–27)) and to the hydrolysis of phenylbutazone (opening of the pyrazolidine-3,5-dione ring in position N₂-C₃ (18, 28, 29) or dihydrouracil derivatives (30, 31). Therefore, as in the case of the hydrolysis of barbiturates, also in this special case the mechanism of hydrolysis itself is not influenced by the degree of ionization, but rather by the character of the substituents.

The isolation and identification of the alkaline hydrolysis products of compounds I – IV confirmed the assumptions that the C₁-N₂ bond is particularly susceptible to the action of hydroxyl ions. In all the cases, the analysis of two-dimensional NMR spectra (COSY, HSQC, HMBC; Figs. 2, 3) confirmed the long-range correlations (HMBC: H-1'H-C) between the carbon from the carboxyl group (c) and the only one hydrogen atom within the pyridine aromatic ring (a) (Fig. 3; c – 168.6 ppm, a – 6.98 ppm), as well as between the carbon (e) and the hydrogen (f) from the amide group (Fig. 3; e – 167.0 ppm, f – 7.92 ppm). At the same time, short-range correlations (HSQC: H-1C) between the hydrogen (a) and the carbon (a) atoms of the pyridine aromatic ring (Fig. 3; 6.98 ppm, 115.4 ppm) were observed. The analysis of the COSY spectra demonstrated a correlation between the hydrogen atom of the amide group and the hydrogen atoms of the methyl group of the alkyl chain (Fig. 2; f – 7.92 ppm, CH₂ – 3.18 ppm). This interpretation proves the isonicotinic acid structure of the isolated derivatives (A; Scheme 1). Based on these findings and previous publications (10–13), it is reasonable to assume that the cleavage of the N₂-C₃ bond in the acidic environment takes place, resulting in the formation of the nicotinic acid derivative (B; Scheme 1). The equilibrium between these two reactions depends on the pH of the reaction medium.

In the next phase of 3-(4-phenylpiperazin-1-yl)propyl derivative hydrolysis, the hydrolysis of the amide group and the formation of 3-(4-phenylpiperazin-1-yl)propylamine derivative (C) and 6-methyl-2-alkoxypyridine-3,4-dicarboxylic acid (D), capable of two-stage decarboxylation, occurs (Scheme 1) (10–12). Ions with an appropriate m/z value were confirmed by the HPLC-MS-based analysis of products formed during the acidic and alkaline hydrolysis, providing evidence in favor of the proposed reaction scheme (10–12). The direction and the reversible character of the observed hydrolysis reaction of derivatives containing the 3-(4-phenylpiperazin-1-yl)propyl substituent is dependent on the pH value of the medium. However, the reaction rate and the possibility of the observation of subsequent reaction products is therefore a power function of reaction temperature (10–16).

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