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Isolation and identification of an alkali-catalyzed hydrolysis product of nitrazepam

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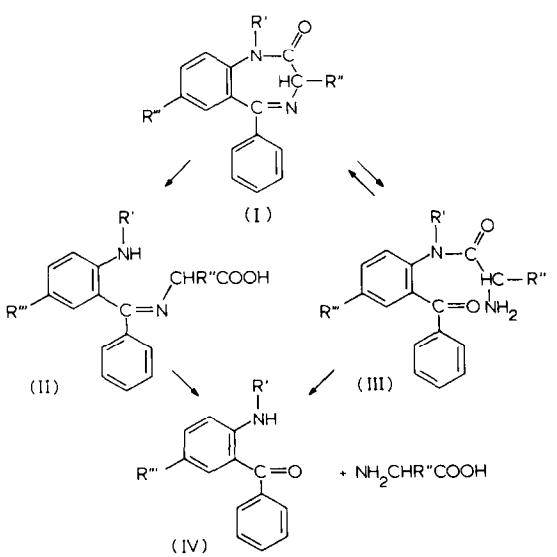
Summary

Spectral evidence is presented for the formation of an intermediate product during alkali hydrolysis of nitrazepam. The compound is obtained in maximum yield by heating a solution of nitrazepam in 0.1 M sodium hydroxide at 75°C for 15 min. A fast extraction technique has been developed that allows the isolation of the compound by solvent extraction with negligible hydrolysis to 2-amino-5-nitrobenzophenone. Structural elucidation by mass spectrometry, infrared spectrometry, proton magnetic resonance spectrometry, ultraviolet-visible spectrophotometry and elemental analysis has shown that the compound is *N*-(α-(2-amino-5-nitrophenyl)benzylidene glycine formed by cleavage of the 1,2 bond of nitrazepam.

Introduction

The hydrolysis of 1,4-benzodiazepines (**I**; Scheme 1) to the corresponding benzophenone (**IV**) proceeds via an open-ring intermediate by scission either of the 1,2 amide bond (**II**) or the 4,5 azomethine bond (**III**), the preferred pathway depending on the nature of the substituents on the benzodiazepine nucleus. For example, oxazepam (**I**; R′ = H, R″ = OH, R‴ = Cl) involves the formation of both intermediate products **II** and **III**. At pH values below the pK_{a1}, the two routes are of equal importance but at pH values above the



Scheme 1. The hydrolysis reactions of 1,4-benzodiazepines.

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pK_{a1}, hydrolysis of the 1,2 bond appears predominant owing to the recyclization of intermediate **III** (but not **II**) (Han et al., 1977a). These findings have been amplified by Reif and De Angelis (1983) who reported that hydrolysis products of oxazepam (additional to **II** and **III**) in acid and alkali are a glyoxanilide and an aldehyde, respectively.

Only one intermediate product (**III**) has been observed during the hydrolysis of diazepam (**I**; $R' = CH_3$, $R'' = H$, $R''' = Cl$), attributed to the methyl substituent on the amide nitrogen inhibiting 1,2 bond hydrolysis. Recyclization of the intermediate **III**, as in oxazepam, occurs rapidly at pH values above the pK_a (3.3) of the intermediate (Han et al., 1977a).

Nitrazepam (**I**; $R' = R'' = H, R''' = NO_2$) has been reported to undergo hydrolysis in aqueous solution by a two-step sequential reaction in which **III** is the only intermediate: this is attributed to the preferential activation for hydrolysis at the azomethine bond by the nitro group. Recyclization of the intermediate **III** of nitrazepam, like those of diazepam and oxazepam, also occurs at pH values above the pK_a of the intermediate product (Han et al., 1977b).

Spectral measurements made using a difference spectrophotometric assay during the alkali hydrolysis of nitrazepam indicated the presence of a previously unrecognised intermediate (Davidson and Li, 1989). The present study was undertaken in order to isolate and to identify this compound.

Experimental

Materials

Nitrazepam and 2-amino-5-nitrobenzophenone were donated by Roche Products Ltd.

All chemicals and solvents (BDH) were of analytical reagent grade quality.

Apparatus

Absorption and difference absorption spectra were recorded as previously described (Davidson and Li, 1989).

Gas-liquid chromatography-mass spectrometry was carried out on a Hewlett-Packard GC/MS System 5988A with a 25 m × 0.22 mm i.d. × 0.33 mm film thickness HP-1 cross-linked methyl silicone column. The carrier gas, helium, flowed through the column at 1 ml min^{-1}. The oven was programmed as follows: 100 °C for 1 min then 10 °C min^{-1} to 310 °C then held for 5 min. The injector, source and interface temperatures were 250, 140 and 280 °C, respectively; the ionisation energy was 70 eV.

Isolation of the intermediate product

Nitrazepam (1 g) dissolved in 0.1 M sodium hydroxide (250 ml) preheated to 75 °C was kept at this temperature for 15 min. The solution was cooled rapidly and extracted with chloroform (3 × 200 ml) to remove unhydrolysed nitrazepam and 2-amino-5-nitrobenzophenone. The aqueous solution was added to a separating funnel containing 1 M hydrochloric acid (25 ml), 0.1 M phosphate-citrate buffer (pH 4) (McIlvaine, 1921) (200 ml) and chloroform (500 ml) (NB: the order of addition is important). The solution was immediately shaken vigorously for 30 s and after 2 min the chloroform layer was evaporated on a rotary film evaporator. The solid residue (yield approx. 500 mg) was recrystallised from chloroform (yield approx. 300 mg).

The methyl derivative of the intermediate product was formed by treatment for 24 h at room temperature with an ethereal solution of diazomethane prepared in an Aldrich Mini Diazald apparatus using 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich Technical Information Bulletin No. AL-121).

Results and Discussion

The protonated, neutral and deprotonated forms of nitrazepam exhibit different UV-visible absorption spectra which form the basis of a pH-induced difference spectrophotometric assay involving the measurement of the absorbance at 282 nm of a solution at pH 1 relative to that of an equimolar solution at pH 13. The assay is selective for nitrazepam in the presence of 2-amino-5-nitrobenzophenone, the final hydrolysis product (**IV**; Scheme 1) (Davidson and Li, 1989).

Evidence for the formation of an intermediate product during hydrolysis of nitrazepam (20 μg ml^{-1}) in 0.01 M borate buffer (pH 9.18 at 25 °C) at 75 °C was first obtained by comparing the difference absorption spectra of solutions removed at different times. Although the λ_{max} at 282 nm remained constant, there was a gradual shift of the isosbestic point from 330 to 342 nm and of the λ_{min} from 372 to 380 nm. These findings were unexpected as it was assumed that only 2-amino-5-nitrobenzophenone existed in solutions of nitrazepam hydrolysed at alkaline pH (Han et al., 1977b).

Formation of an additional hydrolysis product at alkaline pH was confirmed by the observation that a yellow coloured product remained in the aqueous phase after repeated extraction with chloroform (both nitrazepam and 2-amino-5-nitrobenzophenone are readily extractable under these conditions). The polar nature of this intermediate in alkaline solution, and its absorption characteristics (Fig. 1), suggested that the yellow substance was an intermediate formed by hydrolysis at the 1,2 amide bond to yield an ionised carboxylic acid derivative.

To determine the optimum conditions for its formation, solutions of nitrazepam (30 μg ml^{-1}) in aqueous buffers of different pH (9.2–13) were heated to 75 °C. Samples were removed at accurately timed intervals of 5–10 min, cooled quickly to room temperature, and extracted with two equal volumes of chloroform. The absorbance of the aqueous phase was measured at 381 nm, the λ_{max} of the intermediate product in alkaline solution. The results in Fig. 2 show that the highest concentration of the intermediate product is achieved by heating a solution of nitrazepam for 15 min at 75 °C in 0.1 M sodium hydroxide solution (pH 13 at room temperature).

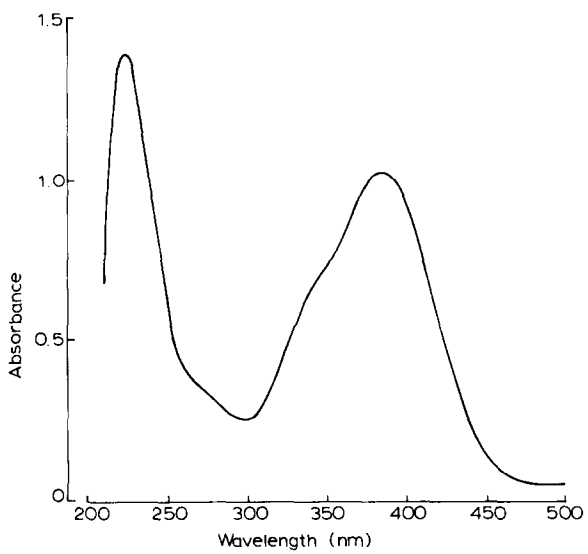


Fig. 1. The ultraviolet-visible absorption spectrum of the intermediate product (7.5×10^{-5} M) formed during the hydrolysis of nitrazepam in 0.1 M sodium hydroxide at 75 °C.

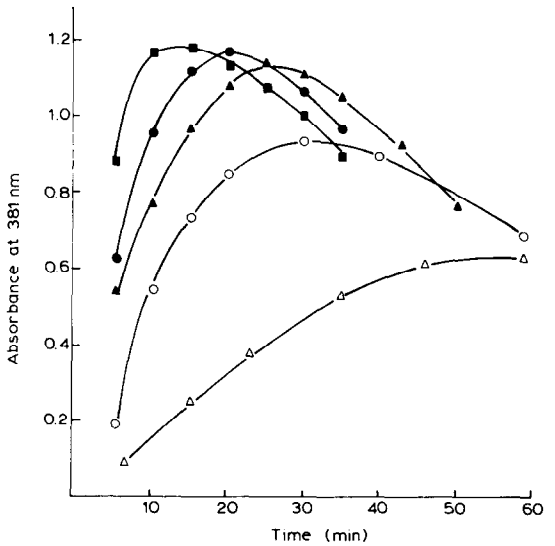


Fig. 2. The effect of pH on the formation of the intermediate product, measured as the absorbance at 381 nm after the solvent extraction of 2-amino-5-nitrobenzophenone and unhydrolysed nitrazepam from a solution of nitrazepam (30 μg/ml) heated at 75 °C. (■———■) pH 13; (●———●) pH 12; (▲———▲) pH 11; (○———○) pH 10; (△———△) pH 9.2.

The stability of the intermediate was monitored by following the changes in its absorption spectrum after adjusting the pH to various values within the range 2–13. At pH 2–4, the spectrum changed almost immediately to give a band with a λ_{max} at 362 nm and a yellow product was extractable into chloroform, that could not be re-extracted into pH 9.2 buffer. The absorption spectra and partition properties of the yellow product are identical with those of 2-amino-5-nitrobenzophenone and its identity was confirmed by thin-layer chromatography (British Pharmaco-

poeia, 1988) by comparison of the R_F with that of an authentic sample.

At pH 6, the shift of the λ_{max} from 381 to 362 nm occurred more slowly and after extraction of the aqueous solution with chloroform, both phases were observed to be yellow. In this case, a yellow product in the chloroform extract could be re-extracted into pH 9.2 buffer. These observations indicated that two products were extracted into chloroform, viz. 2-amino-5-nitrobenzophenone which is not extractable into pH 9.2 buffer, and the intermediate which could be re-extracted into pH 9.2 buffer owing to reversible dissociation.

To extract the intermediate product with the minimum of decomposition to 2-amino-5-nitrobenzophenone, a 'fast-extraction' procedure was developed that involved the simultaneous adjustment of pH and extraction with chloroform as described in Experimental.

To determine the optimum pH for extraction, the chloroform extract of aqueous solutions of the intermediate adjusted to various pH values in the range 2–7 were re-extracted after 2 min with an equal volume of borate buffer (pH 9.2). The absorbances at 351 nm (the λ_{max} of 2-amino-5-nitrobenzophenone) of the chloroform layer and at 381 nm (the λ_{max} of the intermediate product) of the borate buffer extract were measured. The pH giving the highest recovery of the intermediate in the final pH 9.2 extract and the lowest concentration of 2-amino-5-nitrobenzophenone in the final chloroform layer was found to be 4 (Fig. 3).

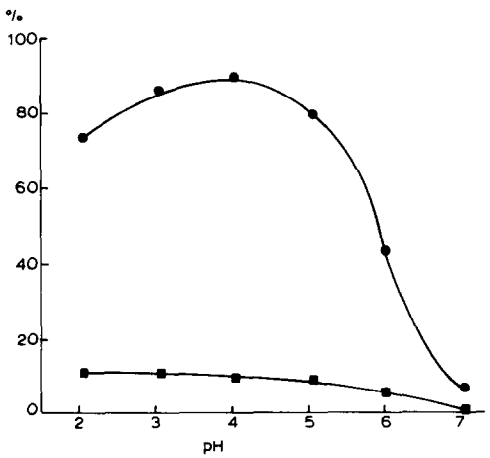


Fig. 3. The effect of pH on the % recovery (●———●) and % decomposition (■———■) of the intermediate product by the fast-extraction procedure.

Evaporation of the chloroform layer obtained by the fast-extraction technique resulted in a yellow solid which was recrystallised from chloroform. The melting point of the yellow amorphous solid was 139 °C with effervescence on liquefaction. The methyl ester, prepared by treatment with diazomethane and recrystallised from carbon tetrachloride, was a pale yellow crystalline solid that melted at 172 °C. Elemental analysis of the methyl ester gave the following results. Found: C, 61.1; H, 4.79; N, 13.55; $C_{16}H_{15}N_3O_4$ requires: C, 61.4; H, 4.83; N, 13.42. Attempted analysis of the intermediate product itself gave variable results due to concomitant thermal decarboxylation.

Structure elucidation

The identification of the recrystallised yellow solid as *N*-(α-2-amino-5-nitrophenyl)-benzylidene glycine (**II**; Scheme 1) is based on the following:

(i) The IR spectrum (recorded using a Perkin-Elmer 781 spectrometer; KBr) had characteristic bands assigned as follows:

Wave number (cm^{-1})	Vibration mode
3430	Aromatic N–H stretch
3200–2800	O–H stretch of COOH
1750	C=O stretch of COOH
1610	C=N stretch
1480	NO_2 stretch, asymmetrical
1445, 1436	Aromatic C–H
1390	O–H in-plane bend
1310	NO_2 stretch, symmetrical
1170	C–O stretch

When the disc was heated at 120 °C for 1 h, loss of the bands at 3100 (broad), 1750, 1390 and 1170 cm^{-1} occurred indicating that the substance had undergone thermal decarboxylation. This has been attributed to the stabilisation of the carbanion formed by decarboxylation, by the presence of the β-unsaturated azomethine group (Hanson, 1987).

(ii) The high-resolution mass spectrum was recorded (using an MSS double focussing spectrometer) by direct probe insertion at 130 °C

TABLE 1

Principal m/z values in the mass spectrum of the isolated product

m/z [a]	Formula	Relative abundance (%)	Fragmentation
299	$C_{15}H_{13}N_3O_4$	0.1	M^+
298	$C_{15}H_{12}N_3O_4$	2.2	M^+-H
281	$C_{15}H_{11}N_3O_3$	6.6	M^+-H_2O
255	$C_{14}H_{13}N_3O_2$	73.3	M^+-CO_2
254	$C_{14}H_{12}N_3O_2$	100.0	M^+-CO_2H
208	$C_{14}H_{12}N_2$	45.3	$M^+-CO_2H-NO_2$
178	$C_8H_8N_3O_2$	17.4	$M^+-CO_2-C_6H_5$
132	$C_8H_8N_2$	23.0	$M^+-CO_2-C_6H_5-NO_2$
118	C_8H_8N	53.6	$M^+-CO_2-C_6H_5N_2O_2$
105	C_7H_7N	8.1	$M^+-CO_2-C_6H_5-NO_2-HCN$

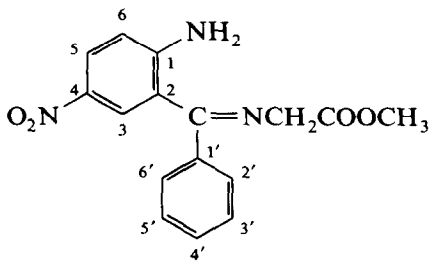
[a] Only the integral m/z values are reported. The accurate values, determined to six places of decimals, agree with the calculated mass of the proposed structure to within 20 ppm.

(ionisation energy 70 eV). The results are listed in Table 1.

Although the molecular ion at m/z 299 was very weak, its presence confirms the molecular formula proposed for the intermediate product. Although this is satisfied by either of **II** or **III** (Scheme 1) the formation of a methyl derivative (see below) clearly indicates **II**. In addition, the base peak at m/z 254 and the fragmentation pattern confirm the presence of the carboxyl group which is readily lost by ionisation.

The methyl ester gave a molecular ion at m/z 313.1064 (69.9% relative abundance, corresponding to $C_{16}H_{15}N_3O_4$; theory m/z 313.1062) and a base peak at m/z 312 resulting from the loss of a hydrogen atom. A sample of this ester gave two small peaks in the gas chromatogram, at 17.4 and 18.2 min, identified from their mass spectra as 2-amino-5-nitrobenzophenone (M^+ 242) and the decarboxylated derivative of the intermediate (M^+ 254), respectively. The major peak at 21.75 min was identified from its mass spectrum (M^+ 313 and base peak 312) as the methyl ester of the carboxylic acid formed by hydrolysis of the 1,2 bond of nitrazepam.

(iii) The ^{1}H-NMR spectrum of the methyl ester of the intermediate product was recorded in deuterated chloroform: δ. 3.70 (3H, s, OCH_3), 4.09 (2H, s, $-CH_2-$), 6.63 (1H, d, $J = 9$ Hz, H-6), 7.12 (2H, m, H-2, H-6), 7.45 (3H, m, H-3, H-4, H-5), 7.78 (1H, d, $J = 2$ Hz, H-3), 7.95 (1H, dd, $J = 9$ Hz, 2 Hz, H-5). A broad singlet at 7.1δ disappeared on shaking with D_2O, indicating the N-H group.



(iv) The UV-visible spectrum of the compound in ethanol exhibited a λ_{max} at 229 nm ($\epsilon = 1.99 \times 10^4$) and at 368 nm ($\epsilon = 1.58 \times 10^4$) consistent with a structure having a crossed conjugated system similar to that of ketimines derived from a benzophenone (Bell et al., 1964).

Conclusions

The present results amend the findings of Han et al., (1977b) who reported that the only intermediate hydrolysis product formed during acid-base hydrolysis of nitrazepam is a ring-opened compound resulting from scission of the azomethine bond.

The conditions described here permit the isolation of *N*-(α-(2-amino-5-nitrophenyl)benzylidene

glycine formed by cleavage of the 1,2 bond of nitrazepam.

Acknowledgements

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