Transformations of the Natural Cytokinin Zeatin in Aqueous Acidic Media

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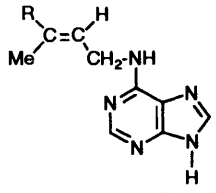
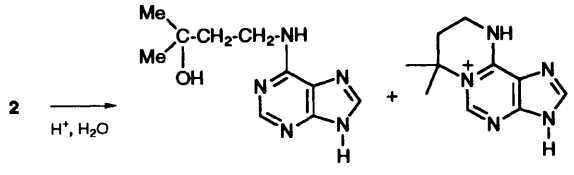
The behaviour of the naturally occurring cytokinin zeatin **1** in aqueous acidic conditions has been studied, after it was observed that this compound was relatively more stable than another related cytokinin, 6-(3-methylbut-2-enylamino)purine **2**. Zeatin **1** reacted readily in 1 mol dm^{-3} aqueous HCl at 100 °C. Three products were characterized, a diol resulting from the hydration of the double bond of the aliphatic chain, and two cyclized products, a hydroxypyrrolidine and a dihydropyrrole. A scheme is proposed to explain the formation of these products. However, zeatin was found to be stable in 0.1 mol dm^{-3} aqueous HCl at 100 °C and in 1 mol dm^{-3} aqueous HCl at 50 °C, conditions which allow the hydrolysis of zeatin riboside.

The plant hormones zeatin **1** and 6-(3-methylbut-2-enylamino)purine **2**, of the cytokinin family, are present in plants



1 R = CH_2OH
2 R = Me

not only as the free bases, but also as the 9-β-D-ribofuranosides, as the corresponding 5'-nucleotides and as various *N*-glucosides. In the case of **1** and of its riboside, *O*-glycosyl conjugates are also found.[1] It has been shown that **2** is particularly unstable in HCl solution[2,3] and the products resulting from the reactivity of the chain have been fully characterized[2] (Scheme 1). Acidic treatment of zeatin **1** with HCl (1 mol dm^{-3})



Scheme 1

at 100 °C,[4,5] gave rise to two products, one of them being a hydration product of the chain double bond, the second being unknown. The question of the stability of the allylic chain of cytokinins in acidic media has to be considered, since acidic treatments are commonly performed during their isolation and purification from plant tissues, in particular the hydrolysis of the *N*-glucosyl conjugates when a quantitative determination of the hormone pool is required.

We describe here the study of the transformation of zeatin **1** in aqueous HCl which allowed us to characterize additional products not seen in the previous studies,[4,5] and to define acidic conditions under which zeatin is stable and which can therefore be used to hydrolyse zeatin *N*-glycosides, especially zeatin riboside.

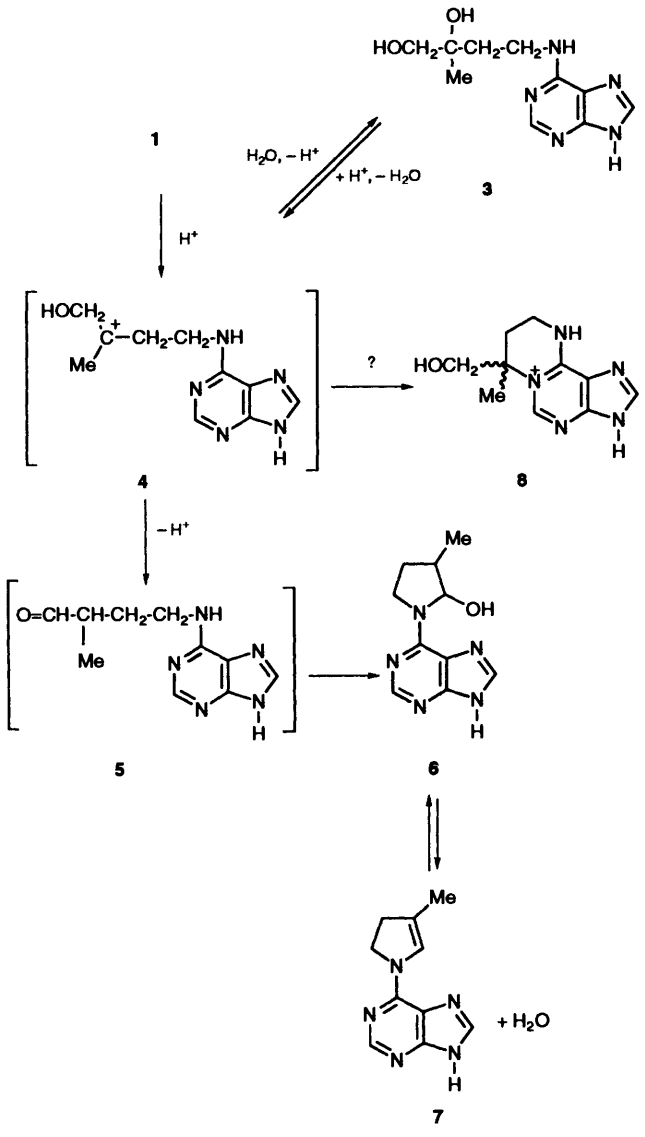
Results and Discussion

In 0.1 mol dm^{-3} aqueous HCl, at 100 °C, zeatin **1** was found to be stable, less than 1% of this compound being degraded after being heated for 1 h. A similar result was observed in 1 mol dm^{-3} aqueous HCl at 50 °C, while at 100 °C, zeatin **1** was quickly transformed. Analysis of the reaction medium by HPLC showed two main peaks, corresponding to products more and less polar than zeatin **1**, respectively, and two small very close peaks (zeatin and unknown compound **A**). Two fractions were recovered from the mixture of products by preparative reversed phase chromatography. From the first fraction, chromatography on silica gel allowed us to isolate 6-(3,4-dihydroxy-3-methylbutyl)aminopurine[6] **3**, and a small amount of impure zeatin **1**. The impurity was probably compound **A**, however, neither its isolation nor characterization proved possible. TLC analysis of the second fraction showed the presence of two products (**B** and **C**) with very close chromatographic properties. The UV spectrum of the mixture exhibited a peak at λ_{max} 276 nm in ethanol, which was shifted to 282 nm in acidic media and remained at 276 nm with a shoulder at 290 nm in basic media. The range of absorption maxima and variations with pH was close to those observed for 6-(dibenzylamino)purine.[7] This suggested that the major product in the mixture might be an N^6,N^6-disubstituted adenine. Shoulders at about 310 nm in the above three spectra revealed the presence of a secondary product bearing a more conjugated chromophore. The EI mass spectrum of the mixture exhibited a tiny molecular peak at 219 (the same mass as zeatin), with a base peak at 201 (M − H_2O).

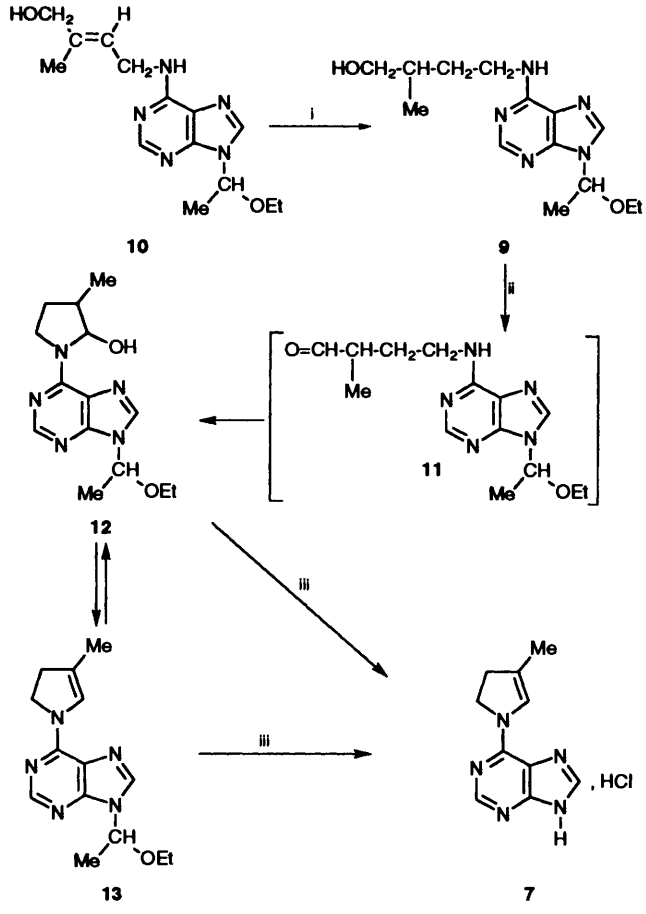
A kinetic study was undertaken on a 10^{-3} mol dm^{-3} solution of zeatin in aqueous 1 mol dm^{-3} HCl heated at 100 °C. The reaction followed first order kinetics ($k = 10^{-4}$ s^{-1}, $t_{1/2} = 2$ h). The amount of the diol **3** first increased before decreasing, while the amounts of the other products increased steadily.

The reaction of the diol **3** in 1 mol dm^{-3} aqueous HCl was monitored by HPLC. The peaks observed were very similar to those observed for zeatin in the same conditions, but the reaction was slower ($t_{1/2} = 10$ h), and also followed first order kinetics ($k = 1.9 \times 10^{-5}$ s^{-1}). Prolonged heating caused the appearance of polar products.

Scheme 2 tentatively explains the reaction of zeatin **1**. Both zeatin **1** and the diol **3** are expected to give the carbocation **4**. This undergoes a prototropic rearrangement to provide the aldehyde **5** which, as a γ-amino aldehyde, quickly cyclizes,[8] giving the hydroxypyrrolidine **6** (product **B**), an N^6,N^6-disubstituted adenine. Loss of H_2O from this species produces the dihydropyrrole **7** (compound **C**), which was detected as the long-wavelength absorbing product in the UV spectra of the mixture of compounds **B** and **C**. The cyclization of the carbocation **4** to the tricyclic compound **8**, which was expected by analogy with the reaction of the 6-(3-methylbut-2-enylamino)-purine **2** (Scheme 1), was not proven. However, this compound



Scheme 2



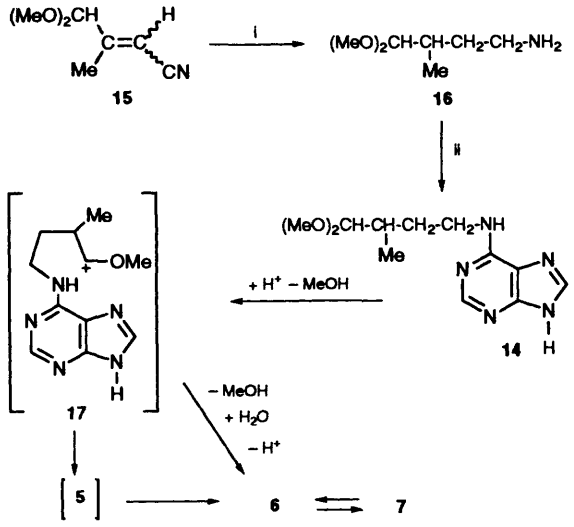
Scheme 3 *Reagents and conditions:* i, H_2, Raney Ni; ii, $BaMnO_4$, CH_2Cl_2, 50 °C; iii, HCl, EtOH–H_2O

8 might be the unidentified product **A**. In any case, this cyclization would appear to be a minor process.

In order to prove that the aldehyde **5** is an intermediate for obtaining the cyclized compounds **6** and **7**, we attempted to prepare it independently. The first method was by $BaMnO_4$[9] oxidation of the 9-protected dihydrozeatin **9** (Scheme 3). This compound was prepared by catalytic reduction of the corresponding 9-substituted zeatin[10] **10**. Its complete oxidation by $BaMnO_4$ in refluxing dichloromethane required many weeks, and the expected aldehyde **11** was not observed, in spite of the neutral reaction conditions. Instead, we obtained the hydroxypyrrolidine **12**, which was characterized by its IR and ^1H NMR spectra. Its UV spectra were in accordance with those expected for an N^6,N^6-disubstituted purine,[7] but a shoulder around 310 nm revealed the presence of the dihydropyrrole **13**. The EI mass spectrum of **12** gave a very small molecular peak (*m/z* 291), the following peak being 273 (M − H_2O, M^+ of **13**). Treatment of the crude compound **12** with a 0.5 mol dm^{-3} aqueous HCl at room temperature allowed elimination of the 9-protecting group. The residue obtained after evaporation of the solvent gave, by recrystallization from ethanol, the pure dihydropyrrole **7**, as the hydrochloride. The mother liquors, after evaporation, left an impure sample of the hydroxypyrrolidine **6**. The EI mass spectrum of **7** exhibited the expected molecular ion at *m/z* 201 as the base peak, and a pseudomolecular ion (M − H) at *m/z* 200 (60% of base peak) which was consistent with the presence of an enamine function.[11] The ^1H NMR spectrum was in accordance with the proposed structure, while the UV spectra at various pH gave absorption maxima between 310 and 320 nm, corresponding to the shoulders observed in the above-mentioned spectra of the hydroxypyrrolidine **6**. When the UV spectrum of the hydrochloride of **7** was recorded in water, the peaks of absorption of the hydroxypyrrolidine **6** slowly appeared, thus revealing that the formation of compound **7** from **6** is reversible.

We further attempted to characterize the aldehyde **5** by exploring the hydrolysis of the acetal **14** (Scheme 4). Thus, compound **14** was prepared from the ethylenic nitrile[12] **15**, which, after catalytic reduction, gave rise to the primary amine **16**. This latter compound reacted with 6-chloropurine to give the expected compound **14**. Smooth hydrolysis conditions were tested for this compound. On wet silica gel,[13] no reaction occurred. Addition of oxalic acid[13] allowed the reaction to proceed, and HPLC controls showed the formation of the hydroxypyrrolidine **6** as the major product, and of a small amount of the dihydropyrrole **7**. The formation of the expected intermediate aldehyde **5** was not proven. In this reaction, the compounds **6** and **7** could be alternatively obtained *via* cyclization of the cationic intermediate **17** followed by hydrolysis. Isolation of the hydrochloride of the dihydropyrrole **7** and of the hydroxypyrrolidine **6** were finally achieved from the acetal **14** by hydrolysis in dilute aqueous HCl.

In conclusion, the N^6-aliphatic chain of zeatin **1** is relatively stable under moderately acidic conditions (0.1 mol dm^{-3}) at



Scheme 4 *Reagents and conditions:* i, H_2, Raney Ni; ii, 6-chloropurine

100 °C. These conditions are sufficient for regenerating zeatin **1** from zeatin riboside and most of its glycosides. Acid hydrolysis, under these conditions, of radiolabelled ribosylzeatin[14] is quantitative (data not shown) and, therefore, constitutes a new access to radiolabelled zeatin. If conditions have to be forced, for example for the hydrolysis of some unreactive *N*-glycosides, extensive transformation of zeatin **1** to at least four products may be expected, three of which have been identified in this study.

Experimental

M.p.s were determined on a Kofler block and are uncorrected. IR spectra were recorded on a Beckman Acculab II spectrophotometer from Nujol mulls for solids, and thin films for liquids. ^{1}H NMR spectra were recorded on a Varian EM 360 spectrometer (60 MHz), and the chemical shifts were measured in δ from Me_4Si; J values are given in Hz. UV spectra were obtained on a Kontron-Uvikon 810 spectrophotometer from solutions of the products studied in 95% ethanol. Water (5 cm^3) aqueous HCl (2 mol dm^{-3}; 5 cm^3) or aqueous NaOH (2 mol dm^{-3}; 5 cm^3) were added to ethanol (95 cm^3) to obtain the samples in neutral, acidic or basic solutions, respectively. EI MS were recorded on a Varian MAT 112 spectrometer. Elemental analyses were obtained at the Service Central d'Analyse du CNRS, Lyon (France). HPLC analyses were performed on Lichrosorb RP 18 columns using methanol–water mixtures containing 2% acetic acid and sodium heptanesulfonate (0.005 mol dm^{-3}); the wavelength of detection was 254 nm.

Hydrolysis of Zeatin [(E)-2-*Methyl*-4-(*purin*-6-*ylamino*)*but*-2-*en*-1-*ol*] **1**. *Preparative Study.*—Zeatin **1** (275 mg, 1.25 mmol) was heated at reflux in aqueous HCl (1 mol dm^{-3}; 100 cm^3) for 4 h. HPLC analysis of the mixture after this time revealed the presence of two major components eluted in the first and last positions, whose peaks were on either side of two nearby small peaks, corresponding to the remaining zeatin **1** and an unknown compound **A**. The solvent was evaporated and the residue chromatographed through a Lichroprep RP 8 column (310 × 25 mm) with MeOH–H_2O (35:65) as eluent. Two fractions were collected. TLC on silica gel ($CHCl_3$–MeOH, 80:20) of the first fraction gave two spots (R_f 0.23 and 0.38), the second corresponding to zeatin **1**, and possibly containing compound **A**. Evaporation of the solvent from this first fraction gave a residue which was chromatographed on a silica gel column with $CHCl_3$–MeOH (80:20) as eluent. The only product isolated in a pure state was 2-methyl-4-(purin-6-ylamino)butane-1,2-diol **3** (60 mg, 20%). The isolated sample exhibited spectra identical with those from a sample of **3** prepared according to the method of Leonard *et al.*[6a] δ_H[$(CD_3)_2SO$] 1.08 (3 H, s, CH_3), 1.7 (2 H, t, CCH_2C), 3.23 (2 H, s, CH_2OH), 3.58 (2 H, t, CH_2NH), 7.4 (1 H, t, NH) and 8.06 and 8.2 (2 × 1 H, 2 s, purine 2-H and 8-H); m/z 237 (M^+, 9%), 206 (28.5), 162 (50) and 148 (100). The second fraction from the Lichroprep column, analysed by TLC on silica gel ($CHCl_3$–MeOH, 90:10) showed two very close spots ($R_f \approx 0.43$). The mixture of compounds (unknowns **B** and **C**) was not purified and gave the following spectra: λ_{max}(EtOH 95%)/nm 276 and 310sh; (EtOH 95%, 0.1 mol dm^{-3} HCl) 282 and 310sh; (EtOH 95%, 0.1 mol dm^{-3} NaOH) 276, 290sh and 310sh; m/z 219 (M^+, 0.77%), 201 (100), 200 (54), 186 (45), 185 (26), 174 (12), 160 (11) and 119 (9).

Kinetic Studies.—Zeatin **1** (11 mg, 5 × 10^{-2} mmol) was dissolved in aqueous HCl (1 mol dm^{-3}; 50 cm^3). The solution was heated at 100 °C in an oil bath. The amounts of remaining zeatin **1** and of its hydration product **3** were determined periodically by HPLC. The other products detected were not quantified. The same experiment was performed at 50 °C in aqueous HCl (1 mol dm^{-3}) and at 100 °C in aqueous HCl (0.1 mol dm^{-3}), without significant transformation of zeatin **1** (< 2% in 24 h and < 1% in 1 h respectively).

Hydrolysis of 2-*Methyl*-4-(*purin*-6-*ylamino*)*butane*-1,2-*diol* **3**.—A solution of the diol **3**[6] (10^{-4} mol dm^{-3}; 50 cm^3) in aqueous HCl (1 mol dm^{-3}) was heated at 100 °C in an oil bath. The concentration of **3** was determined periodically by HPLC. The products detected were the same as in zeatin **1** hydrolysis, except that zeatin **1** was not present.

4-[9-(1-*Ethoxyethyl*)*purin*-6-*ylamino*]-2-*methylbutanol* **9**.—9-(1-Ethoxyethyl)zeatin[10] **10** (1 g, 3.4 mmol) was dissolved in absolute ethanol (100 cm^3) and Raney nickel (1 g) was added. The suspension was shaken in a hydrogenation apparatus under hydrogen at atmospheric pressure for 24 h. After filtration through Celite, the solvent was evaporated, and the expected compound **9** was obtained as a viscous oil (900 mg, 90%), which was not purified in order to avoid deprotection of N-9, $\delta_H(CDCl_3)$ 0.96 (3 H, d, J 7, CH_3CHCH_2OH), 1.18 (3 H, t, J 9, CH_3CH_2), 1.74 (6 H, m, CH_3CHO and $CHCH_2CH_2$), 3.2–4 (6 H, m, CH_2OH, CH_2O and CH_2N), 4.95 (1 H, s, OH), 5.96 (1 H, q, J 8, CH_3CHO), 7.1 (1 H, t, J 6, NH) and 8.1 and 8.4 (2 × 1 H, 2 s, purine 2-H and 8-H).

Oxidation of 4-[9-(1-*Ethoxyethyl*)*purin*-6-*ylamino*]-2-*methylbutanol* **9**.—Compound **9** (600 mg, 2 mmol) was dissolved in dichloromethane (100 cm^3). Barium manganate (30 g, 117 mmol) was added, and the suspension was stirred and heated at 50 °C for 1 week. Additional $BaMnO_4$ (20 g, 78 mmol) and CH_2Cl_2 (20 cm^3) were added and the reaction allowed to continue for a further 18 days. CH_2Cl_2 (100 cm^3) was added and then the mixture was filtered through Celite. The solvent was evaporated, and the residue chromatographed through a silica gel column with CH_2Cl_2–MeOH (95:5) as eluent. Some starting material **9** was recovered along with impure 9-(1-ethoxyethyl)-6-(2-hydroxy-3-methylpyrrolidin-1-yl)purine **12** (240 mg, 45%), as an oil, $\delta_H(CDCl_3)$ 1.2 (3 H, t, CH_3CH_2O), 1.28 (3 H, d, CH_3CHCH_2N), 1.75 (3 H, d, CH_3CHO), 2.2 (2 H, m, CH_2CH_2N), 2.5 (1 H, m, CH_2CHCH_3), 3.4 (2 H, m, CH_2O), 3.85 (2 H, t, CH_2CH_2N), 5.5 (1 H, s, $HOCHN$), 6.01 (2 H, m, CHO and $HOCHN$) and 8.0 and 8.4 (2 × 1 H, 2 s, purine 2-H and 8-H); ν_{max}(neat)/cm^{-1} 3600–3200 (OH), 1600 and 1590 (C=C, C=N); m/z 291 (M^+, 0.5%), 273 (22), 228 (5), 201 (100), 200 (48), 186 (58), 185 (26) and 174 (16); λ_{max}(EtOH 95%)/nm

278, 310sh. The crude product **12** (200 mg, 0.68 mmol) was dissolved in ethanol (10 cm^3) and aqueous HCl (1 mol dm^{-3}; 10 cm^3) was added to it. The mixture was left for 1 h at room temperature and the solution was concentrated and then cooled. The precipitate obtained was filtered and recrystallized in ethanol after charcoal treatment giving the hydrochloride of 6-(3-methyl-1*H*-2,3-dihydropyrrol-1-yl)purine **7** (90 mg, 56%), m.p. 228 °C (Found: C, 50.3; H, 5.1; N, 29.5. $C_{10}H_{11}N_5$, HCl requires C, 50.5; H, 5.1; N, 29.5%); δ_H[$(CD_3)_2SO$] 1.87 (3 H, d, *J* 1.25, CH_3), 2.50 (2 H, t, *J* 8.3, CH_2C=), 4.22 (2 H, m, CH_2N), 7.72 (1 H, m, HC=dihydropyrrole) and 8.28 and 8.35 (2 × 1 H, 2 s, purine 2-H and 8-H); *m/z* 201 (M^+ − HCl, 100%), 200 (53), 186 (65), 185 (28), 174 (17), 160 (14), 159 (12.5), 135 (10), 120 (21) and 119 (33); λ_{max}(EtOH 95%)/nm 312; (EtOH 95%, 0.1 mol dm^{-3} HCl) 318; (EtOH 95%, 0.1 mol dm^{-3} NaOH) 312; (H_2O) 312 and 280.

4,4-*Dimethoxy*-3-*methylbutylamine* **16**.—4,4-Dimethoxy-3-methylbut-2-enenitrile[12] **15** (8.5 g, 0.06 mol) was dissolved in methanol (50 cm^3), and Raney nickel (1 g) was added to it. The suspension was shaken in a hydrogenation apparatus under hydrogen at atmospheric pressure for 4 h. The mixture was filtered through Celite and then the solvent evaporated. The oily residue was distilled and the first fraction gave **16**, a hygroscopic product which was not further purified (4.5 g, 51%), δ_H($CDCl_3$) 0.9 (3 H, d, *J* 7, CH_3CH), 1.17 (2 H, s, NH_2), 1.3–2.3 (3 H, m, $CHCH_2$), 2.75 (2 H, t, *J* 7, CH_2N), 3.99 (6 H, s, 2 × CH_3O) and 4.06 (1 H, d, *J* 7, CH); ν_{max}(neat)/cm^{-1} 3360–3295 (NH), 1680 and 1560 (NH).

6-(4,4-*Dimethoxy*-3-*methylbutylamino*)*purine* **14**.—The amine **16** (2.5 g, 17 mmol) and 6-chloropurine (1 g, 6.8 mmol) were dissolved in absolute ethanol (20 cm^3) and the solution was heated overnight at 80 °C, with stirring. After cooling to room temperature, the solution was concentrated, and then cooled in an ice-bath. The precipitate which appeared was filtered off and crystallized from ethanol, with charcoal treatment of the hot solution, to yield the title compound **14** (960 mg, 56%), m.p. 168 °C (Found: C, 54.0; H, 7.2; N, 26.4. $C_{12}H_{19}N_5O_2$ requires C, 54.3; H, 7.2; N, 26.4%); δ_H[$(CD_3)_2SO$] 0.89 (3 H, d, *J* 6.5, CH_3CH), 1.9 (3 H, m, $CHCH_2$), 3.27 (6 H, s, 2 × CH_3O), 3.53 (2 H, m, CH_2N), 4.09 (1 H, d, *J* 6, OCH), 7.57 (1 H, t, *J* 6, NH) and 8.14 and 8.24 (2 × 1 H, 2 s, purine 2-H and 8-H); *m/z* 265 (M^+, 12.5%), 250 (8), 234 (12), 218 (14), 203 (15), 202 (38), 201 (40), 200 (15), 186 (19), 185 (7), 161 (60), 160 (51), 148 (67) and 75 (100).

Hydrolysis of 6-(4,4-*Dimethoxy*-3-*methylbutylamino*)*purine* **14**.—The compound **14** (500 mg, 1.8 mmol) was dissolved in a mixture of methanol (50 cm^3) and aqueous HCl (0.1 mol dm^{-3}; 50 cm^3). The solution was left at room temperature for 24 h. The solvents were evaporated under reduced pressure and the residue was dissolved in hot butanol. On cooling, a precipitate appeared, which was filtered off and dried *in vacuo*. The hydrochloride of **7** was obtained (160 mg, 45%). The filtrate was evaporated to dryness under reduced pressure, giving impure hydroxypyrrolidine **6**, which was characterized by its ^1H NMR and UV spectra.

References

1 K. Koshimizu and H. Iwamura, *The Chemistry of Plant Hormones*, ed. N. Takahashi, CRC Press, Boca Raton, 1986, pp. 153–195.
2 D. M. G. Martin and C. B. Reese, *J. Chem. Soc. C*, 1968, 1731.
3 (*a*) K. Biemann, S. Tsunakawa, J. Sonnenbichler, H. Feldmann, D. Dütting and H. G. Zachau, *Angew. Chem., Int. Ed. Engl.*, 1966, **5**, 590; (*b*) R. H. Hall, M. J. Robins, L. Stasiuk and R. Thedford, *J. Am. Chem. Soc.*, 1966, **88**, 2614.
4 D. S. Letham, J. S. Shannon and I. R. C. McDonald, *Tetrahedron*, 1967, **23**, 479.
5 J. Van Staden and S. E. Drewes, *J. Plant Physiol.*, 1984, **115**, 137.
6 (*a*) N. J. Leonard, S. M. Hecht, F. Skoog and R. Y. Schmitz, *Isr. J. Chem.*, 1968, **6**, 539; (*b*) D. S. Letham, *Phytochemistry*, 1973, **12**, 2245.
7 N. J. Leonard, K. L. Carraway and J. P. Helgeson, *J. Heterocycl. Chem.*, 1965, **2**, 291.
8 O. T. Cerwinka, *Enamines, Syntheses, Structures and Reactions*, ed. A. G. Cook, Dekker, New York, 1969, pp. 270–272.
9 H. Firouzabadi and E. Ghaderi, *Tetrahedron Lett.*, 1978, 839.
10 M. Haidoune, R. Mornet and M. Laloue, *Tetrahedron Lett.*, 1990, **31**, 1419.
11 H. J. Jakobsen, S. O. Lawesson, J. T. B. Marshall, G. Schroll and D. H. Williams, *J. Chem. Soc. B*, 1966, 940.
12 S. C. Chen, *Experientia*, 1981, **37**, 543.
13 F. Huet, A. Lechevallier, M. Pellet and J. M. Conia, *Synthesis*, 1978, 63.
14 M. Laloue and J. E. Fox, *Phytochemistry*, 1987, **26**, 987.

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