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THE SYNTHESIS AND CYTOKININ ACTIVITIES OF *N*-(PURIN-6-YL)AMINO ACIDS*

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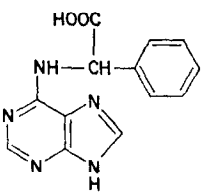
Abstract—A series of *N*-(purin-6-yl)amino acids have been synthesized. The cytokinin activities of these purines have been determined in the excised radish cotyledon bioassay and compared with the activities of related compounds lacking a carboxyl group. Cytokinin activity was greatly reduced by the presence of an unesterified carboxylic acid group in the substituent of a 6-(substituted amino)purine. The purinylamino acid which showed greatest activity was *N*-(purin-6-yl)-α-phenylglycine; the activity of this compound did not appear to be the consequence of conversion to 6-benzylaminopurine. The recently isolated amino acid 2-amino-5-methylhex-4-enoic acid, which was obtained as a synthetic intermediate, inhibited growth in several bioassays.

INTRODUCTION

ALTHOUGH many 6-(substituted amino)purines have been tested for cytokinin activity,[1–5] little is known of the activity of *N*-(purin-6-yl)amino acids. Unidentified compounds of this type with weak cytokinin activity have been purified from plant tissue.[6] *N*-(Purin-6-yl)-aspartic acid has been reported to be inactive in the tobacco pith tissue culture bioassay for cytokinins[1] and the suggestion has been made that 6-*N*-substituted adenines will be inactive if the substituent carries a polar group.[1] However, *N*-(purin-6-yl)-α-phenylglycine (I), which



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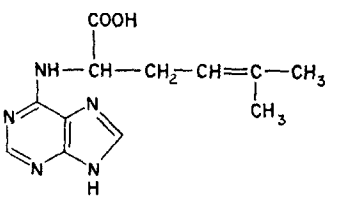

(IX)

* Part X in the series "Regulators of Cell Division in Plant Tissues".

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[4] K. ROTHWELL and S. T. C. WRIGHT, *Proc. R. Soc.* (B). **167**, 202 (1967).
[5] H. YOUNG and D. S. LETHAM, *Phytochem.* **8**, 1199 (1969).
[6] D. S. LETHAM, unpublished results.

does not appear to have been tested in the conventional cytokinin bioassays, has been reported to evoke in intact plants certain growth responses characteristic of cytokinins. For example, this purine breaks bud dormancy very effectively,[7] induces parthenocarpic set of emasculated apple flowers,[8] modifies apple fruit shape[9] and releases lateral buds from apical dominance.[10]

In the investigation now reported, the cytokinin activities of a series of *N*-(purin-6-yl)-amino acids have been determined and related to the activities of analogues lacking a arboxyl group.

RESULTS AND DISCUSSION

The activities of various *N*-(purin-6-yl)amino acids in the radish cotyledon cytokinin assay are detailed in Table 1. The activities of related compounds which contain either a

TABLE 1. MEAN INCREMENTS IN RADISH COTYLEDON WEIGHT INDUCED BY VARIOUS *N*-(PURIN-6-YL)AMINO ACIDS AND RELATED COMPOUNDS

Compounds	Concentrations and increments induced in cotyledon wt. (mg)	
	50 μM	5 μM
N-(*Purin-6-yl)amino acids*		
N-(Purin-6-yl)-DL-α-phenylglycine (I)	6·0	3·2
N-(Purin-6-yl)-L-leucine (II)	2·1	0·4
N-(Purin-6-yl)-L-valine (III)	1·8	0·2
DL-2-(Purin-6-ylamino)-5-methylhex-4-enoic acid (IV)	3·3	1·1
4-(Purin-6-ylamino)butanoic acid (V)	1·4	0·5
5-(Purin-6-ylamino)pentanoic acid (VI)	2·2	0·9
6-(Purin-6-ylamino)hexanoic acid (VII)	1·5	0·6
Methyl esters of N-(*purin-6-yl)amino acids*		
N-(Purin-6-yl)-L-leucine methyl ester (VIII)	12·1	—
N-(Purin-6-yl)-L-valine methyl ester (IX)	8·3	—
Methyl 5-(purin-6-ylamino)pentanoate (X)	5·2	—
Related compounds		
L-6-(2-Hydroxy-1-phenylethylamino)purine (XI)	8·5	4·9
6-Benzylaminopurine (XII)	14·1	12·2
L-6-(1-Hydroxymethyl-3-methylbutylamino)-purine (XIII)	10·0	8·2
6-(3-Methylbutylamino)purine (XIV)	11·2	8·0
6-(3-Methylbut-2-enylamino)purine (XV)	16·3	14·1
6-(5-Hydroxypentylamino)purine (XVI)	5·4	2·3
6-(6-Hydroxyhexylamino)purine (XVII)	6·5	1·9

hydroxymethyl group or a hydrogen atom instead of a carboxyl group are listed for comparison. The activity of each (purin-6-yl)amino acid was less than that of the corresponding hydroxymethyl analogue. This difference in activity was observed both when the carboxyl

[7] J. BENES, K. VERES, L. CHVOJKA and A. FRIEDRICH, *Nature* **206**, 830 (1965).
[8] M. W. WILLIAMS and D. S. LETHAM, *Hort. Science* **4**, 215 (1969).
[9] M. W. WILLIAMS and E. A. STAHLY, *J. Am. Soc. Hort. Sci.* **94**, 17 (1969).
[10] M. W. WILLIAMS and E. A. STAHLY, *Hort. Sci.* **3**, 68 (1968).

group was α to the exocyclic nitrogen (cf. compounds I and II with XI and XIII respectively) and when the carboxyl group was at the terminal position in the side chain (cf. compounds VI and VII with XVI and XVII respectively). Compounds II and XIII exhibited the greatest difference in activity. Compounds I and II were much less active than the corresponding purines (XII and XIV respectively) with a hydrogen atom in place of a carboxyl group. 6-(3-Methylbut-2-enylamino)purine, a naturally occurring cytokinin, was the most active compound listed in Table 1 and was also highly active in other bioassays. It is noteworthy that the closely related purinylamino acid, 2-(purin-6-ylamino)-5-methylhex-4-enoic acid (IV), exhibited only very weak activity. The three methyl esters, VIII, IX and X, were prepared by esterification of the corresponding purinylamino acids with diazomethane. *N*-Methylation, previously reported[11] to occur when some purines are reacted with diazomethane, did not occur or was insignificant. Esterification of the carboxylic acid group markedly enhanced activity (Table 1).

Because I was the most effective purinylamino acid tested in the radish cotyledon assay, the activity of this cytokinin was assessed in other assays. In promoting *Spirodela* growth in darkness, I and XII were compared at 0·05 μM, 0·5 μM and 5 μM. While XII markedly promoted growth at all three concentrations, I exhibited very slight activity at 5 μM and was inactive at lower concentrations. As a senescence retardant for Chinese cabbage leaf disks,[12] I was also much less effective than XII. However, in promoting expansion of disks of immature Chinese cabbage leaves,[13] the two compounds were approximately equally effective.

The results presented in this communication indicate that cytokinin actively is usually very greatly depressed by the presence of an unesterified carboxylic acid group in the substituent of a 6-(substituted amino)purine. This observation raises the possibility that enzymic oxidation *in vivo* of the hydroxymethyl group of naturally occurring cytokinins such as zeatin to yield purinylamino acids could be a mechanism of cytokinin inactivation.

The activity of I could be a consequence of decarboxylation to yield the highly active cytokinin XII. In an attempt to ascertain if this was likely, the metabolism of I by Chinese cabbage leaf disks was briefly investigated. Disks excised from young Chinese cabbage leaves were floated on a solution of tritiated I (231 mc/mM; 100 mg/l.) for 3 days, then washed with water and finally extracted with 95% ethanol. The ethanol was evaporated and an aqueous solution of the residue was fractionated by extraction with ethyl acetate to yield three fractions—a fraction extracted by ethyl acetate at pH 7·5 (A), a fraction then extracted by ethyl acetate at pH 3 (B), and the aqueous solution after extraction (C). The ratio dis/min in A : dis/min in B : dis/min in C was 1 : 16·0 : 6·5. Chromatograms of fractions A and B were subjected to autoradiography. XII and undegraded I would occur in fractions A and B respectively. The radioactivity in fraction B was due almost entirely to undegraded I; no XII was detected in fraction A. Hence the suggestion that the activity of I was a consequence of conversion to XII was not supported by this investigation. The radioactivity in the aqueous phase after extraction with ethyl acetate at pH 3 and pH 7·5 indicated considerable formation of highly polar metabolites from I. The identity of these products was not investigated.

During the present investigation, 2-amino-5-methylhex-4-enoic acid was obtained as a synthetic intermediate by hydrolysis of ethyl 2-acetamido-2-cyano-5-methylhex-4-enoate.

[11] R. K. Robins, in *Heterocyclic Compounds* (edited by R. C. Elderfield), Vol. 8, pp. 162–442, John Wiley, New York (1967).
[12] D. S. Letham, *Planta* **74**, 228 (1967).
[13] M. V. Berridge and R. K. Ralph, *Biochim. Biophys. Acta* **182**, 266 (1969).

This unsaturated amino acid, previously prepared by a less convenient method,[14] is now known to occur in plants[15] and was therefore tested for growth regulatory activity. The growth of excised immature radish cotyledons (medium contained 5 μM 6-benzylaminopurine), carrot secondary phloem explants (medium contained 0·5 μM 6-benzylaminopurine) and sections of wheat coleoptiles (medium contained 5·7 μM indol-3-ylacetic acid) was inhibited approximately 50% by 0·7, $1{\cdot}4 \times 10^{-2}$ and 1·4 mM concentrations respectively of DL-2-amino-5-methylhex-4-enoic acid. At these concentrations, DL-leucine, a closely related amino acid, did not influence growth. In the radish cotyledon growth assay, 2-amino-5-methylhex-4-enoic acid was much more inhibitory than coumarin. The above observations indicate that this amino acid, and possibly related unsaturated amino acids, could be of value as growth retardants.

EXPERIMENTAL

Bioassay Methods

The excised radish cotyledon assay was performed as previously described.[16]

In the *Spirodela* assay, three-frond units of *Spirodela oligorrhiza* were cultured in darkness in the presence and absence of cytokinin using the medium previously employed[12] modified by the addition of calcium orthophosphate (0·25 g/100 ml) as a solid buffer. The media and $Ca_3(PO_4)_2$ were autoclaved separately and mixed aseptically. The addition of $Ca_3(PO_4)_2$, but not $CaCO_3$, to the medium enhanced cytokinin-induced increments in culture weight and frond area but did not appreciably affect frond number. The addition of $Ca_3(PO_4)_2$ maintained the pH of the medium at 5·6; in the absence of this additive, the pH dropped to approximately 3·6 at the end of the 3-week culture period.

Synthesis of N-(*Purin-6-yl*)*amino Acids and Related Compounds*

The following compounds were prepared by published methods: *N*-(purin-6-yl)-DL-α-phenylglycine,[17] *N*-(purin-6-yl)-L-leucine,[18] *N*-(purin-6-yl)-L-valine,[18] 6-(3-methylbut-2-enylamino)purine,[12] 6-(5-hydroxypentylamino)purine,[19] 6-(6-hydroxyhexylamino)purine.[19] The following two compounds were synthesized by Koshimizu *et al.*[20] and kindly supplied by Dr. K. Koshimizu of Kyoto University: XI and XIII.

N-(*Purin-6-yl*)-DL-*α-phenylglycine-*$^3H(G)$. The sodium salt of *N*-(purin-6-yl)-DL-α-phenylglycine (0·40 g) and Pt catalyst (200 mg) were stirred with tritiated water (3 ml) containing about 100 C for *ca.* 20 hr under reflux. Solvent and labile tritium were then removed. The crude product (0·36 g) was dissolved in water and the pH of the solution adjusted to 3·4. The precipitate which formed was washed with water and then dissolved in water by addition of the minimum volume of aq. NaOH. The resulting solution (pH 6) was concentrated and subjected to preparative TLC on silica gel (Merck PF_{254}) in *n*-BuOH–H_2O. The purified compound was eluted with 70% methanol and crystallized from water to yield *N*-(purin-6-yl)-DL-α-phenylglycine-^{3}H(G) (130 mg), 231 mC/mM.

2-*Amino-5-methylhex-4-enoic acid.* Ethyl cyanoacetamido acetate (8·5 g) was added to a stirred solution of Na (1·27 g) in dry ethanol (75 ml) under N_2 at room temp. After 1 hr, 3-methylbut-2-enyl bromide (9·9 g) was slowly added and the mixture was then heated to 60° for 3 hr. The residue obtained by removal of ethanol under vacuum was partitioned between water (50 ml) and Et_2O (200 ml). The ether layer was washed with water (50 ml), dried ($MgSO_4$) and evaporated to yield crude *ethyl 2-acetamido-2-cyano-5-methylhex-4-enoate* as a white solid (11·7 g). TLC (sliica gel; separated components located with iodine vapour) revealed the presence of only traces of impurity. A small portion was recrystallized from hexane–CH_2Cl_2 yielding crystals, m.p. 118–119°.* (Found: C, 60·5; H, 7·8; N, 11·7. $C_{12}H_{18}N_2O_3$ required: C, 60·5; H, 7·6; N, 11·8%); ν_{max} 1752, 1655, 1550 cm^{-1}. NMR ($CDCl_3$) δ 1·32 (3H, triplet, J = 7 c/s, —CH_2—$C\underline{H}_3$), 1·68 (3H, singlet, C=C—$C\underline{H}_3$), 1·77 (3H, singlet, C=C—$C\underline{H}_3$), 2·05 (3H, singlet, CO—$C\underline{H}_3$), 2·85 and 5·16 (2H heptuplet and 1H triplet respectively; ABX system; J_{AB} = 14, J_{AX} = 7, J_{BX} = 7; C=C$\underline{H}$—$C\underline{H}_2$—C), 4·30 (2H, quartet, J = 7 c/s, O—$C\underline{H}_2$—CH_3), 6·40 (1H, broad singlet, —N$\underline{H}$—)ppm.

* All m.ps are corrected.

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[18] D. N. WARD, J. WADE, E. F. WALBORG and T. S. OSDENE, *J. Org. Chem.* **26**, 5000 (1961).
[19] D. S. LETHAM, R. E. MITCHELL, T. CEBALO and D. W. STANTON, *Australian J. Chem.* **22**, 205 (1969).
[20] K. KOSHIMIZU, A. KOBAYASHI, T. FUJITA and T. MITSUI, *Phytochem.* **7**, 1989 (1968).

Crude ethyl 2-acetamido-2-cyano-5-methylhex-4-enoate (11·7 g) was heated under reflux for 25 hr with water (470 ml) containing $Ba(OH)_2$ (31·8 g). The resulting solution was cooled, saturated with CO_2 to precipitate Ba, and filtered. The filtrate was adjusted to pH 5 with 2N H_2SO_4, filtered after several hours, concentrated to 50 ml and left at 2° to yield DL-2-amino-5-methylhex-4-enoic acid (2·0 g), m.p. 215–216° (dec) (lit.[14] 220°); ν_{max} 1592, 1490 cm^{-1}. NMR (D_2O) δ 1·68 (3H, singlet, C=C—C$\underline{H}_3$), 1·77 (3H, singlet, C=C—C$\underline{H}_3$), 2·63 (2H, apparent triplet, apparent J = 7 c/s, C=CH—C$\underline{H}_2$—CH(NH_2)), 3·78 (1H, triplet, J = 5·8 c/s, —C$\underline{H}$(NH_2)—), 5·13 (1H, triplet broadened by allylic coupling, J = 7·5 c/s, C=C$\underline{H}$—CH_2) ppm.

Reduction of the pH of the above mother liquor to 4 yielded crystals of DL-2-*acetamido*-5-*methylhex*-4-*enoic acid* (2·16 g), m.p. 109–110°. (Found: C, 58·7; H, 8·2; N, 7·6; O, 25·6. $C_9H_{15}NO_3$ required: C, 58·4; H, 8·2; N, 7·7; O 25·3%); ν_{max} 1720, 1600, 1545 cm^{-1}. NMR ($CDCl_3$) δ 1·73 (3H, singlet, C=C—C$\underline{H}_3$), 1·83 (3H, singlet, C=C—C$\underline{H}_3$), 2·17 (3H, singlet, —CO—C$\underline{H}_3$), 2·68 (2H, unresolved multiplet, C=CH—C$\underline{H}_2$—CH), 4·73 (1H, multiplet, CH_2—C$\underline{H}$—NH), 5·18 (1H, unresolved triplet, C=C$\underline{H}$—CH_2), 6·67 (1H, broad doublet, —N$\underline{H}$—) ppm.

DL-2-(*Purin*-6-*ylamino*)-5-*methylhex*-4-*enoic Acid* (IV). A mixture of 6-chloropurine (0·50 g), DL-2-amino-5-methylhex-4-enoic acid (0·68 g) and Na_2CO_3 (0·30 g) was stirred in 10 ml of water at 60° for 6 hr, the solution pH being maintained at 8·5 by periodic addition of saturated $NaHCO_3$. Formic acid (50%) was added to the cooled reaction solution to reduce the pH to 5 and the mixture was then left at 4° overnight. The precipitate was filtered off, suspended in water (5 ml) and dissolved by the addition of the minimum amount of 2N NH_4OH. The purinylamino acid was then precipitated by the slow addition of dilute formic acid and this purification step was repeated to yield DL-2-(*purin*-6-*ylamino*)-5-*methylhex*-4-*enoic acid* (80 mg), m.p. 221–222°. (Found: C, 55·0; H, 6·0; N, 26·7; O, 12·4. $C_{12}H_{15}N_5O_2$ required: C, 55·2; H, 5·8; N, 26·8; O, 12·3%); ν_{max} 1650 (broad) cm^{-1}.

4-(*Purin*-6-*ylamino*)*butanoic acid* (V). A mixture of 4-aminobutanoic acid (0·4 g), 6-chloropurine (0·50 g), Na_2CO_3 (0·35 g) and water (6 ml) was heated under reflux for 3 hr. Adjustment of the pH of the cooled solution to 4·2 with 50% formic acid yielded a precipitate. After 24 hr at room temp. this was filtered off and recrystallized twice from aq. ethanol to yield 4-(*purin*-6-*ylamino*)*butanoic acid* (0·45 g), decomposed 250–300°. (Found: C, 48·8; H, 5·1; N, 31·8; O, 14·6. $C_9H_{11}N_5O_2$ required: C, 48·9; H, 5·0; N, 31·7; O, 14·5%); ν_{max} 1685 (broad), 1620 (broad) cm^{-1}.

5-(*Purin*-6-*ylamino*)*pentanoic acid* (VI). A mixture of 6-chloropurine (0·50 g), 5-aminopentanoic acid hydrochloride (0·70 g), $NaHCO_3$ (0·92 g) and water (20 ml) was heated under reflux with stirring for 44 hr. The precipitate obtained by adjusting the pH of the cooled solution to 4 with formic acid was filtered off and dissolved in the minimum volume of 2N NH_4OH. Dowex 1 (formate form) was added to the resulting solution until it became colourless. Adjustment to pH 4 with formic acid and cooling at 4° yielded cream crystals (50 mg), m.p. 249–250°. The mother liquid was evaporated and the residue dissolved in water (50 ml) by addition of the minimum amount of conc. NH_4OH. After the resulting solution had been decolourized by the addition of Dowex 1 (formate), the solution pH was adjusted to 4 with formic acid yielding crystals of 5-(*purin*-6-*ylamino*)*pentanoic acid* (0·40 g), m.p. 250–251°. (Found: C, 51·0; H, 5·7; N, 29·7; O, 13·8. $C_{10}H_{13}N_5O_2$ required C, 51·1; H, 5·6; N, 29·8; O, 13·6%); ν_{max} 1700, 1634, 1608 cm^{-1}.

6-(*Purin*-6-*ylamino*)*hexanoic acid* (VII). A mixture of 6-chloropurine (0·50 g), 6-aminohexanoic acid (0·50 g), $NaHCO_3$ (0·32 g) and water (20 ml) was stirred at 70° for 48 hr. A further 0·15 g $NaHCO_3$ was then added and the mixture was heated under reflux for 24 hr. The precipitate obtained by adjusting the cooled filtered solution to pH 3·5 (formic acid) was recrystallized from dimethylformamide to yield 6-(*purin*-6-*ylamino*)*hexanoic acid* (0·20 g), m.p. 240–241°. (Found: C, 52·9; H, 5·9; N, 28·1; O, 12·9. $C_{11}H_{15}N_5O_2$ required: C, 53·0; H, 6·1; N, 28·1; O, 12·8%); ν_{max} 1680 (broad), 1615 (broad) cm^{-1}.

N-(*Purin*-6-*yl*)-L-*valine methyl ester* (IX). An ether solution of CH_2N_2 was added slowly to a solution of *N*-(purin-6-yl)-L-valine (68 mg) in methanol (45 ml) until a small excess became apparent. The solution was then immediately evaporated and the residue subjected to preparative TLC (Merck silica gel PF_{254} in MeCOEt saturated with H_2O) to free the product from minor impurities. The eluted compound (70 mg) was crystallized from EtOAc–EtOH to yield N-(*purin*-6-*yl*)-L-*valine methyl ester*, m.p. 163–164° (dec). (Found: C, 53·1; H, 6·1; N, 28·1. $C_{11}H_{15}N_5O_2$ required: C, 53·0; H, 6·1; N, 28·1%); ν_{max} 1742, 1633, 1565 cm^{-1}. NMR ($CDCl_3$) δ 1·07 (6H, doublet, J = 6·5 c/s, CH(—C$\underline{H}_3$)$_2$), 2·38 (1H, broad multiplet, C$\underline{H}$(—CH_3)$_2$), 3·78 (3H, singlet, COOC$\underline{H}_3$), 5·17 (1H; broad signal sharpened slightly on D_2O exchange; NH—C$\underline{H}$—), 6·37 (1H, broad doublet, J = 9 c/s, —N$\underline{H}$—; signal disappears on D_2O exchange), 8·03 (1H, singlet, purine —C$\underline{H}$=) and 8·45 (1H, singlet, purine —C$\underline{H}$=) ppm.

Methyl 5-(*purin*-6-*ylamino*)*pentanoate* (X). This ester was prepared by esterification of 5-(purin-6-ylamino)-pentanoic acid with CH_2N_2 by the method described above. Crystallization of the chromatographed product from EtOAc–EtOH yielded *methyl* 5-(*purin*-6-*ylamino*)*pentanoate*, m.p. 153·5–154°. (Found: C, 52·7; H, 6·1; N, 28·2. $C_{11}H_{15}N_5O_2$ required: C, 53·0; H, 6·1; N, 28·1%); ν_{max} 1735, 1620, 1604 cm^{-1}. NMR ($CDCl_3$) δ 1·80 (4H, multiplet, CH_2—C$\underline{H}_2$—C$\underline{H}_2$—CH_2), 2·62 (2H, broad, C$\underline{H}_2$—COOC$\underline{H}_3$), 3·68 (3H, singlet, COOC$\underline{H}_3$), 3·77 (2H, broad and partially obscured by 3·68 ppm peak, NH—C$\underline{H}_2$—), 6·43 (1H, triplet, J = 5 c/s, —N$\underline{H}$—), 8·02 (1H, singlet, purine —C$\underline{H}$=) and 8·67 (1H, singlet, purine —C$\underline{H}$=) ppm.

N-(*Purin*-6-*yl*)-L-*leucine methyl ester* (VIII). Esterification of the corresponding purin-6-ylamino acid with CH_2N_2 and crystallization of the chromatographed product from EtOAc yielded N-(*purin*-6-*yl*)-L-*leucine*

methyl ester, m.p. 190–192°. (Found: C, 54·9; H, 6·5; N, 26·5. $C_{12}H_{17}N_5O_2$ required: C, 54·7; H, 6·5; N, 26·6%); ν_{max} 1740, 1638 (shoulder), 1628, 1570 cm^{-1}; λ_{max} 275·5 nm (pH 2), 267 nm (pH 8), 272·5 with shoulder at 282 nm (pH 11). NMR ($CDCl_3$) δ 0·97 (6H, slightly broadened doublet, J = 5 c/s, $CH(—C\underline{H}_3)_2$), 1·82 (3H, broad multiplet, J = 5 c/s, $C\underline{H}_2—C\underline{H}(—CH_3)_2$), 3·76 (3H, singlet, $COOC\underline{H}_3$), 5·15 (1H; unresolved multiplet, poorly resolved triplet on D_2O exchange; —NH—C<u>H</u>—), 6·63 (1H, doublet, J = 8 c/s, —N<u>H</u>—; signal disappears on D_2O exchange), 8·01 (1H, singlet, purine —C<u>H</u>=) and 8·42 (1H, singlet, purine —C<u>H</u>=) ppm. Condensation of L-leucine methyl ester with 6-chloropurine in refluxing *n*-butanol containing NEt_3 yielded an identical product.