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BIOSYNTHESIS AND TURNOVER OF *N,N*-DIMETHYLTRYPTAMINE AND 5-METHOXY-*N,N*-DIMETHYLTRYPTAMINE IN *PHALARIS TUBEROSA*

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Key Word Index—*Phalaris tuberosa;* Gramineae; alkaloid; biosynthesis; alkaloid turnover; *N,N*-dimethyltryptamine; 5-methoxy-*N,N*-dimethyltryptamine.

Abstract—The biosynthesis and turnover of *N,N*-dimethyltryptamine and 5-methoxy-*N,N*-dimethyltryptamine have been studied in the pasture grass *Phalaris tuberosa* by a combination of feeding and trapping experiments using radioisotopically labelled compounds. Their turnover has been demonstrated by feeding tryptophan and tryptamine. The combined results of the feeding and trapping experiments indicate that at least five pathways could be operating in the biosynthesis of 5-methoxy-*N,N*-dimethyltryptamine.

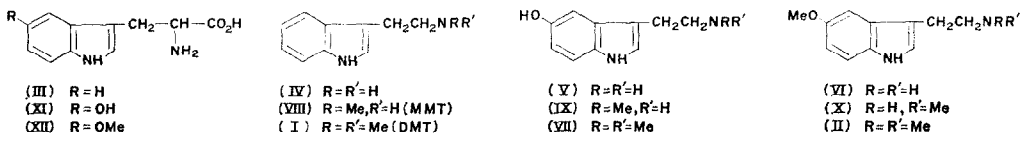
INTRODUCTION

Phalaris tuberosa is a major winter pasture grass in Australia and is known to contain a number of tryptamine alkaloids.[1] The major alkaloids are *N,N*-dimethyltryptamine (DMT) and 5-methoxy-*N,N*-dimethyltryptamine (5MeODMT), while 5-hydroxy-*N,N*-dimethyltryptamine (bufotenin) and several other indole bases are present in smaller quantities. In spite of its importance as a pasture grass *P. tuberosa* is sometimes unpredictably toxic to sheep causing three related neurological disorders of the central nervous system referred to as 'Phalaris Staggers', one of which can be fatal.[2] The known physiological activity of the tryptamine alkaloids led to the suggestion[2,3] that these could be the cause of the disease, and pharmacological tests were able to produce some of the symptoms. Accordingly, a plant breeding programme to produce varieties which produced lower levels of alkaloids was undertaken by C.S.I.R.O.[4] but recent reports[5] indicate that there is no correlation between the disease and the alkaloid level. In the course of this work many interesting results on the factors influencing alkaloid production emerged. For example, factors affecting nitrate nitrogen were shown[6] to effect concentrations of the alkaloids. This was also true with increased day–night temperatures and the amount of shade in which the plants were grown.[7] There are also reports of large diurnal variations in the alkaloid content.[3] All this work seemed an excellent background to studying the biosynthesis and the turnover of the alkaloids which are reported in the present paper.

[1] C. C. J. CULVENOR, R. DAL BON and L. W. SMITH, *Austral. J. Chem.* **17,** 1301 (1964).
[2] C. H. GALLAGHER, J. H. KOCH and H. HOFFMAN, *Austral. Vet. J.* **42,** 279 (1966).
[3] C. H. GALLAGHER, J. H. KOCH and H. HOFFMAN, *New Scientist* **24,** 412 (1966).
[4] R. N. ORAM and J. D. WILLIAMS, *Nature, Lond.* **213,** 946 (1967).
[5] R. N. ORAM, *Proc. XI Intern. Grasslands Congr.* 785 (1970).
[6] R. M. MOORE, J. D. WILLIAMS and J. CHIA, *Proc. X Intern. Grasslands Congr.* 524 (1966).
[7] R. M. MOORE, J. D. WILLIAMS and J. CHIA, *Austral. J. Biol. Sci.* **20,** 1131 (1967).

RESULTS AND DISCUSSION

Alkaloid synthesis in *Phalaris tuberosa* (cv. Australian Commercial) is directed towards the production of two alkaloids, *N,N*-dimethyltryptamine (I) and 5-methoxy-*N,N*-dimethyltryptamine (II). If alkaloid synthesis is considered as starting from tryptophan (III), and it is assumed that a free amino group is necessary for decarboxylation, then the metabolic grid in Fig. 1 shows possible intermediates in the biosynthesis of I and II.



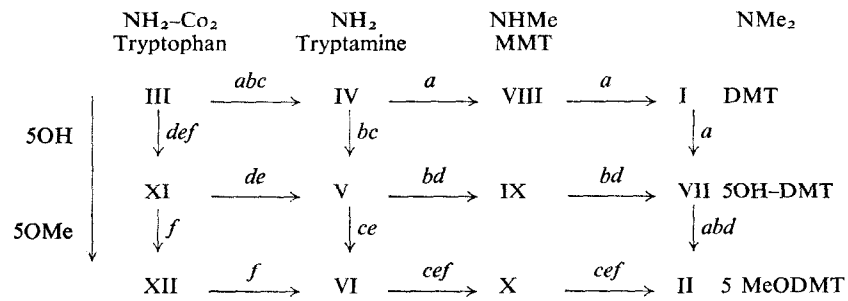


FIG. 1. POSSIBLE PATHWAYS FOR THE BIOSYNTHESIS OF I AND II IN *P. tuberosa*. THE SIX PATHWAYS ARE LABELLED *a* TO *f*.

Of the 12 compounds in this grid seven could be detected in the plant. In addition to I and II, which in this variety were present to the extent of 0·1 and 0·05% (based on dry wt) respectively, trace amounts of III, tryptamine (IV), 5-hydroxytryptamine (V), 5-methoxytryptamine (VI) and bufotenin (VII) could be detected by TLC of plant extracts. In the same weight of seedlings (0·18 g fr. wt) only I and II could be detected by GLC. No trace of the three mono *N*-methyl derivatives, *N*-methyltryptamine (VIII), *N*-methyl-5-hydroxytryptamine (IX) or *N*-methyl-5-methoxytryptamine (X) could be detected in plant extracts using sodium nitroprusside,[8] though enzymic evidence shows that the two *N*-methylations occur in discrete steps.[9] If it is assumed that *in vivo* the second methylation follows the first one immediately, then there are only six pathways, as shown in Fig. 1, to be considered.

Time-course Experiments with Methylene-^{14}*C-L-Tryptophan*

The rate of uptake of ^{14}C-tryptophan, at least in concentrations up to 3·2 μmol/0·18 g of seedlings, was apparently independent of the amount fed. Thus, when either 10 nmol (see Fig. 2) or 3·2 μmol (see Table 1) were fed there was 71% uptake after 24 hr and 88% after 48 hr. This uptake of radioactivity was paralleled by a rapid incorporation of radioactivity into the alkaloids. The incorporation of ^{14}C-III was dependent on the amount fed. When 10 nmol were fed 0·13 nmol (1·3%) were converted to alkaloids (Fig. 2) which only increased to 1·3 nmol (0·04%) when 3·2 μmol were fed (Table 1). The turnover of the alkaloids was followed after feeding 10 nmol of ^{14}C-III (Fig. 2). There was a maximum incorporation of

[8] F. FEIGL, *Organic Applications*, Vol. II, p. 189, Elsevier, Amsterdam (1954).
[9] J. P. G. MACK and M. SLAYTOR, unpublished results.

radioactivity into the alkaloids at 19 hr after which it rapidly declined as the alkaloids were turned over. This pattern was not followed when higher amounts of tryptophan were fed as can be seen in Table 1.

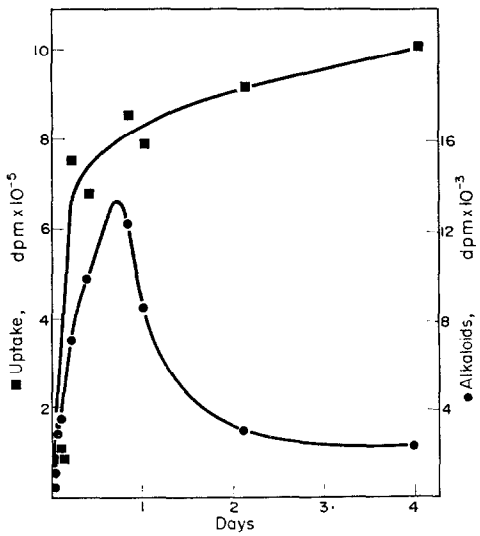


FIG. 2. UPTAKE OF ^{14}C-L-TRYPTOPHAN AND ITS INCORPORATION INTO I AND II. THE ALKALOIDS WERE ESTIMATED FROM TLC.

β-^{14}C-Tryptamine is also rapidly taken up by the seedlings (Fig. 3). When 4 μmol were fed, maximum incorporation was attained in I and II 2 days after feeding, but after a further 2 days 80% of this maximum amount had been turned over (Fig. 3).

After separation of I and II by GLC, the specific activity of the two alkaloids was always the same, i.e. both are synthesized and turned over at the same rate. In addition to showing the uptake of ^{14}C-tryptamine and the turnover of the alkaloids, Fig. 3 shows the turnover of exogenous tryptamine. Figure 3 also indicates that the major pathway for tryptamine metabolism is not via I. The turnover of endogenously formed tryptamine can be demonstrated (Fig. 4) by allowing the seedlings to take up 2 μmol of unlabelled tryptamine and then feeding 10 nmol of ^{14}C-III. In this way radioactivity is trapped in IV, but this radioactivity reaches a maximum after 1 day and cannot be detected after 3 days when all the endogenously formed ^{14}C-tryptamine has been turned over. The results in Figs. 2 and 3 show that exogenously supplied III and IV can reach the site of alkaloid synthesis quite efficiently.

Although alkaloid synthesis could be demonstrated within 10 min of feeding 10 nmol of ^{14}C-tryptamine (Fig. 1) none of the proposed intermediates contained any radioactivity. it appears that 10 min is already too long a time for this approach to work with *P. tuberosa.*

Feeding Experiments with Labelled Precursors

In studying the biosynthesis of the alkaloids all the compounds in Fig. 1, except the 3 mono *N*-methyltryptamines, were fed. All compounds were fed in equimolar amounts. In practical terms this meant diluting some of the labelled substrates to non-physiological amounts so that incorporations expressed as percentages appear low. With the exception

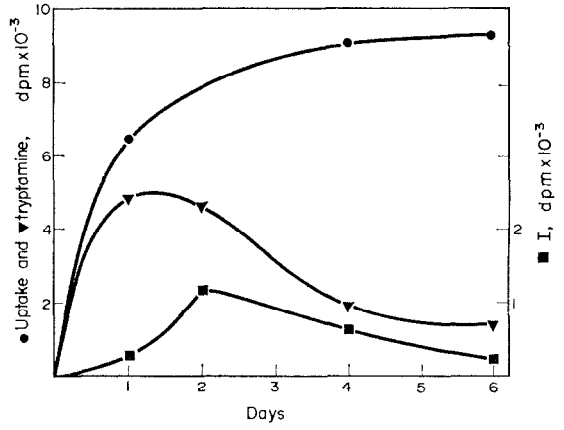


FIG. 3. UPTAKE AND TURNOVER OF ^{14}C-TRYPTAMINE AND ITS INCORPORATION INTO I AND II. THE ^{14}C-TRYPTAMINE WAS ESTIMATED FROM TLC AND I WAS SEPARATED BY GLC (QF1 COLUMN).

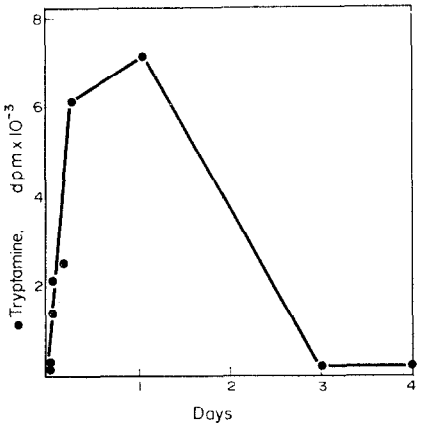


FIG. 4. TURNOVER OF ENDOGENOUSLY FORMED ^{14}C-TRYPTAMINE AFTER FEEDING 10 nmol OF ^{14}C-L-TRYPTOPHAN AND 5 μmol OF UNLABELLED TRYPTAMINE. THE ^{14}C-TRYPTAMINE WAS ESTIMATED BY TLC.

of I, all compounds fed were shown to be incorporated into II. The results of the feeding experiments in Table 1 show that in all cases there is a slight increase in incorporation from 1 to 2 days. The exception is I which shows no incorporation in this time although it is efficiently taken up by the seedlings.

TABLE 1. FEEDING EXPERIMENTS WITH *Phalaris tuberosa*

Substrate	Specific activity (μmCi/mmol)	Time fed (days)	μmol taken up	Radioactive I formed (nmol)	Radioactive II formed (nmol)
^{14}C-III	103	1	3·2	1·3	0·7
		2	3·9	3·0	1·6
^{3}H-XI*	70	1	2·3	ne	0·2
		2	4·0	ne	0·7
^{3}H-XII*	1140	1	2·6	ne	6·3
		2	2·8	ne	7·3
^{14}C-IV	20	1	4·0	12·8	4·4
		2	4·6	15·2	6·9
^{14}C-V	94	2	1·7	0·0	11·7
^{3}H-VI*	144	1	3·2	ne	7·8
		2	3·6	ne	8·5
^{14}C-I	56	1	2·3	—	0·0
		2	3·1	—	0·0
^{3}H-VII	1690	1	0·4	0·0	6·9
		2	2·7	0·0	8·6

The total incorporation into I + II using 0·18 g of plants was estimated from TLC and based on the amount and the specific activity of radioactive substrate. The relative amounts were then determined by GLC except where ne (not estimated) is indicated.

* Prepared from the unlabelled compounds and $^{3}H_2O$ according to the method of Daly and Witkop.[10]

[10] J. W. DALY and B. WITKOP, *J. Chem. Am. Soc.* **89**, 1032 (1967).

After ^{14}C-I had been fed to the seedlings for 14 days 85% of it had not been metabolized. A comparison between this result and the turnover of the alkaloids in Figs. 2 and 3 strongly suggests that I is being transported to the site of alkaloid synthesis at a much lower rate than either III or IV. Perhaps for similar reasons I is not incorporated efficiently into psilocybin in *Psilocybe cubensis*.[11] It is interesting that feeding IV produces I and II in the same ratio (2:1) as when III is fed which is the same ratio as is found normally in the plant. This is consistent with IV being a normal precursor of both alkaloids. However, XI, XII, V, VI and IX are all incorporated into II indicating that either the pathways *d*, *e* and *f* do normally operate or that the enzymes in pathways *a*, *b* and *c* are non-specific.

Trapping Experiments with methylene-^{14}C-L-Tryptophan and Unlabelled Traps

Trapping experiments were designed to give more direct evidence for the existence of the compounds in the grid as intermediates. In these experiments one of the compounds in the grid was fed unlabelled to the plant followed 24 hr later by 10 nmol of ^{14}C-tryptophan. After varying times the radioactivity in the trap was determined. The results are shown in Table 2.

TABLE 2. LABELLED ALKALOIDS PRODUCED IN TRAPPING EXPERIMENTS AFTER FEEDING ^{14}C-METHYLENE-L-TRYPTOPHAN

Alkaloids in the plant	Control*†‡	Trap (dpm) IV†	VI†	V†‡	I*	IV + VI‡	IV + VI + V†‡
I	550	36	75	70	1120	245	0
II	300	72	120	140	200		
IV	Trace	792	430	170	0	1205	
VI	Trace		455	220	0		1400
V	Trace		0	290	0		
Total dpm§ in alkaloids in aliquot	850	890	1080	890	1320	1450	1400

* Alkaloids separated by GLC on W-98 column.
† Alkaloids separated by GLC on QF-1 column.
‡ Alkaloids separated by TLC.
§ Analyses were carried out on 5% aliquots.

In all trapping experiments there was, as expected, a large reduction in the amount of labelled alkaloids synthesized. Where measured, the total amount of radioactivity in the alkaloids and traps was greater than that in the alkaloids in the control experiment, i.e. where no trap is used and only ^{14}C-tryptophan is fed.

When IV was used as a trap some labelled I was formed indicating an inefficiency in the trapping method. After feeding IV for 24 hr as a trap the ratio of radioactivity was IV: I: II (22:1:2). Figure 4 indicates IV is ineffective as a trap after 3 days so that it is reasonable to suppose that some of the trapped ^{14}C-IV will have metabolized to I after 24 hr.

[11] S. AGURELL and J. L. G. NILSSON, *Acta Chem. Scand.* **22**, 1201 (1968).

The production of labelled II when using either IV, V or VI as traps again shows that all 6 pathways could be operating. One double trapping experiment, using IV and VI as traps showed that II could still be synthesized either through pathway *d* or by processes similar to those considered when using only IV as a trap. No labelled alkaloids were formed when IV, V and VI were used simultaneously as traps and all the traps contained label. The most interesting results from the trapping experiments are where label has accumulated not only in the expected trap but in other compounds in the grid. For example, VI acts as a trap but label also accumulates in IV. Again, V acts as a trap and causes accumulation of label in both IV and VI. There are a number of possible explanations of which the most attractive is that the *N*-methyltransferases are non-specific for the tryptamines and that the feeding of large amounts of any one of the tryptamines, i.e. the traps, will saturate the enzymes and cause accumulation of the other tryptamines.

In summary the trapping experiments provide evidence that the plant is capable of making IV, V and VI from tryptophan and strengthens the TLC evidence for the existence of these compounds in *P. tuberosa*.

The results from the feeding and trapping experiments are an excellent illustration of Bu'Lock's idea[12] that the more information which can be obtained from precursor-incorporation experiments then the fewer clear-cut conclusions there are to be drawn from them. The main conclusion is that II can be synthesized by at least 5 of the 6 pathways *a* to *f* whose relative importance cannot be distinguished by feeding and trapping experiments.

P. tuberosa is an ideal plant to work with enzymically and it is hoped that some of the problems indicated here will be solved by cell-free studies. To date, enzymic activity in cell-free systems has been demonstrated for *N*-methylation,[9] *O*-methylation[9] and decarboxylation.[13]

EXPERIMENTAL

General. M.ps were determined on a Kofler block and are uncorrected. All chemicals and solvents were of AR grade or were purified before use. All indole derivatives were purchased commercials. The following compounds were purchased from the Radiochemical Centre, U.K.; methylene-^{14}C-L-III, 3-^{14}C-V creatinine SO_4 and ^3H_2O. $^{14}CH_2O$ and 2-^{14}C-indole were purchased from I.C.N., U.S.A. ^{14}C-toluene and ^{3}H-toluene were obtained from the Packard Instrument Company, U.S.A.

Plant material. Seeds of *Phalaris tuberosa* (cv. Australian Commercial) from Ramsay's Seeds, Sydney, were grown in a temperature controlled greenhouse at 21–24°, daylight being supplemented with fluorescent lighting set on a 16-hr day. 10- to 14-day-old seedlings grown either in sand or on a nylon mesh frame over a solution of Aquasol (Hortico Ltd., Port Melbourne) were used for feeding and trapping experiments. The convenience in handling seedlings of this age was the factor of choice.

Feeding experiments. Seedlings were transferred to small vials (12 plants per vial) containing substrates dissolved in water. Tryptamines insoluble in water were fed as soluble salts. The amount taken up by the plants was assumed to be the difference between the amount fed and that remaining in the vial at the end of the feeding experiment. With ^{14}C-DMT feeding experiments the plants were transferred to fresh water every 48 hr.

Trapping experiments. 5 μmol of each trap was fed to 12 seedlings in water for 24 hr. In all cases approximately 40% of the trap had been taken up. The amount not taken up was measured spectrophotometrically. The seedlings were then transferred to a solution of ^{14}C-L-tryptophan (10 nmol, specific activity 56·5 mCi/mmol) for 24 hr when the alkaloids were extracted and separated on TLC or GLC. In time-course trapping experiments with tryptamine groups of seedlings were harvested at time intervals from 20 min to 13 days. Tryptamine was then estimated by GLC on a QF-1 column.

Isolation and detection of alkaloids. Seedlings were homogenized in 80% EtOH in a Ten Broeck homogenizer and the homogenate heated for 15 min on a steam bath and then centrifuged. The basic and amphoteric compounds were exchanged on a cellulose phosphate cation exchange column (H^+ form) and eluted with EtOH–conc. NH_3 (4:1). The EtOH was removed and the alkaloids separated from the amphoteric compounds by extraction with EtOAc. The alkaloids were separated on 2D TLC using Merck A1 precoated

[12] J. D. BU'LOCK *The Biosynthesis of Natural Products*, p. 141, McGraw-Hill, London (1965).
[13] C. R. BAXTER and M. SLAYTOR, *Phytochem.* **11**, 2763 (1972).

plates with silica gel F_{254} (0·25 mm). The first solvent was isoPrOH–conc. NH_3–H_2O (8:1:1) and the second solvent was *n*-BuOH–AcOH–H_2O (12:3:5). The following spray reagents were used for detection. Ehrlich's oversprayed with 4·5% $NaNO_2$ for all III and IV derivatives (blue colour): Xanthydrol for distinguishing between unsubstituted indoles (pink colour) and 5-substituted indoles (blue colour);[6] diazotised sulphanilic acid for phenolic indoles (pink colour); Na nitroprusside for *N*-methyltryptamines (blue colour). From feeding and trapping experiments alkaloids were also detected by autoradiography.

GLC. GLC was carried out on an F and M Biomedical Gas Chromatograph model 400 with a flame ionization detector, a Speedomax G model S (60 000 series) recorder and an effluent splitter using He (flow rate 80 ml/min), H_2 (65 ml/min) or air (600 ml/min). Samples were collected for counting in glass capillary tubes at 1 min intervals. A W-98 column (3·8% on Diatoport S 80–100 mesh) at 160–180° was used to separate I from II. It did not separate I from IV or II from VI. A QF-1 column (5% on Diatoport S 60–80 mesh) at a temperature programme of 160° for 15 min and then 10°/min until 210° was used to separate IV, I, VI and II. Standards were added to plant extracts when necessary for detection.

Counting of radioactivity. A Packard Tri-carb liquid scintillation spectrometer model number 3375 was used for counting samples containing ^3H or ^{14}C. The samples were dissolved in 15 ml of scintillant (containing 4 g of PPO and 100 mg of POPOP per l. of toluene. Areas scraped off TLC plates were suspended in Thixotropic gel (0·5 g/vial). All samples were internally standardized using ^{14}C-toluene or ^3H-toluene.

Chemical syntheses. N,N-*Dimethyl*-2-^{14}C-*tryptamine.*[14,15] 2-^{14}C-indole (0·1 mCi; 150 mg) was dissolved in dry ether (5 ml) and cooled in an ice-salt bath. The reaction was kept moisture free. Excess oxalyl chloride (1 ml) was added over 20 min while N_2 was blown through the reaction mixture. Yellow crystals of the indole-3-glyoxylyl chloride precipitated out. Dry Me_2NH was blown with N_2 through the acid chloride suspension. After 15 min a white ppt formed and the mixture was left for 3 hr at 5°. The ppt was filtered, washed with water, and dried over silica gel and P_2O_5. The crude indole 3-*N,N*-dimethyl-glyoxylamide (250 mg) was dissolved in hot dry dioxane (5 ml) and added dropwise to $LiAlH_4$ (280 mg in hot dioxane) and the mixture was refluxed for 16 hr. Wet dioxane was added cautiously with stirring and the white ppt was filtered and washed with hot dioxane. Solvent was removed from the filtrate to yield a yellow oil. I was purified from this as the picrate (m.p. 172°) from which the picric acid was extracted. The ^{14}C-I was recrystallized from benzene–hexane to give colourless crystals m.p. 49° (reported 49°);[14] yield 92 mg (38%); specific activity, 56 μCi/mmol. The I was chromatographed on TLC (solvent 1). Autoradiography showed a single spot.

β-^{14}C-*tryptamine.* Me_2NH (0·2 ml 26% w/v aq.) was cooled in an ice bath and HOAc (0·5 ml) followed by CH_2O (0·1 mCi, 0·14 ml 37% w/v aq.) added. This solution was poured onto indole (99 mg) and allowed to stand overnight at 20°. Excess NaOH solution (8 M, 3 ml) was added and after 2 hr at 0° the ppt was filtered off and washed with water. The gramine was then dried and converted to the indolyl acetonitrile via the methiodide.[16] This was then reduced to β-^{14}C-tryptamine according to Staskun and van Es.[17] The crude tryptamine (91 mg) was sublimed at 100–120° and then recrystallized (benzene) to yield pure β-^{14}C-tryptamine, 27 mg (20% based on indole), m.p. 113–116° (reported 116–117°)[18] activity, 16·8 μCi/mmol. 2D TLC and autoradiography showed a single radioactive spot.

Acknowledgement—This work was supported in part by the Australian Research Grants Committee.

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[16] K. I. POTTS and R. ROBINSON, *J. Chem. Soc.* 2675 (1955).
[17] B. STASKUN and T. VAN ES, *J. Chem. Soc.* **C,** 531 (1966).
[18] J. THESING and F. SCHULDE, *Chem. Ber.* **85,** 324 (1952).