Phytochemistry, 1976, Vol. 15, pp. 153–155. Pergamon Press. Printed in England.

THE SYNTHESIS OF A RADIOACTIVE CYTOKININ WITH HIGH SPECIFIC ACTIVITY

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(*Received* 23 *June* 1975)

Key Word Index—Tritiated benzyladenine; high specific radioactivity cytokinin; volatile purine tritium; *para*-bromobenzyladenine; palladium catalyzed dehalogenation.

Abstract—6-benzylaminopurine-[*p*-^{3}H-benzyl] at a specific activity of 10 Ci/mmol was synthesized by reacting *p*-bromo-6-benzylaminopurine with carrier-free tritium gas in the presence of 10% Pd/C. A radiochemical purity of 97% was obtained by a one-step purification of the tritiated reaction product using cellulose TLC. This simple procedure yields the highly active cytokinin, 6-benzylaminopurine, with tritium at near maximum specific activity in a known, stable position.

INTRODUCTION

Receptors for some animal hormones [1] and efficacious drugs [2] are present in tissues at low concentrations, and the binding of ligands to these receptors is more readily detected if a ligand of high specific radioactivity is available. This is especially true if one wishes to detect a small number of high affinity binding sites in the presence of a large excess of low affinity sites. Ligands labelled with ^{14}C cannot be obtained at sufficiently high specific activity, hence most studies employ tritiated or iodinated ligands.

Previously published attempts to introduce a stable tritium label into biologically active cytokinins by the Wilzbach method or by catalytic exchange in an aqueous solution [3–6] and by reduction of *p*-benzoylaminopurine with lithium aluminum tritiide [7] have failed to produce a specific activity greater than 231 mCi/mmol. In this report we describe a new and simple method for synthesizing a radioactive cytokinin, 6-benzylaminopurine-[*p*-^{3}H-benzyl] (*p*-[^{3}H]BA) with tritium in a stable, known position at near maximum specific activity. The method employs catalytic dehalogenation (hydrogenation) of a halogenated precursor, *p*-bromo-6-benzylaminopurine (*p*BrBA).

RESULTS

Synthesis and purity of p*BrBA*. The procedure used to synthesize *p*BrBA was that of Elion *et al.* [8] as described by Okumura *et al.* [9]. Though the synthesis of the *o*-, *m*-, and *p*-Cl analogues of 6-benzylaminopurine (BA) had already been reported, we chose to synthesize the previously unreported Br analogues since exchange with tritium is considered easier with this halogen. The *p*-position was chosen simply to reduce any possibilities of steric hindrance in the synthesis and dehalogenation reactions.

A preliminary Beilstein test of the recrystallized reaction product indicated the presence of halogen, which was confirmed by obtaining a green color after heating a sample on a copper wire over a gas flame. The irregularly shaped crystals changed to needles before melting at 288–289°. Elemental analysis found: C, 47·34; H, 3·38; N, 22·96; Br, 26·34%. $C_{12}H_{10}N_5Br$ requires: C, 47·39; H, 3·31; N, 23·03; Br, 26·27%. Identity of the compound was further confirmed by its UV, IR and mass spectra. The UV spectrum is indistinguishable from that of BA at acidic, neutral and basic pH. The IR spectrum (in KBr discs) is also similar to BA in showing (1) an unsubstituted purine N-9 position (absorption peaks at 2400–2800 cm^{-1}), and (2) the absence of the strong primary amine N–H stretching absorption peak at 3380 cm^{-1} seen in *p*-bromobenzylamine. Finally, the assigned structure was confirmed by MS and GLC–MS. The natural abundance of the two isotopes of Br, Br^{79} and Br^{81}, in the ratio 100:97·5 permits facile identification of Br containing fragments. The fragmentation pattern of this compound is similar to BA. The only peaks showing the presence of Br are the molecular ion, at *m/e* 303, 305 and fragments at *m/e* 169, 171 and 184, 186 identified respectively as the Br substituted tropylium ion ($C_7H_5Br^+$; analogous to *m/e* 91 in BA) and a species ($C_6H_5BrCH_2N^+H$; *m/e* 106 in BA) formed by the loss of the purine ring system. In addition, intense peaks are observed at *m/e* 89 and *m/e* 90 corresponding to the loss of HBr and Br, respectively, from the tropylium ion. All other major peaks were accounted for as arising from the purine ring.

The purity of *p*BrBA was checked by TLC on silica gel, cellulose and aluminum oxide (Table 1). In all instances, only a single zone was detected, using several visualization techniques. In particular, the absence of potentially reducible unreacted *p*-bromobenzylamine was verified by the absence of ninhydrin positive spots in the above TLC systems. Absence of impurities was also

* In partial fulfillment of the requirements for Ph. D. degree.

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Table 1. TLC R_f values of compounds examined

	Support		
	Silica gel	Cellulose	Aluminum oxide
BA*†	0·36	0·64	0·72
*p*BrBA*†§	0·36	0·54	0·72
6-(methylmercapto)purine*‡	0·36	0·44	—
p-bromobenzylamine¶	0·09	0·50	—
Benzylamine¶	0·07	0·80	—

See Experimental for solvent systems used.

Visualization methods: * quenching of fluorescence induced by excitation at 254 nm after spraying with 0·1% fluorescein in EtOH; † purple fluorescence after excitation at 254 nm; ‡ yellow fluorescence after excitation at 254 nm; ¶ ninhydrin stain; § silver chromate stain for purines [13] using commercial plastic-backed Si gel and cellulose TLC.

demonstrated by GLC on a 3% SE-30 column in which only a single peak was observed. As expected from comparison with the effect of bromine substitution on the retention time of benzylamine derivatives, *p*BrBA had a retention time 2–3 times that of BA.

Dehalogenation reaction. The dehalogenation reaction with H_2 gas was performed by us on the same small scale as the later reaction with T_2 gas, in order to allow more direct comparison between the 2 reactions. The time course of the reaction as measured by GLC showed that after 1 hr, less than 10% of the original *p*BrBA was left, and the reaction was essentially completed after 2 hr. This conclusion was confirmed by the absence of a *p*BrBA zone upon cellulose TLC of the 2-hr reaction product. The identity of the product was verified by co-chromatography with authentic BA on cellulose TLC, GLC and by GLC–MS.

For dehalogenation with T_2 gas, a sample (10 mg) of *p*BrBA was sent to Mallinkrodt Chemical Works (Box 5439, St. Louis, Mo. 63160), and the reaction was performed under conditions similar to those used in the reaction with H_2 gas (see Experimental) but with carrier-free T_2 gas (sp act 59 Ci/mmol). Volatile ^3H was removed from the radioactive product, and the sample was purified on preparative cellulose TLC as described for the nonradioactive reaction. Again, only a single fluorescent zone at the R_f of BA was observed. The sample was eluted from the cellulose with 50% EtOH and subsequently stored in this solvent at −18° at a concentration of 3–30 × 10^9 dpm/ml. The chemical concentration of this sample was measured by UV absorbance at 270 nm and by comparison with the absorbance of standard authentic ^1H-BA solutions, the sp act was calculated to be 10 Ci/mmol. The UV spectrum for the radioactive sample was found to be identical to BA at acid, neutral and basic pH. In addition, the radioactivity co-chromatographed with BA in 3 TLC systems and on Sephadex LH-20 column chromatography, as described below.

The amount of volatile tritium in the sample purified by cellulose TLC was calculated to be less than 2–3%. After autoclaving for 20 min in aqueous buffers at pH 2, pH 7·5 and pH 10, this value rose to 10, 18 and 15%, respectively. Under similar conditions, other workers [10] have reported for adenine-2-[^3H], values of 2% at pH 1, 42% at pH 9–10 and for adenine-8-[^3H], 5% at pH 1, 53% at pH 9–10. Since the exchange of ^3H in purine-labelled adenine is considered alkali mediated [11], this experiment indicates that ^3H in *p*-[^3H]BA prepared by the dehalogenation reaction is located in a less labile position.

Purity of p-[^3H]*BA.* The radiochemical purity of this compound was shown to be at least 97% by chromatography using 3 TLC systems. Re-chromatography on silica gel TLC raised this value to 99%. With some commercial silica gel plates (Merck pre-coated glass TLC plates), 5–10% of the radioactivity stayed at the origin. Since re-chromatography of the BA zone in the same system gave the same percentage of non-moving radioactivity, and since it was not observed on other silica gel TLC preparations (Brinkman Polygram Sil C pre-coated plastic sheets) it was considered to be an artifact produced by that particular silica gel preparation. Because the trailing of both BA and *p*BrBA on cellulose TLC did not permit their complete resolution and a 5–10% contamination with radioactive or non-radioactive *p*BrBA might therefore have gone undetected, another chromatographic system was desired. Since the BA sample is of such high sp act, GLC, which gives complete separation of BA and *p*BrBA, could not be safely used to quantitate a small amount of non-radioactive *p*BrBA present in the tritiated sample. Chromatography on Sephadex LH-20 was found to give excellent separation of BA and *p*BrBA. The chromatographic profile showed only the *p*-[^3H]BA peak, without any peaks for radioactive or non-radioactive *p*BrBA in the absence of carrier BA or *p*BrBA, proving the complete absence of *p*BrBA in this sample. The percent of the total radioactivity and $A_{270\,nm}$ migrating as BA in this sample was 91 and 85% respectively. This sample, which had been stored for 13 months after the initial cellulose TLC purification likewise showed 5–10% radioactive impurities on silica gel TLC which were not present prior to the storage period. Finally, constant specific activity (in this experiment, 9 Ci/mmol) over the BA zone was observed, demonstrating further the purity of *p*-[^3H]BA obtained by this procedure.

DISCUSSION

The technique described in this paper is an extremely easy procedure for obtaining a very potent cytokinin radioactively labelled in a specific, known position at a very high sp act and radiochemical purity. The label appears to be quite stable, with less than 5–10% radiochemical decay detected after a 13-month storage at −18° in 50% EtOH at a high radioactive concentration.

Other workers may wish to substitute Sephadex LH-20 chromatography for cellulose TLC in the purification of the dehalogenation reaction product since this Sephadex chromatography was found to give such excellent separation of BA and *p*BrBA and since it is a convenient way of handling the large amounts of radioactivity. In addition, the chromatographic solvent (35% EtOH) provides a convenient solvent to store the labelled cytokinin without the need for subsequent transfer to another solvent.

Caution should be exercised in work with radioactive benzyladenine at the high sp act described herein. We observed the binding of radioactivity in an aqueous solution to the walls of laboratory glassware. This binding can be reduced by the simultaneous presence of a low concentration (5×10^{-7} M) of non-radioactive BA in a manner similar to that described for the binding of insulin to silica and other non-tissue material [12].

EXPERIMENTAL

Synthesis of p-*BrBA.* 6-(methylmercapto)purine (0·3 g) was added to *p*bromobenzylamine (1·25 g) and heated at 120–130° in a closed tube for 18 hr with intermittent agitation. *p*-BrBA was precipitated by the addition of Me_2CO and the ppt. collected by filtration on Whatman GF/C filter paper followed by washing with Me_2CO. The collected and washed ppt. was recrystallized from $HCONME_2$, the crystals collected by filtration, washed with Me_2CO and dried in an oven at 80° overnight. The yield of product was 28%. In a separate experiment, recrystallization from EtOH gave a similar yield.

Dehalogenation reaction with H_2 gas. This entire procedure was performed at room temp. 10% Pd/C (3 mg) was added to $HCONMe_2$ (2 ml) containing 10 μl NEt_3 in a 5 ml reaction vessel containing a Viton stopper and a Teflon magnetic stirring bar. The vessel was evacuated, H_2 gas added and kept at a slight pressure (approximately 0·7 kg/cm² above atm pres) for 30 min to saturate the Pd. The vial was then opened, *p*BrBA (10 mg) added and allowed to dissolve by stirring for 3 min. The vessel was then closed, evacuated and H_2 applied again at the slight pressure for the time denoted.

Silylation and GLC of BA and p*BrBA.* Samples were typically silylated just prior to GLC by the following procedure. The dry sample was dissolved in dry $HCONMe_2$, followed by the addition of a 3–5 fold excess vol of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane. For measuring the time course of dehalogenation, 50 μl samples were withdrawn periodically, filtered to remove charcoal and silylated just prior to GLC injection. All GLC determinations were performed using a 1·8 m × 1·83 mm glass column packed with 3% SE-30; column temp. 205° isothermal, injection port temp. 225°, flame detector temp. 250°, carrier gas He at 87·5 ml/min. Retention times: BA-TMS_1 4·38 min; *p*BrBA-TMS_1 12·00 min, measured from the solvent peak.

MS and GLC–MS. All MS were obtained on an LKB Model 9000 Mass Spectrometer. The most abundant ions in the mass spectrum obtained by direct probe at 70 eV were *m/e* (rel. int.): for BA, 226 ($M^+ + 1$; 19), 225 M^+ (100), 224 ($M^+ - 1$; 44), 120 ($M^+ - 135$; 22), 119 ($M^+ - 136$), 106 ($M^+ - 119$; 80), 93 ($M^+ - 132$; 27), 91 ($M^+ - 134$; 27), 65 ($M^+ - 160$; 38); for *p*BrBA, 306 ($M^+ + 1$; 13), 305 M^+ (96), 304 ($M^+ + 1$, $M^+ - 1$; 40), 303 M^+ (100), 302 ($M^+ - 1$; 27), 224 ($M^+ - 81$, 79; 33), 186 ($M^+ - 119$; 61), 184 ($M^+ - 119$; 69), 171 ($M^+ - 134$; 39), 169 ($M^+ - 134$; 41), 120 ($M^+ - 195$, 193; 52), 119 ($M^+ - 196$, 194; 42), 93 ($M^+ - 212$, 210; 40), 90 ($M^+ - 215$, 213; 56), 89 ($M^+ - 216$, 214; 50). For GLC–MS, the silylated samples were chromatographed on a 1·8 m × 1·83 mm glass column packed with 2·5% SP-2401 with the carrier gas at 30 ml/min on a temp. program. For the H_2 dehalogenation reaction product, the program was 220–245° at 4°/min and the retention time 5 min (BA-TMS_1). For *p*BrBA, the program was 220–240° at 3°/min and the retention time 9 min (*p*BrBA-TMS_1). The MS showed that both compounds were derivatized by TMS at the purine N-9 position.

TLC conditions. Running solvents for Si gel, cellulose and aluminum oxide TLC were, respectively, 10% MeOH in CH_2Cl_2, 0·01 N HCl, and H_2O saturated EtOAc. Cellulose plates were pre-washed with 0·1 N HCl and dried just prior to use. Visualization techniques were as described in Table 1.

UV spectra. *p*BrBA, authentic BA and *p*-[^{3}H]BA had the following, identical spectra: $\lambda_{max}^{50\% EtOH}$ nm (log ε): pH 2, 275·5 (4·26), pH 7, 269·5 (4·28), pH 12, 275·5 (4·28) with *sh* at 280–285 nm.

Acknowledgments—This research was supported by the U.S. Atomic Energy Commission/Energy Research and Development Administration under Contract AT(11-1)-1338. M. Sussman was the recipient of a National Science Foundation Graduate Fellowship. We gratefully acknowledge the support and encouragement of Dr. Hans Kende.

REFERENCES

1. Cuatrecasas, P., Tell, G. P. E., Sica, V., Parikh, I. and Chang, K. (1974) *Nature* **247**, 92.
2. Goldstein, A. (1974) *Life Sci.* **14**, 615.
3. Berridge, M. V., Ralph, R. K. and Letham, D. S. (1970) *Biochem. J.* **119**, 75.
4. Letham, D. S. and Young, H. (1971) *Phytochemistry* **10**, 2077.
5. Elliott, D. C. and Murray, A. W. (1972) *Biochem. J.* **130**, 1157.
6. Walker, G. C., Leonard, N. J., Armstrong, D. J., Murai, N. and Skoog, F. (1974) *Plant Physiol.* **54**, 737.
7. Fox, J. E., Sood, C. K., Buckwalter, B. and McChesney, J. D. (1971) *Plant Physiol.* **47**, 275.
8. Elion, G. B., Burgi, E. and Hitchings, G. H. (1952) *J. Am. Chem. Soc.* **74**, 411.
9. Okumura, F. S., Kotani, Y., Ariga, T., Masumura, M. and Kuraishi, S. (1959) *Bull. Chem. Soc. Japan* **32**, 883.
10. Evans, E. A., Sheppard, H. C. and Turner, J. C. (1970) *J. Labelled Cmpds.* **6**, 76.
11. Shelton, K. R. and Clark, Jr., J. M. (1967) *Biochemistry* **6**, 2735.
12. Cuatrecasas, P. and Hollenberg, M. D. (1975) *Biochem. Biophys. Res. Commun.* **62**, 31.
13. Whitfield, P. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M., eds.), 2nd edn., p. 565. Oxford University Press, New York.