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ANALYSIS OF BIOGENIC AMINES IN THE BRAIN OF THE AMERICAN COCKROACH (*PERIPLANETA AMERICANA*) BY GAS CHROMATOGRAPHY–NEGATIVE ION CHEMICAL IONISATION MASS SPECTROMETRY

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SUMMARY

Biogenic amines in the brain of the American cockroach have been identified and quantified by an extraction–derivatisation procedure involving their reaction with ditrifluoromethylbenzoyl chloride (DTFMB) in the aqueous phase followed by extraction into an organic solvent, hydrolysis of phenolic esters and conversion of free hydroxyl groups to trimethylsilyl (TMS) ethers and subsequent analysis by gas chromatography–negative ion chemical ionisation mass spectrometry. The molecular ion of these DTFMB–TMS derivatives carried most of the ion current which made the method highly specific and gave a potential limit of detection below the picogram level. This method establishes unequivocally that the principal amines in cockroach brain are tyramine, *p*-octopamine, dopamine, 5-hydroxytryptamine and noradrenaline. In contrast to mammalian nervous tissue, the other positional isomers of octopamine, together with the isomeric synephrines, are absent.

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INTRODUCTION

It has been proposed that *p*-octopamine, dopamine and 5-hydroxytryptamine (5-HT) are neurotransmitters in the central nervous system of insects [1–4]. These compounds and the pathways involved in their biosynthesis and metabolism are of interest in relation to the systematic development of insecticides.

Since 1969 it has been possible to investigate the presence of *p*-octopamine in invertebrates by a sensitive radiochemical enzyme assay [1] which has been shown subsequently to be non-specific [5,6]. Recently an elegant method using high-performance liquid chromatography (HPLC) with electrochemical detection has been developed by Downer and co-workers [7,8] for the analysis of a range of biogenic amines in samples of invertebrate nervous tissue. This has an advantage over gas chromatographic–mass spectrometric (GC–MS) methodology since the amines may be analysed without derivatisation but it lacks the high specificity which is inherent in GC–MS namely, that compounds may be identified unequivocally by high-resolution capillary GC combined with monitoring of significant ions in the mass spectrum of a compound: these may afford characteristic ratios of ion intensities or their *m/z* values may be changed in a predictable manner, to provide additional proof of identity, by the preparation of a different derivative of the same chemical class.

In our earlier work [9] we have developed an extraction–derivatisation method suitable for the analysis of picogram amounts of biogenic amines by gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICIMS). In this report we describe an application of our method to the analysis of biogenic amines in the brain of the American cockroach, *Periplaneta americana*. This technique is specific and allows for the first time the unambiguous measurement of such substances: the sensitivity of the method permits their determination in the brains of individual insects (as opposed to the previous use of pooled samples).

EXPERIMENTAL

Gas chromatography–mass spectrometry

GC–MS in the NICI mode was carried out using a Hewlett-Packard 5988A gas chromatograph–mass spectrometer interfaced with an HP RTE-6/VM data system. The following mass spectrometric conditions were used: the instrument was tuned in the NICI mode to the ions at *m/z* 452, 595 and 633 from the perfluorotributylamine (PFTBA) calibrant, source temperature was 140 °C, electron energy 200 eV and methane reagent gas was introduced to give a source pressure ~0.9 Torr. The gas chromatograph was fitted with an HP-1 fused-silica column (either 12.5 m×0.2 mm, I.D. or 25 m×0.2 mm I.D.), helium

carrier gas was used with a head pressure of 0.54 bar for the 12.5 m column or 1.7 bar when the 25 m column was installed.

The GC conditions were as follows: injector temperature 250°C, transfer line temperature 280°C, the oven temperature was maintained at 100°C for 1 min, then programmed at 10°C min^{-1} to 300°C. Injections were made using a Grob splitless injection system.

Materials and reagents

All solvents used in extraction and derivatisation were HPLC grade (Rathburn Chemicals, Walkerburn, U.K.). Chemicals were obtained from the following sources: 3,5-ditrifluoromethylbenzoyl (DTFMB) chloride from Fluorochem (Glossop, U.K.); bistrimethylsilylacetamide (BSA), *tert.*-butyldimethylsilyl chloride (TBDMSCl), imidazole, tyramine, dopamine, *p*-octopamine, adrenaline, noradrenaline, 5-hydroxytryptamine (5-HT), deuterium chloride, deuterium bromide, deuterium oxide, deuterioacetic acid and chloroacetylchloride from Aldrich (Gillingham, U.K.); deuterium gas from M.S.D. Isotopes, Cambrian Gases (Croydon, U.K.); dihydroxybenzylamine (DHB) from Sigma (Poole, U.K.).

Synthesis of deuterium-labelled internal standards

[1,2,6-$^2H_3$]Dopamine deuteriochloride and [3,5-$^2H_2$]tyramine deuteriochloride were synthesised by established procedures [10]: briefly the products were obtained by heating the unlabelled compounds with 20% ^2H_1Cl in a sealed tube at 130°C for 40 h. [$^2H_3$]Dopamine deuteriochloride was obtained in 73% yield (m.p. 240–247°C decomp.; lit [11] m.p. 241°C decomp.[a]). The ^1H NMR spectrum showed that signals for the aromatic protons were absent and GC–NICIMS with selected-ion monitoring (SIM) of the M^- cluster of the DTFMB–trimethylsilyl (TMS) derivative gave the isotope composition as: $^2H_3$ 92.6%, $^2H_2$ 6.9%, $^2H_1$ 0.4% and $^2H_0$ 0.1%. [$^2H_2$]Tyramine was obtained in 92% yield (m.p. 245–250°C decomp.; lit. [11] m.p. 269°C[a]). The ^1H NMR spectrum gave no detectable signal for aromatic protons at C-3 and C-5 and GC–NICIMS with SIM of the M^- cluster of the DTFMB–TMS derivative gave the isotope composition as: $^2H_2$ 88.9%, $^2H_1$ 10.6% and $^2H_0$ 0.5%. [α,α,β,3,5-$^2H_5$]-*p*-Octopamine deuteriobromide was prepared as described in our previous work [12], with certain modifications. Anisole was subjected to Friedel–Crafts acylation with chloroacetyl chloride and the product reacted with dibenzylamine to give 2-dibenzylamino-1-(4-hydroxyphenyl)ethanone hydrochloride (67.4%, m.p. 235–241°C decomp.; lit. [12] m.p. 232–233°C decomp.). This compound (100 mg) was dissolved in [$^2H_1$]ethanol–20% ^2H_1Br–[$^2H_4$]acetic acid (0.9:2:0.8, v/v) and the solution heated in a sealed tube (110°C, 160 h) to give colourless crystals (95 mg, 95%, m.p. 235–241°C) after removal

[a]This refers to the m.p. of the non-deuteriated compound.

of the solvent. The product (1.48 g) from several batches was dissolved in [$^2H_1$]ethanol–^2H_2O–[$^2H_4$]acetic acid (16.6:3.4:3.4, v/v) and reduced by stirring the solution with palladium on charcoal (10%, 334 mg) in an atmosphere of deuterium (190 ml) until the absorption of deuterium ceased (160 h). The product was recrystallised from ethanol–ethyl acetate (1:20) yielding [$^2H_5$]-*p*-octopamine deuteriobromide (594 mg, 70%, m.p. 164–165°C; lit. [11] *p*-octopamine hydrochloride m.p. 170°C decomp.[a]). The ^1H NMR spectrum indicated that protons in the α-, α-, β-, 3- and 5-positions were absent. GC–NICIMS analysis using M^- of the compound (as the DTFMB–TMS derivative) gave $^2H_5$ 42.1%, $^2H_4$ 26.4%, $^2H_3$ 14.2%, $^2H_2$ 8.1%, $^2H_1$ 7.6% and $^2H_0$ 1.6%.

Extraction and derivatisation

Adult cockroaches were kept at −20°C for 20 min in a freezer to anaesthetise them and were then transferred to a container and kept in ice until dissection. The brain (cerebral ganglion) was removed directly from the anaesthetised insect and either processed immediately or frozen by placing it on a piece of dry ice followed by storage at −20°C until required. Three different methods of extraction were used (the derivatisation procedure being the same in each case) and, in all cases, internal standards were added to the extraction medium prior to homogenisation.

(1) The tissue was rapidly added to boiling water (1.0 ml) followed by manual homogenisation in a ground-glass homogeniser before boiling the homogenate for a further 2 min. The suspension was then transferred to a centrifuge tube and centrifuged for 30 min (2500 *g*). The supernatant liquid was transferred to a sample tube (3.5 ml) and the pH adjusted to 7.2 with an equal volume of 1 *M* potassium phosphate buffer (pH 7.2). The solution was then shaken with DTFMBCl (2 μl) for 10 min, extracted with ethyl acetate (2×1.5 ml) and the organic layer shaken with aqueous ammonia (0.5 ml, 10 *M*) on a wrist-action shaker for 10 min to hydrolyse phenolic ester groups [13]. The organic layer was dried by passing it through anhydrous sodium sulphate and the solvent removed under a stream of nitrogen. The residue was then reacted (10 min, 70°C) with BSA (30 μl), after which the reagent was removed under a stream of nitrogen and the residue redissolved in ethyl acetate (50 μl). Alternatively, in order to form TBDMS derivatives, the residue was reacted (15 min, 70°C) with TBDMSCl and imidazole (each 1 *M* in dimethylformamide, 30 μl). The reaction mixture was then diluted with ethyl acetate (100 μl) followed by hexane (900 μl) and passed through Sephadex LH20 (ca. 3 cm) in a Pasteur pipette; the LH20 was then washed with hexane (1 ml) and the eluate concentrated to 50 μl for GC–MS analysis.

(2) The tissue was homogenised as before but ice-cold 0.3 *M* perchloric acid (1 ml) was used as the medium: after centrifugation (8000 *g*, 5 min) to remove

[a]This refers to the m.p. of the non-deuteriated compound.

protein the acid was neutralised with aqueous $KHCO_3$ (0.1 *M*, 2 ml). The mixture was centrifuged (8000 *g*, 5 min) again to remove precipitated potassium perchlorate and the supernatant was then treated as described above before GC–MS analysis.

(3) The tissue was homogenised in 1 ml of 0.1 *M* hydrochloric acid. The extract was centrifuged for 30 min at 2500 *g* and the supernatant was treated as described in (1) before GC–MS analysis.

All of the deuteriated internal standards (20 ng each) were subjected to the extraction procedures – which did not change their isotopic composition appreciably. A linear response was obtained for the ion current carried by $M^{\cdot}$ of *p*-octopamine, *p*-tyramine and dopamine (over the range 0.5–50 ng) against that observed for $M^{\cdot}$ of the corresponding deuteriated isotopomers after dissolving the compounds in the appropriate extraction medium, followed by the addition of phosphate buffer and then the usual derivatisation procedure. Standard mixtures (20 ng) of deuteriated and undeuteriated amines (1:1) were derivatised and analysed when each batch of samples was processed and blanks were also performed on the reagents on each occasion.

RESULTS AND DISCUSSION

Fig. 1 compares the selected-ion trace containing the $M^{\cdot}$ ions of the DTFMB–TMS derivatives (*m/z* 451, 542, 523 and 540) of [$^2H_2$]tyramine, [$^2H_5$]octopamine, dihydroxybenzylamine and [$^2H_3$]dopamine, respectively with that of a selected-ion trace containing the molecular ions for the same derivatives of endogenous *p*-tyramine, *p*-octopamine, dopamine, noradrenaline and 5-HT (*m/z* 449, 537, 537, 625 and 560, respectively) extracted from cockroach brain. We have shown previously [9] that DTFMB–TBDMS derivatives of biogenic amines yield mass spectra where the molecular ion (together with its isotope peaks) carried > 60% of the ion current under NICI conditions; this is illustrated by Fig. 2 which shows the mass spectrum for the DTFMB–TMS derivative of *p*-octopamine. The method of selective formation of DTFMB amides of biogenic amines has the additional advantage that the silylating reagent may be changed in order to shift both the *m/z* value of the molecular ion and the retention time of the derivative to ensure that its identification is unequivocal. Thus we have used various alkylated dimethylsilyl chlorides to produce a change in these parameters; this procedure afforded high specificity in the identification of nanogram amounts of biogenic amines. Fig. 3 compares the selected-ion traces of the DTFMB–TBDMS derivatives of deuteriated internal standards and the corresponding undeuteriated amines from cockroach brain. Table I indicates the retention indices and base peak (in each case the molecular ion) for a number of biogenic amines as their DTFMB–TMS and DTFMB–TBDMS derivatives. 5-HT could not be detected after reaction of the extract of brain with TBDMSCl–imidazole reagent, although we did con-

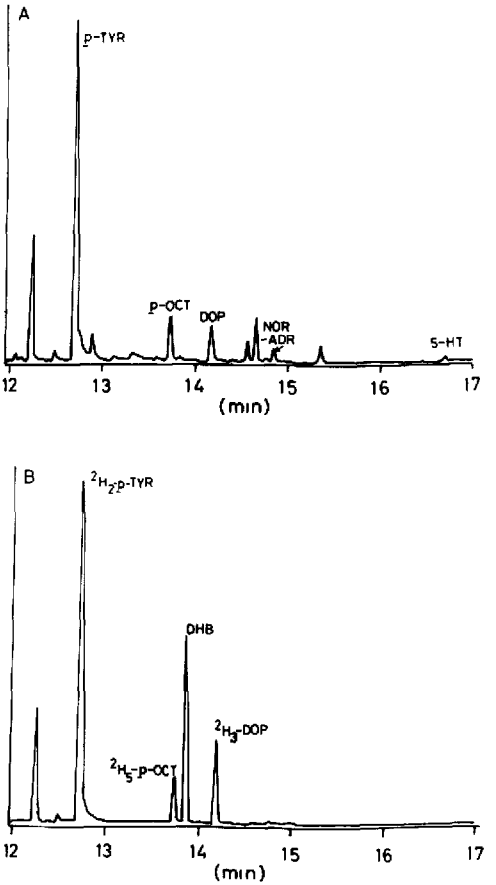

Fig. 1. Selected-ion traces from cockroach brain (with addition of 20 ng of each internal standard) after treatment to yield a DTFMB–TMS derivative. (A) Selected ions for the DTFMB–TMS derivatives of endogenous *p*-tyramine (*m/z* 449), *p*-octopamine (*m/z* 537), dopamine (*m/z* 537), noradrenaline (*m/z* 625) and 5-hydroxytryptamine (*m/z* 560). (B) Selected ions for the DTFMB–TMS derivatives of the internal standards [$^2H_2$]-*p*-tyramine (*m/z* 451), [$^2H_5$]-*p*-octopamine (*m/z* 542), dihydroxybenzylamine (*m/z* 523) and [$^2H_3$]dopamine (*m/z* 540).

firm its presence using the method of Markey et al. [14], which involves the formation of the spirocyclic pentafluoropropionyl (PFP) derivative.

The average amounts per brain of most of the amines estimated show good agreement with the estimates for such compounds in cockroach brain previously obtained by different methods (Table II). Concentrations of *p*-octopamine, noradrenaline and 5-HT are very similar to previous figures, although the concentration of dopamine was rather lower than that found by both Martin et al. [7] and Sloley and Owen [15]. The coefficients of variation of the present estimates were generally higher than those found by other methods, an inevitable consequence of working with individual, rather than pooled, in-

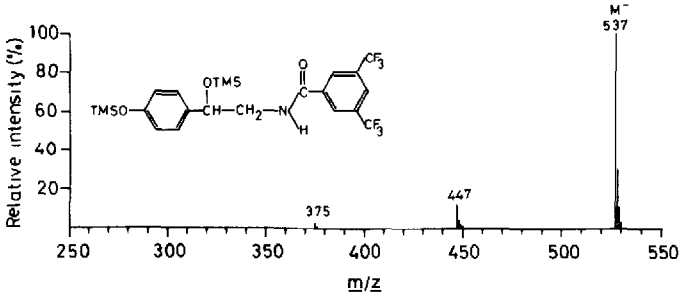

Fig. 2. NICI mass spectrum of *p*-octopamine ditrifluoromethylbenzylamide-ditrimethylsilyl ether derivative.

sect brains. Attempts to limit this variation by relating concentration to weights of protein in extracted tissues did not diminish the standard deviation. Consequently, concentrations are expressed as amount per brain. In any case, as discussed by Evans [16], this may be a more valid means of expression.

Only in the case of tyramine do the present results differ sharply from those obtained previously. The concentration of 3.4 ng per brain is more than ten times that found by Martin et al. [7]. The literature does not seem to offer any other recent, relevant estimates of this amine, in which hitherto there has been little interest. The work of Mir and Vaughan [17], with the locust brain, showed the importance of tyramine in the biosynthesis of both octopamine and dopamine. Furthermore, the work in vitro with the isolated ganglia of *Manduca sexta* showed that label from [^{14}C]tyrosine accumulated in tyramine to a greater extent than either dopamine or octopamine [18]. Further work will be required to determine whether or not the high concentration of tyramine represents a function of greater significance than that of a biosynthetic intermediate. Our final results for the levels of biogenic amines in cockroach brain differ from those reported in a preliminary communication [20]. The divergence is most marked for dopamine where our earlier extraction method using boiling water was unsatisfactory for the quantitation of this compound even though the use of a deuterated internal standard should have compensated for differences in the extraction method. Difficulties arose in the quantitation of 5-HT since poor linearity was observed with regard to its response relative to a number of internal standards; [2,4,6,7-^{2}H$_4$]-5-HT was prepared as an internal standard but, although the compound appeared to be satisfactory initially, it did not seem to be very stable and afforded unsatisfactory results after storage (in a desiccator in the dark). Latterly it was established that DHB yielded a satisfactory calibration curve relative to 5-HT.

In contrast to our previous work [6], which showed that all the positional isomers of octopamine (together with *m*- and *p*-synephrine) occur naturally in mammals, *m*- and *o*-octopamine and the isomeric synephrines are absent

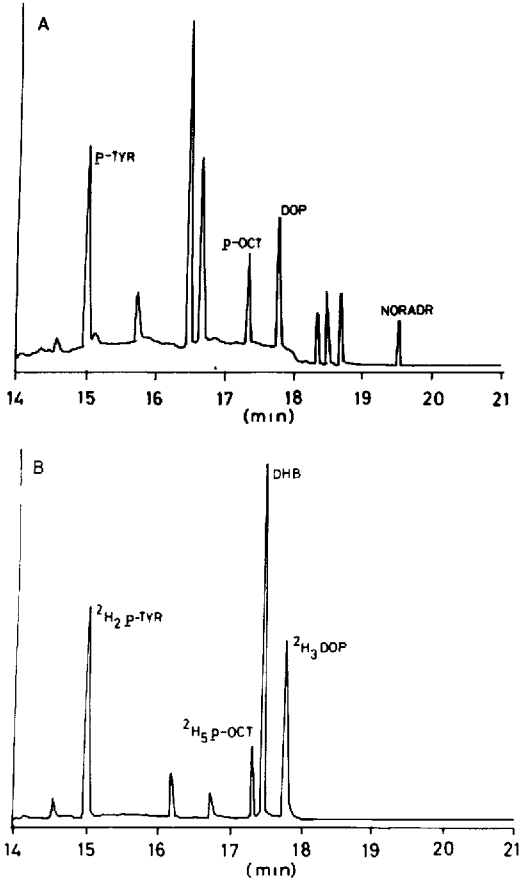

Fig. 3. Selected-ion traces from cockroach brain (with addition of 20 ng of each internal standard) after treatment to yield DTFMB–TBDMS derivatives. (A) Selected ions for the DTFMB–TBDMS derivatives of endogenous *p*-tyramine (*m/z* 491), *p*-octopamine (*m/z* 621), dopamine (*m/z* 621) and noradrenaline (*m/z* 751) (B) Selected ions for the DTFMB–TBDMS derivatives of the internal standards [$^2H_2$]-*p*-tyramine (*m/z* 493), [$^2H_5$]-*p*-octopamine (*m/z* 626), dihydroxybenzylamine (*m/z* 607) and [$^2H_3$]dopamine (*m/z* 624).

from cockroach brain. Two major unidentified peaks (*m/z* 537) were observed in the selected-ion traces; this *m/z* value corresponded to the molecular ion of the DTFMB–TMS derivative of the isomeric octopamines but the retention time of these peaks (both as DTFMB–TBDMS derivatives) did not correspond to those observed for the *o*- and *m*-isomers of octopamine. However it was not possible to rule out the possibility that these unidentified compounds may be isomers of dopamine.

We also compared our method of extraction with boiling water to the more usual extraction of brain tissue with perchloric acid, followed by precipitation of the perchlorate ion as the potassium salt. We found that the latter method

TABLE I

RETENTION INDEX DATA (I) AND BASE PEAKS IN THE NICI MASS SPECTRA OF DTFMB-xTMS AND DTFMB-xTBDMS DERIVATIVES OF BIOGENIC AMINES

Compound	DTFMB-xTMS		DTFMB-xTBDMS	
	I (Kovats indices)	Base peak (m/z)	I (Kovats indices)	Base peak (m/z)
p-Tyramine	2114	449	2367	491
m-Synephrine	2220	551	2615	635
m-Octopamine	2226	537	2615	621
p-Synephrine	2250	551	2688	635
p-Octopamine	2256	537	2692	621
Dihydroxybenzylamine	2300	523	2672	607
Dopamine	2314	537	2765	621
Adrenaline	2386	639	3022	765
Noradrenaline	2391	625	3010	751
5-Hydroxytryptamine[a]	2846	560	–	–

[a]TBDMS derivative will not form readily.

TABLE II

CONCENTRATIONS OF AMINES IN INDIVIDUAL BRAINS (CEREBRAL GANGLIA) OF THE COCKROACH (*PERIPLANETA AMERICANA*)

The figures are the average obtained by the following authors and methods: (a) present authors, GC–NICIMS; (b) Martin et al. [7], HPLC–electrochemical detection; (c) Dymond and Evans [19], radioisotope enzyme assay; (d) Sloley and Owen [15], ion-exchange chromatography–electrochemical detection. The figures of the present authors are the averages ($\pm$S.D.) of the number of estimates on individual insects shown in the parentheses. The other figures are shown (without S.D.) for purposes of comparison.

Amine	Concentration (ng per brain)			
	a	b	c	d
p-Tyramine	3.4 ± 1.6 ($n=34$)	0.3	–	–
p-Octopamine	3.7 ± 1.9 ($n=43$)	3.1	2.1	–
Dopamine	2.7 ± 0.6 ($n=12$)	6.3	4.1	6.4
Noradrenaline	0.8 ± 0.3 ($n=11$)	1.1	0.9	0.8
5-Hydroxytryptamine	3.2 ± 2.3 ($n=12$)	2.6	–	4.9

gave much poorer recoveries of the biogenic amines whilst the method of extraction with boiling water not only gave satisfactory recoveries but also minimised the risk of exchange of deuterium for hydrogen in the internal standards. Moreover, our discovery that the passage of aqueous solutions of very small quantities (5 ng or less) of deuteriated biogenic amines through cationic ion-exchange resins (e.g. Dowex 50W-X8) caused substantial replacement (ca.

90%) of deuterium by hydrogen (even for deuterium in the side-chain) led us to develop extraction–derivatisation procedures for the analysis of these substances. Latterly extraction with 0.1 *M* hydrochloric acid has been adopted as our general method since extraction with boiling water was less satisfactory for the analysis of catecholamines such as dopamine. Extraction with boiling water and with hydrochloric acid gave similar results for the monophenolic amines.

Thus we have developed a highly sensitive and specific method for the identification and quantitation of biogenic amines in biological systems where variation in the derivatisation reagent at the silylation stage allowed a shift in the *m/z* values of characteristic ions and retention time of a given compound. These permit its unequivocal identification in contrast to methods such as HPLC, which rely on retention time alone. These derivatives have the advantage over those previously developed (where pentafluoropropionic anhydride was used to make N- and O-PFP derivatives [5]) in that the molecular ion carried most of the ion current and there were negligible amounts of reagent-specific ions. In the mass spectrum of corresponding PFP derivatives a large amount of the ion current is carried by the reagent-specific ion (due to $C_2F_5CO_2^-$) and the proportion of ion current which it carries is very sensitive to slight changes in the temperature and pressure (of reagent gas) in the ion source. The stability of the molecular ion of DTFMB–silyl derivatives allows for high sensitivity in the detection of compounds and makes quantitation more reliable when an analogue (rather than an isotopomer) is used as the internal standard since the fragmentation pattern is not affected by changes in ion source conditions.

We are currently investigating (using deuteriated precursors) the biosynthesis of biogenic amines in invertebrate nervous tissue and also the determination of the absolute configuration of those endogenous amines using an extension of our extraction–derivatisation procedure employing chiral derivatisation reagents.

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