Pergamon

Tetrahedron 56 (2000) 3303–3307

TETRAHEDRON

Diketopiperazines as Advanced Intermediates in the Biosynthesis of Ecteinascidins

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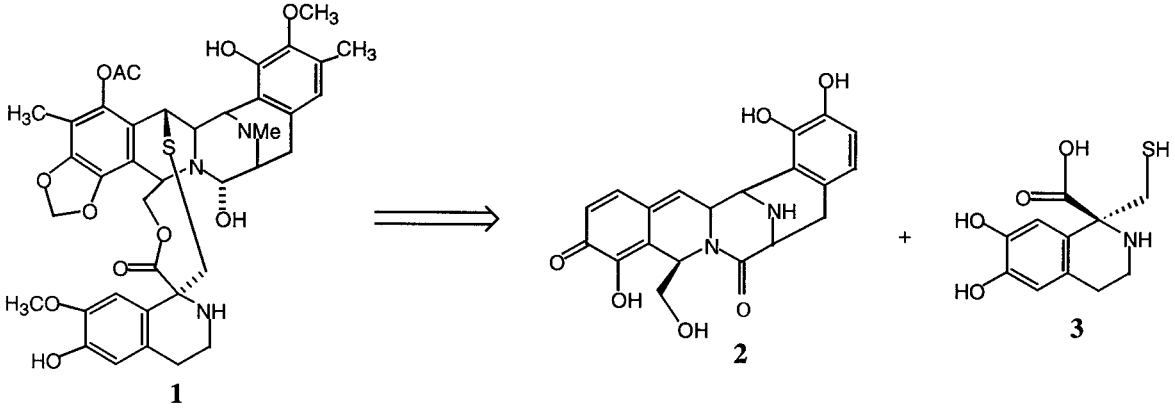
Received 29 January 2000; accepted 21 March 2000

Abstract—The diketopiperazines of phenylalanine, tyrosine and DOPA have been synthesized from ^{14}C-labeled amino acids and tested as intermediates in the biosynthesis of the ecteinascidins. Biosynthetic experiments were performed using an enzyme preparation of the tunicate *Ecteinascidia turbinata,* the source of the ecteinascidins. Tyrosine and DOPA diketopiperazines were both transformed to the ecteinascidins whereas the diketopiperazine of phenylalanine was not modified. It is now apparent that the biosynthesis of the ecteinascidins is initiated by the condensation of tyrosine to its cyclic dipeptide followed by a subsequent oxidation to the diketopiperazine of DOPA. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

The ecteinascidins are a family of tetrahydroisoquinoline alkaloids isolated from the tunicate *Ecteinascidia turbinata*.[1] Ecteinascidin 743 (ET 743) is a candidate for the treatment of several human cancers, in particular lung cancer and melanoma. Ecteinascidins have been shown to kill tumor cells in vitro,[2] inhibit tumor growth in vivo,[2] suppress allograft rejection,[2b] diminish host reactions to tissue grafts and inhibit lymphocyte proliferation.[3] The ecteinascidins are structurally related to the safracins[4] and saframycins[5] from microbes as well as the renieramycins[6] and xestomycin[7] from sponges.

As initially suggested by Rinehart and co-workers,[1b] an inspection of the structure of ecteinascidin 743 (**1**) (Scheme 1) suggests that this metabolite could be derived from intermediates **2** and **3**. (It is, however, conceivable that intermediate **2** reacts first with cysteine [or β-mercaptopyruvic acid] and then with dopamine rather than with intact **3**.) Pentacyclic intermediate **2** appears to be derived from a diketopiperazine of either phenylalanine (**4**), tyrosine (**5**) or DOPA (**6**), likely via intermediates **7** and **8** (Scheme 2).



Scheme 1. Possible biosynthetic origin of ecteinascidin 743.

Keywords: biosynthesis; alkaloids; marine metabolites; antitumor compound.

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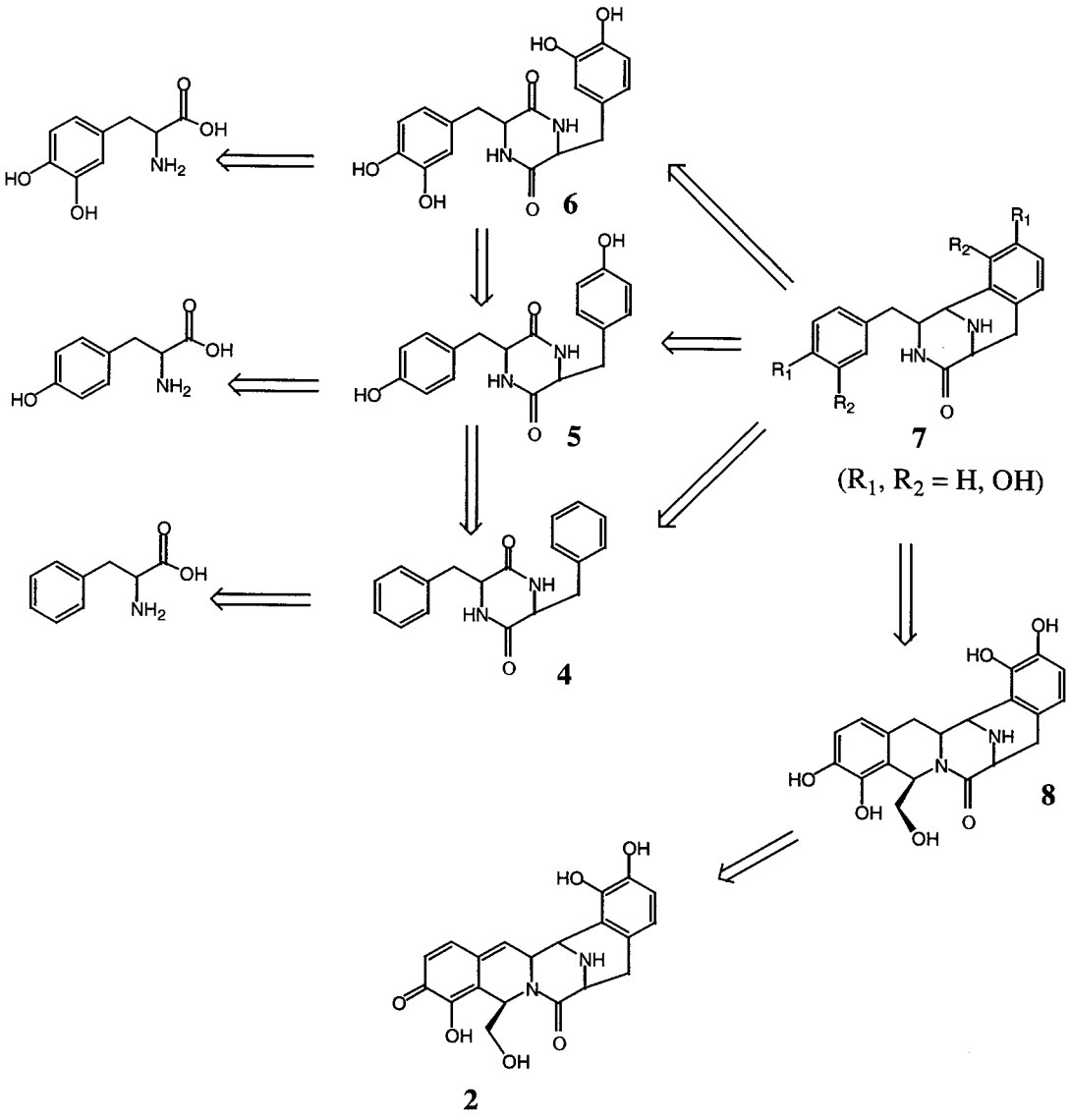
PII: S0040-4020(00)00249-0

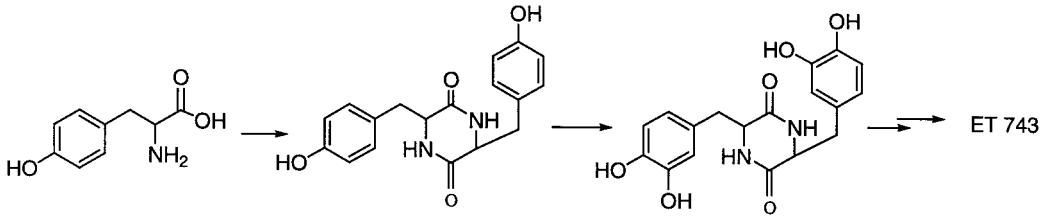


Scheme 2. Potential diketopiperazine intermediates in ecteinascidin biosynthesis.

Results and Discussion

We recently reported in vitro conditions to evaluate the biosynthetic transformation of putative ecteinascidin precursors and demonstrated the conversion of labeled amino acids to ecteinascidin 743.[8] A cell-free extract of *E. turbinata* afforded 3000 dpm ET 743 when incubated with tyrosine (5.5×10^6 dpm) and 2100 dpm ET 743 when incubated with cysteine (11×10^6). Herein, we report the results of incubating radiolabeled diketopiperazines of phenylalanine, tyrosine and DOPA with a cell-free extract of *E. turbinata*.

Diketopiperazines **4**, **5** and **6** were prepared by standard methods.[9] While **4** and **5** are known compounds, **6** had not been previously reported. The syntheses of **4** and **5** proceeded as outlined in Jung's report[9] and proved to be applicable to both medium scale and 1 mg scale preparations. Conditions were established to perform this synthesis on a 1 mg scale to define appropriate techniques for the



Scheme 3. Initial steps of the biosynthesis of ecteinascidin 743.

Table 1. Incorporation of labeled precursors into ecteinascidin 743

Radiolabeled precursor	Total radioactivity used (dpm)	Recovered radioactivity in ET 743 (dpm)	Conversion (%)
[U–^{14}C] Tyrosine	1.21×10^7	2,000	0.016
[U–^{14}C] Phe DKP	6×10^5	Background	0
[U–^{14}C] Tyr DKP	8×10^5	11,530	1.4
[U–^{14}C] DOPA DKP	4×10^5	39,960	11.4

synthesis of radiolabeled compounds required for the biosynthetic studies.

In general, the methyl ester of the amino acid was condensed with an equimolar amount of *t*-BOC protected amino acid using DCC. The acyclic dipeptide was then treated with formic acid to afford the cyclic dipeptide. The medium scale reactions produced cyclic dipeptides **4** and **5** in yields of 93 and 96%, respectively. The synthesis of the diketopiperazine of DOPA (**6**) had not been previously reported and in fact, to our knowledge, there are no reports describing the incorporation of DOPA into peptides.[10] Interestingly, we found that **6** could be readily prepared in an overall yield of 52% if all transformations were performed under argon. NMR analysis of this diketopiperazine revealed the presence of small amounts of dicyclohexylurea, a byproduct of the coupling reaction, which proved difficult to remove without degradation of product.

The ^{14}C-labeled diketopiperazines **4**, **5** and **6** were prepared from 50 μCi each of phenylalanine ethyl ester, tyrosine and DOPA. Radiolabeled tyrosine and DOPA esters are not commercially available and their methyl esters were synthesized by the treatment of tyrosine with acidic 2,2-dimethoxy propane.[11] The cyclic dipeptides were purified by vacuum chromatography over ODS, followed by repeated RPHPLC using an acetonitrile/water (0.1% TFA) gradient to afford radiochemically pure material.

All biosynthetic experiments were performed using a cell-free extract of *E. turbinata*. We found that the activity of these enzyme preparations was greatly dependent upon the handling of the organism following the collection. Optimum results were obtained when the tunicate was immediately flash frozen in liquid nitrogen upon collection. The frozen samples were then transported to the laboratory at −80°C and maintained at this temperature. The tunicate (250 g) was then ground to a fine powder in a chilled mortar and pestle, added to a phosphate buffer (500 mL) at pH 7.7 containing DTT, EDTA and leupeptin, centrifuged at 10,000 g and the supernatant stored in 30 mL aliquots at −80°C.

To assess the viability of the cell-free extract, an aliquot was incubated with [U–^{14}C] tyrosine (2.5 μCi), a known ecteinascidin precursor, as previously described.[8] Recovery of ET 743 with an activity of 2000 dpm (0.017% conversion) indicated a viable extract. As indicated in Table 1, diketopiperazines **5** and **6** were both transformed to ET 743 while **4** was not modified. Radiochemical purity of ET 743 was established by repeated HPLC analysis. The extremely trace quantities of this metabolite precluded degradative work and recrystallization to constant specific activity. In all cases in which radioactive product is reported, the radioactivity of the ecteinascidin HPLC peak was at least 100 times that of the eluent immediately prior to, and immediately following, this fraction.

Diketopiperazine **6** afforded ET 743 with a radiochemical yield of 11.4%, significantly higher than that from **5** (Table 1). The radiochemical transformation of both diketopiperazines was at least two orders of magnitude greater than that for tyrosine as is to be expected for committed biosynthetic intermediates. These data indicate that the diketopiperazine of tyrosine (**5**) is the first committed intermediate in ecteinascidin biosynthesis and suggest that prior to any further cyclization, **5** is oxidized to **6**. It is conceivable that these diketopiperazines are not the actual intermediates but instead are transformed to the ecteinascidins through a reduction to the aminol. Recent analysis of putative peptide synthetases SafB and SafA involved in saframycin biosynthesis suggest that a linear tetrapeptide is a precursor.[12] It has been further suggested that this tetrapeptide is likely to be reductively released to afford Ala-Gly-Tyr-Tyr-CHO prior to an intramolecular cyclization to a heminaminal structure.[13] To address this possibility in ecteinascidin biosynthesis, we examined the conversion of **5** to **6** in our enzyme preparation of *E. turbinata*. This oxidation was confirmed from an analysis of the tyrosine diketopiperazine incubation mixture. Addition of **6** as 'cold carrier' to the quenched incubation of labeled **5** facilitated the purification of radioactive DOPA diketopiperazine (4300 dpm),[14] providing strong support for the direct involvement of diketopiperazines **5** and **6** in ecteinascidin biosynthesis.

We have provided evidence that the biosynthesis of the ecteinascidins is initiated by the condensation of tyrosine to its cyclic dipeptide followed by a subsequent oxidation to the diketopiperazine of DOPA (Scheme 3). Further experiments directed at identifying the fate of **6** are currently underway.

Experimental

General

^{1}H NMR spectra were recorded on a Unity Inova Varian 500 instrument at 500 MHz. IR spectra were obtained on a Mattson 4020 instrument. HPLC purifications were performed on either a PE Series 410 pump equipped with a PE 325 detector or an HP 1090 instrument. Incubations were performed at 27°C in a Labline 4628 Environmental shaker at 220 rpm. Radioactivity was measured using an LKB Wallac 1219 Rackbeta scintillation counter. All solvents used were Optima grade and the phenylalanine methyl ester, tyrosine methyl ester, DOPA methyl ester,

EDTA, BSA, DTT and leupeptin were purchased from Sigma Chemical Co. Radiolabeled amino acids were purchased from American Radiolabeled Chemicals Inc.

Collection of animal material and preparation of the cell-free extract

Colonies of *E. turbinata* were collected at a depth of 0.1–0.5 m in a mangrove community in Long Key, Florida. The freshly collected tunicate was quickly cleaned of extraneous material, flash frozen, and ground to a fine powder in a chilled mortar and pestle. This powder (250 g) was added to 500 mL of a phosphate buffer at pH 7.7 containing DTT (290 mg), EDTA (550 mg), BSA (375 mg) and leupeptin (0.5 mg). The mixture was vortexed for 5 min and then centrifuged at 10,000 g and the supernatant stored in 30 mL aliquots at −80°C.

Synthesis of *cyclo*-(L-Phe-L-Phe) (4)

Medium scale reaction conditions: To a solution of L-phenylalanine methyl ester hydrochloride (323 mg) and N-*t*-BOC-L-phenylalanine (397 mg) in 3 mL DMF and 12 mL acetonitrile at 0°C was added triethylamine (210 μL) followed by DCC (309 mg). The mixture was stirred at 0°C for 2 h and allowed to stand at this temperature for 12 h. The dicyclohexyl urea was filtered and washed with EtOAc. The combined filtrate was evaporated under nitrogen to afford a gummy residue, which was partitioned between EtOAc and H_2O. The organic layer was washed sequentially with 0.5N HCl, H_2O, 0.5N $NaHCO_3$ and brine, and then dried over sodium sulfate, and evaporated to afford a white solid. Purification by flash chromatography (70% EtOAc/hexane) gave a colorless foam of *N*-(*tert*-butoxycarbonyl)-L-phenylalanyl-L-phenylalanine methyl ester. This dipeptide was stirred with formic acid (20 mL) at ambient temperature for 2 h and subsequently evaporated. The residue was dissolved in *sec*-butanol (40 mL) and toluene (10 mL) and the solution refluxed for 2 h. The mixture was concentrated and purified by flash chromatography over ODS using a step gradient of H_2O (0.1% TFA), H_2O (0.1% TFA)/CH_3CN 8:2, H_2O (0.1% TFA)/CH_3CN 6:4, H_2O (0.1% TFA)/CH_3CN 4:6. Final purification with RPHPLC using an Altex Ultrasphere column (10 mm×25 cm), monitoring 230 nm with a linear gradient of 75% H_2O (0.1% TFA): 25% CH_3CN to CH_3CN over 20 min afforded *cyclo*-(L-Phe-L-Phe) (**4**) in an overall yield of 93%. Melting point, IR, ^1H NMR and ^{13}C NMR were in accord with literature values.[9a]

Radiochemical synthesis: To a solution of L-phenylalanine ethyl ester hydrochloride (50 μCi) and *N-t*-BOC-L-phenylalanine (1.1 mg) in 50 μL DMF and 450 μL acetonitrile at 0°C was added triethylamine (10 μL) followed by DCC (1 mg). The mixture was stirred at 0°C for 2 h and allowed to stand at this temperature for 12 h. The dicyclohexyl urea was filtered and washed with EtOAc. The residue was washed sequentially with 400 μL each of 0.5N HCl, H_2O, 0.5N $NaHCO_3$ and brine and the organic layer evaporated under nitrogen. The dipeptide was stirred with formic acid (2 mL) at ambient temperature for 2 h and subsequently evaporated. The residue was dissolved in *sec*-butanol (1.5 mL) and toluene (1 mL) and the solution refluxed for 2 h. The mixture was concentrated and directly purified by RPHPLC using a Vydac column (4.6×150 mm^2), monitoring 230 nm with a linear gradient of 75% H_2O (0.1% TFA): 25% CH_3CN to CH_3CN over 20 min to afford 2.4×10^6 dpm of ^{14}C-*cyclo*-(L-Phe-L-Phe) (**4**).

Synthesis of *cyclo*-(L-Tyr-L-Tyr) (5). The medium scale synthesis of *cyclo*-(L-Tyr-L-Tyr) was performed as described for *cyclo*-(L-Phe-L-Phe). The overall yield was 96% and the melting point and IR, ^1H NMR and ^{13}C NMR were in accord with literature values.[9a]

The radiochemical synthesis of **5** was performed as described for **4** to afford 2.0×10^6 dpm. [^{14}C–U] Tyrosine methyl ester was synthesized as follows: a solution of tyrosine (50 μCi) in 1.5 mL 2,2-dimethoxy propane was treated with 100 μL of HCl (12N) and the mixture stirred for 18 h. The reaction mixture was passed through a column of silica and concentrated to dryness.

Synthesis of *cyclo*-(L-DOPA-L-DOPA) (6). The medium scale synthesis of *cyclo*-(L-DOPA-L-DOPA) was performed as described for *cyclo*-(L-Phe-L-Phe) in an overall yield of 52% with the exception that all transformations were performed under argon: IR (KBr pellet) 3404, 3320, 1671 cm^{-1}; ^1H NMR (d_6-DMSO, 500 MHz) δ 8.7 (s, OH), 8.1 (br. s, NH), 6.55 (dd, 2 H, J=8.5, 1.5 Hz), 6.51 (br. s, 2 H), 6.36 (d, 2 H, J=8.0 Hz), 4.15 (m, 2 H), 2.75 (ddd, 2 H, J=4 Hz), 1.9 (m, 2 H); HRFABMS calcd for $C_{18}H_{19}N_2O_6$ $(M+1)^+$ 359.1238, found 359.1243.

The radiochemical synthesis of **6** was performed (under argon) as described for **4** to afford 6.0×10^5 dpm.

Incubation of ^{14}C-labeled tyrosine and DKPs

The ^{14}C-labeled precursor was added to 30 mL of the cell-free extract fortified with 1 mg each of ATP, NADH and NADPH and the mixture incubated at 200 rpm at 27°C for 24 h. The incubations were quenched by shaking with 30 mL EtOAc, which was subsequently removed in vacuo and the aqueous mixture dried by lyophilization. The ecteinascidins were purified by a reversed phase (C-18) vacuum flash chromatography using a step gradient of H_2O (0.1% TFA), H_2O (0.1% TFA)/CH_3CN 8:2, H_2O (0.1% TFA)/CH_3CN 6:4, H_2O (0.1% TFA)/CH_3CN 4:6. The third fraction was concentrated to dryness and subjected to RPHPLC purification using a C-18 Vydac column (4.6×250 mm^2) and monitoring 230 nm with a mobile phase of H_2O (0.1% TFA)/CH_3CN 82:18.

Acknowledgements

Financial support for this research was provided by a National Sea Grant award (R/LR-MB-6) and the American Cancer Society (RPG-97-170-01). The staff of the Keys Marine Laboratory is gratefully acknowledged for providing field support. A. Wright (Harbor Branch Oceanographic Inst.) is acknowledged for the generous gift of a sample of ecteinascidin 743. We thank S. Kerr for assistance with the preparation of cell-free extracts. Mass spectra were

recorded at the University of Florida and their assistance is acknowledged.

References

1. (a) Wright, A. E.; Forleo, D. A.; Geewananda, P. G.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* **1990**, *55*, 4508–4512. (b) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512–4515.
2. (a) McCumber, L. J.; Trauger, R.; Sigel, M. M. *Int. Symp. Fish Biologics* **1981**, *49*, 289–294. (b) Lichter, W.; Wellham, L. L.; Van der Werf, B. A.; Middlebrook, R. E.; Sigel, M. M. In *Food-drugs from the Sea: Proceedings*; Worthen, L. R., Ed.; Marine Technology Society: Washingnton, D.C., 1972; pp 7–127.
3. Lichter, W.; Lopez, D. M.; Wellham, L. L.; Sigel, M. M. *Proc. Soc. Exp. Mar. Biol. Med.* **1975**, *150*, 475–478.
4. Ikeda, Y.; Matsuki, H.; Ogawa, T.; Munakata, T. *J. Antibiot.* **1983**, *36*, 1284.
5. Trowitzssch, W.; Irschik, H.; Reichenbach, H.; Wray, V.; Hofle, G. *Liebigs Ann. Chem.* **1988**, 475.
6. He, H.-Y.; Faulkner, D. J. *J. Org. Chem.* **1989**, *54*, 5822.
7. Gulavita, N. K.; Scheuer, P. J.; De Silva, E. D. Abstracts, Indo-United States Symposium on Bioactive Compounds from Marine Organisms, Goa, India, 1989; p 28.
8. Kerr, R. G.; Miranda, N. F. *J. Nat. Prod.* **1995**, *58*, 1618–1621.
9. (a) Jung, M. E.; Rohloff, J. C. *J. Org. Chem.* **1985**, *50*, 4909–4913. (b) Nitecki, D. E.; Halpern, B.; Westley, J. W. *J. Org. Chem.* **1967**, *33*, 864–866.
10. Solid-phase peptide synthesis. In *Methods in Enzymology*; Fields, G. B., Ed.; Academic: Orlando, 1997; Vol. 289.
11. Rachele, J. R. *J. Org. Chem.* **1963**, *17*, 2898.
12. Pospiech, A.; Bietenhader, J.; Schupp, T. *Microbiology* **1996**, *142*, 741–746.
13. (a) Ehmann, D. E.; Gehring, A. M.; Walsh, C. T. *Biochemistry*, **1999**, *38*, 6171–6177. (b) Konz, D.; Marahiel, M. A. *Chem. Biol.* **1999**, *6*, 39–48.
14. The diketopiperazines are not present in isolable quantities in the extract of *E. turbinata* and thus the recovered activity represents a high level of specific activity.