Pergamon

Tetrahedron Letters, Vol. 37, No. 46, pp. 8305-8308, 1996

0040-4039/96 $15.00 + 0.00

PII: S0040-4039(96)01943-0

In Vitro Biosynthetic Studies of the Bryostatins, Anti-Cancer Agents from the Marine Bryozoan *Bugula neritina.*

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Abstract. Acetate, S- adenosylmethionine and glycerol have been identified as key building blocks of the anticancer polyketide, bryostatin 1, through a series of *in vitro* biosynthetic experiments. The active enzyme preparation was fortified with ATP, Mg^{2+}, NADH and NADPH.

The bryostatins 1 are a series of biologically active polyketide macrolides present in trace quantities in the marine bryozoan *Bugula neritina*.[1] Bryostatin 1 exhibits selective activity against B-cell lymphomas and leukemias,[2] and directly stimulates bone marrow progenitor cells to form colonies that functionally activate neutrophils.[3] Further, bryostatin 1 activates protein kinase C[4] and has immunomodulatory activity both *in vitro* and *in vivo*.[5] In combination with the vinca alkaloid vincristine, bryostatin 1 inhibits the growth of lymphoma cells without adverse effects on bone marrow cells.[6] Due to these and other activities, this compound is in clinical development as a chemotherapy for various forms of cancer.

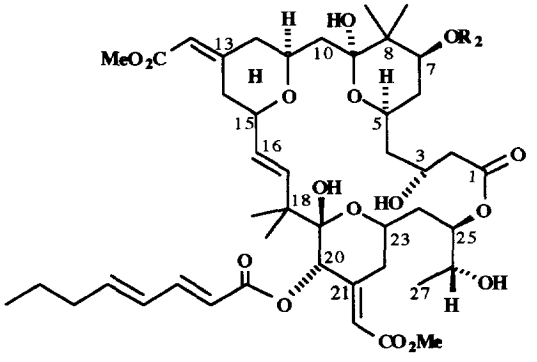

Figure 1 Bryostatin 1

In addition to the biomedical potential of this polyketide, the structure of bryostatin 1 poses intriguing biosynthetic questions. Firstly, the gem dimethyl groups at C-8 and C-18 could arise from S-adenosylmethionine (SAM) methylations as has been documented in the biosynthesis of polyketides such as lankacidin and aplasmomycin.[7] Alternatively, the gem dimethyl groups could be derived from the C-3 of a propionate and a single SAM biomethylation. A further possibility is that two isobutyrates are employed in bryostatin production. The ethylidene substituents at C-13 and C-21 may be derived directly from the addition of acetate to a polyketide chain or alternatively from an intact four carbon unit. There are a number of possible sources of the C25-27 starter unit. Propionate is known to be the polyketide starter in the biosynthesis of the siphonarins[8] and denticulatins,[9] while glycerol has been implicated as fulfilling this role in the macrolide aplasmomycin.[10] A further alternative is n-butyrate which would undergo a post-assembly oxidation-decarboxylation sequence as has been demonstrated in rosaramicin biosynthesis in *Micromonospora rosaria.*[11]

Biosynthetic experiments were carried out using a fortified crude enzyme preparation of the bryozoan. Freshly collected *B. neritina* was cleaned of extraneous material, flash frozen in liquid nitrogen and stored at -80°C. The frozen material was ground to a fine powder in a large, chilled mortar and pestle, and the powder added to a phosphate buffer at pH 7.7 containing Leupeptin (0.1 mM), Pepstatin A (0.1 mM), EDTA (5 mM), DTT (5 mM). Since the bryostatins are trace metabolites, we assumed that the enzymes responsible for their production would also be present in low concentrations, and fortified the supernatant with the coenzymes ATP, Mg^{2+}, NADH and NADPH. NADH has the added benefit of inhibiting consumption of acetate in the Citric Acid Cycle, thus increasing its availability for polyketide production.

The fortified cell-free extract was incubated with radiolabeled precursor for 24 h at 27°C and then quenched by the addition of ethyl acetate. Bryostatin 1 was then purified using a modification of the procedure of Schaufelberger.[12] The ethyl acetate extract of the incubation mixture was coated on diatomaceous earth, applied to a silica cartridge, and eluted with dichloromethane followed by ethyl acetate. The latter fraction was further purified by a reversed phase C-8 cartridge with acetonitrile as eluent. Final purification was achieved by HPLC (C-18) using a gradient elution of 60% acetonitrile/40% water to 100 % acetonitrile. To ensure that this bryostatin 1 is radiochemically pure, 50% of the bryostatin 1 fraction from the HPLC was re-injected using a different gradient (80% acetonitrile/20% water to 100 % acetonitrile). The radioactivity of the remainder of the initial bryostatin 1 peak, and the total bryostatin 1 peak from the second injection were determined using a scintillation counter. In all cases in the Table where we report the recovery of radioactive bryostatin 1, the radioactivity of the first and second HPLC injections agreed within 10% error.

The Table lists the precursors which were incubated with our fortified enzyme preparation.[13] These results indicate that acetate, SAM and glycerol are all used in bryostatin production. In initial single labeling experiments, propionate, n-butyrate, isobutyrate and succinate did not generate radioactive bryostatin 1. Precursors which were not transformed into the bryostatins were re-examined using double labeling studies. To confirm that a precursor is not employed in bryostatin production, it was administered along with acetate bearing a different radiolabel to our fortified enzyme extract. The last four entries of the Table describe the result of incubating ^{3}H-acetate together with ^{14}C-propionate, n-butyrate, isobutyrate and succinate. In all cases, the recovered bryostatin 1 showed significant ^{3}H activity and no ^{14}C activity, demonstrating that these precursors are not employed in bryostatin production.

Table 1: Incubations of cell-free extracts of 25 g *Bugula neritina* with 1.1 x 10^{7} dpm of each isotope. Background activity was taken as < 50 dpm.

Precursor(s)		Recovered radioactivity (dpm)	
		^{3}H activity	^{14}C activity
acetate (^{3}H)		20,000	--
SAM (^{3}H)		31,500	--
glycerol (^{14}C)		--	2,500
propionate (^{14}C)	acetate (^{3}H)	15,400	background
n-butyrate (^{14}C)	acetate (^{3}H)	18,000	background
isobutyrate (^{14}C)	acetate (^{3}H)	21,650	background
succinate (^{14}C)	acetate (^{3}H)	13,500	background

Currently, we are initiating stable isotope *in vitro* biosynthetic experiments to confirm the utilization and identify sites of incorporation of the polyketide building blocks.

ACKNOWLEDGMENTS

Financial support was provided by a grant from the National Institutes of Health (CA 58158-01) and a Henry Dreyfus Teacher-Scholar Award. Bryostatins 1 and 2 were generously provided by the National Cancer Institute and Bristol Myers Squibb. We also thank D. Mendola for generous assistance with the collections of the bryozoan.

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13. All precursors used in the incubations were commercially available with the exception of ^{14}C-isobutyrate. ^{14}C-Isobutyrate was synthesized by treating isopropanol tosylate with ^{14}C-potassium cyanide, followed by hydrolysis.

(*Received in USA* 23 *July* 1996; *revised* 24 *September* 1996; *accepted* 25 *September* 1996)