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Biosynthetic Thiolase from *Zoogloea ramigera*

II. INACTIVATION WITH HALOACETYL CoA ANALOGS*

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The thiolase involved in biosynthesis of poly-β-hydroxybutyrate in *Zoogloea ramigera* generates an acetyl-enzyme species during catalysis. Up to 0.86 [^{14}C] acetyl eq/subunit of this homotetrameric enzyme is accumulated by acid precipitation in the presence of [^{14}C]acetyl-CoA. Gel filtration of the same solutions produced only 7% acetyl-enzyme suggesting hydrolytic lability of the acetyl-enzyme during the 10-min isolation at 4 °C. In an effort to identify active site residues which may function as basic groups to deprotonate at C-2 of acetyl-CoA to generate the required nucleophilic equivalent in carbon-carbon bond formation, we have prepared and tested haloacetyl-thioesters, oxoesters, and amides in the panthetheine pivalate series (Davis, J. T., Moore, R. N., Imperiali, B., Pratt, A. J., Kobayashi, K., Masamune, S., Sinskey, A. J., and Walsh, C. T. (1987) *J. Biol. Chem.* 262, 82–89). The [^{14}C]bromoacetyl-oxoester alkylatively inactivates thiolase irreversibly with stoichiometric incorporation of four labels/tetramer. Determination of amino acid composition of the radiolabeled tryptic peptide indicated trapping of Cys-89 (Peoples, O. P., Masamune, S., Walsh, C. T., and Sinskey, A. J. (1987) *J. Biol. Chem.* 262, 97–102), the same residue modified by iodoacetamide. When the bromoacetyl-thioester was used, inactivation was pH-dependent. The data are consistent with the competition of two processes, acylation, and alkylation. Direct (rather than secondary) alkylation of thiolase by the inactivator accounts for the significant ^{14}C incorporation into thiolase with the thioester labeled with [^{14}C] in the pantetheine pivalate moiety. It appears likely that the haloacetyl analogs described herein should be generally useful for affinity labeling other enzymes using acetyl-CoA as a substrate.

In the preceding article (1) we describe the preliminary structural characterization and general kinetic behavior of the biosynthetic thiolase from *Zoogloea ramigera*. The thermodynamically favored direction is thiolytic cleavage of acetoacetyl-CoA (AcAc-CoA[1] or AcAc-*S*-CoA when emphasis is placed on the thioester moiety) to two molecules of acetyl-CoA (Ac-CoA or Ac-*S*-CoA). The ratio of k_{cat} for cleavage/k_{cat} for condensation is 4100:1, but the AcAc-CoA is drawn off biosynthetically for poly-β-hydroxybutyrate accumulation.

In this biosynthetic Claisen reaction, there is a formal requirement for an Ac-CoA C-2 carbanion as attacking nucleophile in the C–C bond forming sequence. For thiolases from mammalian and bacterial sources there is evidence that a covalent acetyl-*S*(cys)-enzyme intermediate occurs (1–3) and the electrophilic partner in the condensation is then the acetyl-*S*-enzyme as shown below.

$$CH_3COSCoA + E\text{-}SH \rightleftarrows CH_3COS\text{-}E + CoASH$$

$$CH_3COSCoA + E\text{-}B \rightleftarrows {}^{-}CH_2COSCoA + E\text{-}BH^{+}$$

$$CH_3COS\text{-}E + {}^{-}CH_2COSCoA \rightleftarrows CH_3COCH_2COSCoA + E\text{-}S^{-}$$

We have already described proton exchange studies which provided no evidence for formation of a Ac-*S*-CoA carbanion equivalent in the transition state separate from condensation and raised the possibility that C–H cleavage and C–C formation are tightly coupled (1). In this paper we provide preliminary evidence for the *Zoogloeal* acetyl-enzyme intermediate. In an effort to identify active site residues which may function as the catalytic base in the proton abstraction, we have used haloacetyl substrate analogs and find that one of these, bromoacetyl-*S*-pantetheine 11-pivalate acts (BrAc-*S*-pan analog **1**) as an affinity label that apparently inactivates thiolase in two different ways, irreversibly by alkylation and reversibly by bromoacetylation. Both reactions very likely involve the same cysteine of the enzyme.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

[^{14}C]Acetyl-Enzyme Formation from [1-^{14}C]Acetyl-CoA—Several enzymes which use Ac-CoA as a substrate utilize a covalent acetyl-enzyme intermediate at some stage in their reaction coordinates. Based on literature precedent with other thiolases, it is likely that the biosynthetic *Z. ramigera* thiolase utilizes the formation of this intermediate as a means of distinguishing the first Ac-CoA molecule (as an electrophilic acetyl-*S*-enzyme equivalent) from a second, and thus, the two

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[1] The abbreviations used are: AcAc-CoA or AcAc-*S*-CoA, acetoacetyl-CoA; Ac-CoA or Ac-*S*-CoA, acetyl-CoA; BrAc-*S*-Pan, bromoacetyl-*S*-pantetheine 11-pivalate; DCC, *N,N*-dicyclohexylcarbodiimide; DTNB, dithionitrobenzoate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; KP_i, KH_2PO_4 + K_2HPO_4; ME, mercaptoethanol; THF, tetrahydrofuran; TNB, thionitrobenzoate; XAc-*O*-Pan, halo-(*X* = Br, bromo; *X* = Cl, chloro) acetyl-pantetheine 11-pivalate *O*-ester.

[2] Portions of this paper (including "Experimental Procedures" and Schemes 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-312, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

molecules of Ac-CoA become a differentiable electrophile-nucleophile pair to form AcAc-CoA in the condensation reaction that occurs at the active site. One expects that the acetyl-thiolase may be a hydrolytically stable species in equilibrium with Ac-CoA and AcAc-CoA, and in some cases the intermediate accumulates substantially. For instance, incubation of pig heart biosynthetic thiolase (homotetramer) with [1-^{14}C]Ac-CoA (11 eq/subunit), followed by precipitation with acetone-HCl led to a protein which contained 0.8 eq of ^{14}C per subunit (2). The *ato* and *fad* thiolases from *Escherichia coli* are biosynthetic and degradative enzymes, respectively. Duncombe and Freeman (4) succeeded in isolating the corresponding acetyl-enzymes from both thiolases. In the case of the former (*ato*) two isolation methods, trichloroacetic acid precipitation and gel filtration were used, providing ^{14}C incorporation of 0.25 eq (precipitation) and 0.22 eq (Sephadex gel filtration) per thiolase subunit. Apparently, very little hydrolysis of acetyl-enzyme occurred during the gel filtration interval. More recently, Gilbert *et al.* (3), working on pig heart degradative thiolase, found that the extent of ^{14}C incorporation after gel filtration was dependent on the concentration of Ac-CoA used, reaching a limiting value of 0.8 eq/subunit. The acetyl-enzyme slowly hydrolyzed at 28 °C (pH 6.8) with $k = 2.6 \times 10^{-4}$ s^{-1} and the equilibrium constant for acetyl-enzyme formation from Ac-CoA and free enzyme was determined to be $(1 \pm 0.5) \times 10^{-2}$.

Incubation of the *Z. ramigera* thiolase with 55-fold molar excess of [1-^{14}C]Ac-CoA (pH 7.0) at 0 °C followed by acetone-HCl treatment led to precipitation of protein containing, in duplicate studies, 0.86 and 0.77 eq of ^{14}C per subunit. However, the ^{14}C/subunit ratio decreased to 0.072 or 0.043 when gel filtration was carried out at 0–4 °C and at 10–15 °C, respectively, in order to separate native acetyl-thiolase from small molecules. Elution of the protein with 0.1 M KP_i buffer (pH 6.8) required approximately 10 min, during which time substantial amounts (roughly 95% of acetyl-thiolase) must have hydrolyzed (at 10–15 °C). Therefore, its half-life is approximated to be on the order of 2–3 min, significantly shorter than that (44 min at 25 °C) of the acetyl-thiolase from pig heart (3).

Inactivation of Thiolase by Haloacetyl-O-Ester Substrate Analogs (1–6)—We have documented in the preceding paper that iodoacetamide reacts with cysteine 89 of *Z. ramigera* (1), but we do not yet know if this is the same cysteine involved in formation of acetyl-enzyme. Biosynthetic thiolase from pig heart (2) has been shown to involve the same cysteine in both alkylation and acetylation. However, the kinetic lability of native acetyl-thiolase from *Z. ramigera* has slowed this investigation. Meanwhile we resorted to reactive substrate analogs to substantiate the iodoacetamide labeling results. Although iodoacetamide shows kinetic specificity, it bears no structural similarity to acetyl-CoA. We have prepared novel bromoacetyl-thioester **1**, oxoesters **2–6**, and amide **7** as analogs of acetyl-pantetheine 11-pivalate, as documented in the Synthetic section (Miniprint). These analogs were expected to be efficient affinity labels capable of alkylating (or possibly acylating) the putative active site cysteine, since AcAc-*S*-pan analog and Ac-*S*-pan analog are excellent substrates (1). In a first set of inactivation experiments, we selected BrAc-*O*-pan analog **2** and ClAc-*O*-pan analog **3** instead of BrAc-*S*-pan analog **1** in order to avoid complications which might arise from the possible acyl transfer reaction of **1**, which is a thioester. The acyl-oxoesters are, in general, much less susceptible to thiolytic cleavage and should be sluggish or inert in thiolase-mediated acyl transfer. Therefore, both **2** and **3** would be useful in accumulating Michaelis-type enzyme bound haloacetyl oxoesters, which might undergo preferential alkylation of an active-site residue rather than acylation of the active site cysteine sulfhydryl. Analogs **2** and **3** inactivate enzyme with pseudo-first order kinetics at both pH 7.0 and 8.1. Fig. 1 shows plots of $t_{1/2}$ *versus* 1/[I] and reveals limiting k_{inac} values of 1.75 min^{-1} for BrAc-*O*-pan analog **2** and 0.175 min^{-1} for ClAc-*O*-pan analog **3**. These values are consistent with bromide being a better leaving group for S_N2-type alkylation than chloride by one order of magnitude.

As a further test for alkylative inactivation occurring from a haloacetyl-CoA analog kinetically trapped in a Michaelis complex at the active site, (prior to acyl transfer to form a covalent haloacetyl-*S*-cysteinyl enzyme), the sulfur of the pantetheine moiety was replaced by a nitrogen atom to yield a haloacetyl amide which has such a thermodynamically stable acyl group that it has a negligible chance of acetyl transfer to the active site cysteine. As shown in Table I, the bromoacetyl amide **7** is still a saturable time-dependent inactivator of *Z. ramigera* thiolase with an extrapolated limiting k_{inac} of 0.03 min^{-1}. The 50 times lower rate of alkylation with the amide **7** as compared to **2** is consistent with expectations for lower rates of α-haloamides *versus* α-halo ester displacement reactions.

We have pursued three other haloacetyl oxoester pantetheine pivalates in this phase of the work, the trichloroacetyl analog **4**, the bromacetyl-homopantetheine analog **5**, and the corresponding bromoacetyl-norpantetheine analog **6**. The latter two species were designed to map the inactivating consequences of moving the same bromoacetyl group one methylene distance along the acetyl-CoA-binding site in either direction from the site occupied by Ac-CoA itself.

As summarized in Table I, these analogs are time-dependent affinity labeling reagents. In the event the nor-*O*-compound **6**, due to its hydrolytic instability, gave results that were not accurate for determination of k_{inac} due to competing nonenzymic destruction of the compound (but qualitatively it reacted at a rate 100 times slower than **2**), while oxoester **5** was 10 times slower in inactivation, perhaps consistent with

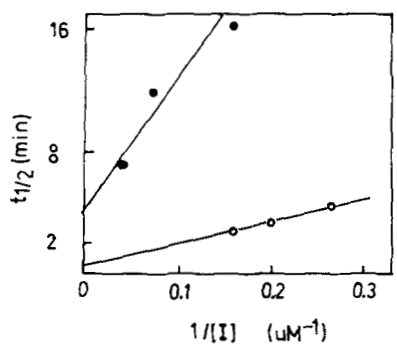


FIG. 1. **Inactivation of thiolase (0.625 μM) by haloacetyl-*O*-pan analogs 2 (○——○) and 3 (●——●).** Half-life of inactivation *versus* 1[I] are shown. All inactivations were done in 0.2 M Tris (pH 7.0) at room temperature.

TABLE I

Kinetic data for affinity-labeled inactivation reactions (0.2 M Tris, pH 7.0) using compounds 1–7

Compound	k_{inac}	Relative k_{inac}
	min^{-1}	
BrAc-*S*-pan analog **1**	2.3	1.00
BrAc-*O*-pan analog **2**	1.75	0.76
ClAc-*O*-pan analog **3**	0.175	0.08
BrAc-*O*-pan homolog **5**	0.158	0.07
BrAc-*O*-pan nor-analog **6**		0.01
BrAc-*N*-pan analog **7**	0.03	0.01

less than optimal orientation toward the alkylating amino acid side chain.

To test the enzyme microgeographic consequences of inactivation, both the normal and *homo* pantetheine bromoacetyl oxoesters **2** and **5** were prepared with [^{14}C]bromoacetyl groups, and on full inactivation labeled tetrameric enzyme was obtained with 0.98 and 1.20 eq of ^{14}C per mol of subunit, respectively. Tryptic digestion and separation of radiolabeled peptides from thiolase (120 nmol) inactivated with ^{14}C analogs **2a** and **5a** gave peptides **2a**-A (42.5 nmol) and **2a**-B (2.0 nmol), and peptides **5a**-A (19.5 nmol) and **5a**-B (34.5 nmol), respectively.

Having obtained sizable amounts of three peptides, namely **2a**-A, **5a**-A, and **5a**-B, we determined their amino acid compositions. Peptides **2a**-A and **5a**-A, the faster moving peptides off the HPLC columns, had identical compositions to that of the peptide isolated earlier by iodo[1-^{14}C]acetamide labeling (1) (Table II). The peptide **5a**-B, the more nonpolar peptide, had an amino acid composition equivalent to the other peptides except that it contained approximately 3 eq of alanine and substoichiometric amounts of additional amino acids. This could easily reflect the contamination of **5a**-B with another peptide. Amino acid standards indicate that alanine and β-alanine, a component of pantetheine, have an identical retention time. This result may indicate that peptide **5a**-B contains an unhydrolyzed oxoester linkage connecting the pantetheine ester to the same peptide as **2a**-A and **5a**-A. Since we isolated such a small amount of **2b**-B, no analysis was done on this peptide.

Given that peptides **2a**-A and **5a**-A are identical to the peptide isolated earlier from the iodoacetamide inactivation, it is likely that the cysteine 89 displaces the activated bromide and that both oxoesters are capturing this active site cysteine. The similarity in amino acid composition of peptide **5a**-B to the others indicates that it is also likely generated from the same alkylation process.

The active site of thiolase must be comprised of at least two amino acid residues directly participating in the enzymatic reaction: one is the cysteine that becomes acylated during catalysis and the other is a residue acting as a base to deprotonate Ac-CoA once the acetyl-enzyme is formed (1). The above results prove that Cys-89 is near or at the active site but does not *per se* establish which function, nucleophile toward the first Ac-CoA or base toward the second Ac-CoA, it has in turnover.

Inactivation Studies with Bromoacetyl-S-Pantetheine Pivalate, 1 and Evidence for Bromoacetylation of Enzyme—We next turned our attention to BrAc-*S*-pan analog **1** to determine whether this thiolester also attacks cysteine 89 or a different amino acid side chain; the latter alternative should occur if Cys-89 is the reactive thiol and if acylation to form bromoacetyl-*S*-enzyme precedes alkylative inactivation. We note that in turnover with excess Ac-CoA about 90% of the enzyme accumulates as acetyl-enzyme. If bromoacetyl-*S*-enzyme similarly accumulates subsequent alkylative, inactivation would capture a second residue.

Analog **1** caused a time-dependent loss of enzyme activity, as expected. Interestingly, this loss of enzyme activity was dependent on both the pH of the incubation and the presence of nucleophilic species such as ME or Tris buffer.

For example, with **1** in 10-fold molar excess over thiolase (6.25 μM *versus* 0.625 μM subunit) at pH 8.1 in 0.2 M Tris, curve 1 of Fig. 2 was obtained. Activity drop was rapid ($t_{1/2}$ = 0.5 min) to about 20% residual activity, then there was a slow regain of thiolysis activity over 90 min to about 90% catalytic activity. Since the stock solution of enzyme contained 5 mM ME as an anti-oxidant, the incubation mixture had a final concentration of 250 μM thiol. Curve 2 shows the results of inactivation with an incubation mixture without ME as the only variable changed. In curve 2 the regain of activity is a much slower process, and after 150 min a maximum of 56% catalytic activity is regained. In contrast, when thiolase inactivation was effected at pH 7.0 in 0.2 M Tris containing 250 μM ME, curve 3 was obtained (Fig. 2). A $t_{1/2}$ of 0.5 min was observed at the same 10-fold molar excess over enzyme and now the enzyme remained approximately 90% inactivated for at least 4 h.

In order to eliminate complications apparently caused by the presence of nucleophiles in the incubation buffer, all subsequent inactivations with **1** were performed in thiol-free 0.2 M KP_i buffers. The amount of residual enzyme activity was a function of length of time of exposure of enzyme to this inactivator. When 5 molar eq of **1** were incubated at pH 7 with enzyme for 2, 5, 15, 30, and 60 min, followed by dilution with an equal volume of 0.25 M Tris (pH 9.0) to a final pH of 8.1 and residual activity measured 60 min later (to allow full reactivation), the profile shown in Fig. 3 was obtained. Thus, prolonged exposure at pH 7 eventually inactivates the enzyme irreversibly. Noteworthy and somewhat curious was the reactivation in basic media after short incubations with **1** (see Figs. 2 and 3).

It is known that acyl thioesters are hydrolytically labile in even mildly alkaline solutions. We determined the nonenzymatic rates of hydrolysis of bromoacetyl-thioester **1** using DTNB which upon reaction with a thiolate (or thiolates) liberates TNB with A_{412} = 13.6 mM^{-1}. Fig. 4 shows that in 0.2 M KP_i k (at pH 7.0) = 0.019 min^{-1} and k (at pH 8.1) = 0.113 min^{-1}. It is sufficient to note at this point that while **1** is nonenzymatically rather stable in the inactivation experiment

TABLE II

*Amino acid composition of peptides **2a**-A, **5a**-A, and **5a**-B compared to peptide isolated from iodoacetamide inactivation and DNA sequence prediction*

Amino acid	Iodoacetamide	DNA	2a-A	5a-A	5a-B
Gly	3	3	3	3	4
Met	1	1	1	0.7	0.7
Asp (Asn)	2	2	1.7	2.1	2.1
Cys[a]	1	1			
Glu (Gln)	1	1	1.1	1.0	1.5
Leu	2	2	1.5	1.8	1.7
Ser	1	2	0.7	0.9	1.1
Arg	1	1	1.0	1.2	1.0
Ala	0	0	0.0	0	2.8

[a] Carboxymethyl cysteine not analyzed for in composition.

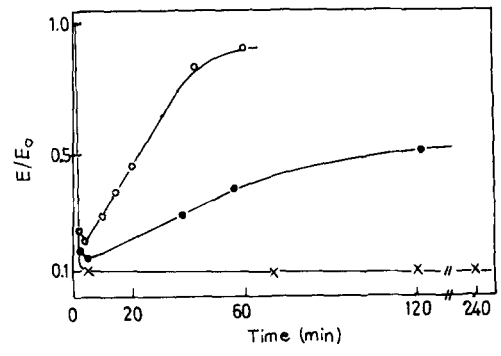


FIG. 2. **Inhibition of thiolase with 1.** Curve 1 (O—O): 10 molar eq of **1** (6.25 μM) to thiolase subunits (0.625 μM) in 0.2 M Tris (pH 8.1), 250 μM ME. Curve 2 (●—●): same as curve 1, except no ME. Curve 3 (×—×): same conditions as curve 1 except 0.2 M Tris (pH 7.0).

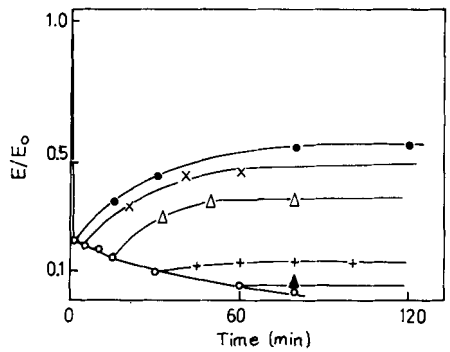


FIG. 3. **Reactivation of inactivated thiolase by base hydrolysis.** Thiolase (0.58 nmol subunit) was incubated with **1** (4.6 nmols) in 0.1 M KP_i (pH 7.0) (O---O). At the indicated times (t = 2, 5, 15, 30, 60 min) an aliquot was removed and diluted with 0.25 M Tris (pH 9.0) to bring the pH to 8.1. The regains are shown by (●—●), (×---×), (Δ---Δ), (+---+), and (▲—▲).

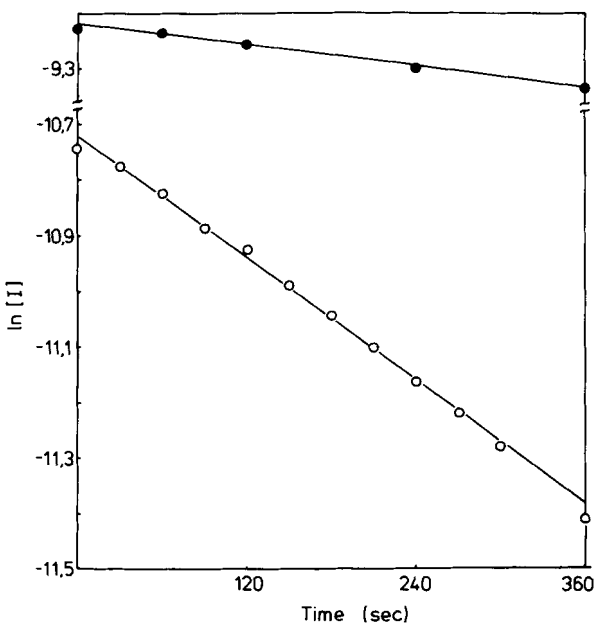


FIG. 4. **Nonenzymatic hydrolysis of 1 at pH 7.0 and 8.1 in 0.2 M KP_i solution.** Curve 1 (O---O); kinetics at pH 7.0; curve 2 (●---●), kinetics at pH 8.1. See "Methods" (Miniprint) for experimental detail.

at pH 7.0, it hydrolyzes rapidly at pH 8.1, at a rate slightly faster than thiolase regains its activity at this same pH after initial inactivation at pH 7.0.

It is natural to assume that a major reaction of thiolase with thioester **1** is the formation of bromoacetyl-enzyme which is stable at pH 7.0 and in equilibrium with **1**. Since the thioester **1** has a half-life of only 6 min at pH 8.1, not only does this equilibrium shift during incubation to generate active enzyme, but at the same time bromoacetyl-enzyme undergoes hydrolytic cleavage at least as fast as that of **1** at this basic pH (see below). The enzyme inactivated at pH 7.0 can regain its activity in this way. As a means of estimating the degree of bromoacetylation, we measured the liberation of pantetheine 11-pivalate when thiolase is incubated with **1** at pH 7.0 (Fig. 5).

Two control experiments provided curves 1 and 2, using thiolase (4.5 μM) and inhibitor (18 μM) separately. The reaction rate of DTNB with thiolase is insignificant. Curve 3 plots the amount of pantetheine pivalate liberated during the inactivation reaction. The difference between curves 3 and 2, shown by the *dotted line*, can be interpreted as the maximum

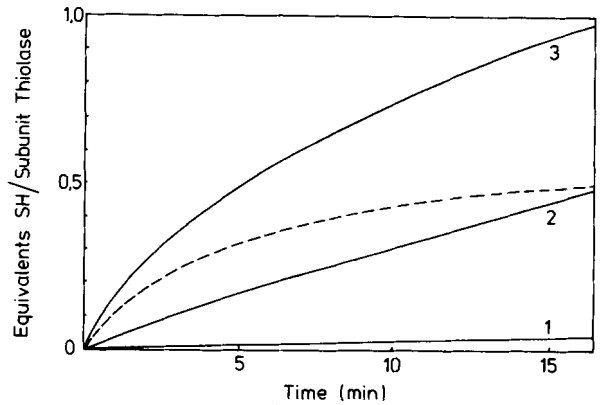


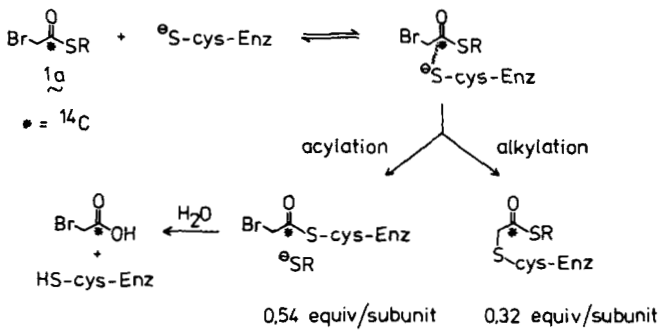
FIG. 5. **Release of pantetheine 11-pivalate upoan incubation of thiolase with BrAc-*S*-pan analog 1.** Curve 1 is a control with 48 μM DTNB and 4.5 μM thiolase. Curve 2 consists of 48 μM DTNB and 18 μM BrAc-*S*-pan analog **1**. Curve 3 is thiolase (4.5 μM) mixed with 48 μM DTNB and 18 μM BrAc-*S*-pan analog **1**. The *dotted curve* is (curve 3)-(curve 2). Absorbance at 412 nm was followed and converted to thiol equivalents by using 13.6 mM^{-1} cm^{-1} as the extinction coefficient for the thionitrobenzoate anion.

amount of pantetheine 11-pivalate liberated by the process of enzyme actylation by **1**. A rapid release of thiol is noted, with 2 to 2.5 mol per tetramer being generated within 10 min. This result supports the involvement of acylation as a major reaction course of inactivation during the early incubation periods.

Quantification of the Extent of Enzyme Acylation Versus Alkylation—Earlier studies with pig heart thiolase had found that bromoacetyl-CoA and analogs were effective inactivators: Gehring *et al.* (5) had found that bromoacetyl-pantetheine inactivated biosynthetic thiolase at pH 7.0 (5). Holland *et al.* (6) used bromoacetyl-CoA to inactivate thiolase at pH 8.1. However, no evidence for bromoacetylation of the enzyme had been advanced. Our initial studies, consistent with significant bromoacetylation of the active-site cysteine, were more similar to results recently obtained for another CoA utilizing enzyme, HMG-CoA synthase. Miziorko and Behnke (7) have found that β-chloropropionyl-CoA inactivated HMG-CoA synthase via both alkylation and acylation. The synthase is also inactivated by succinyl-CoA, which forms a covalent succinyl-enzyme linkage (8).

It was clear that bromoacetylation of thiolase was not the sole process of inactivation, but alkylation was also occurring concurrently. In order to gain further information on this aspect, we designed an experiment to quantify the extents of the two processes. Thiolase was inactivated to 14% residual activity by incubation with 10 eq of [^{14}C]bromoacetyl-thioester **1a** at pH 7.0 (0.1 M KP_i) for 5 min. At this time, the incubation mixture was divided evenly into three separate portions and each portion was treated differently.

One portion was diluted with chilled acetone-HCl so as to precipitate all protein instantaneously at pH 7.0. A stoichiometry of 0.86 eq of ^{14}C per subunit was obtained. This is the same value as that obtained for the [^{14}C]acetyl-CoA incubation described earlier. A second portion of the reaction mixture was diluted with 0.25 M Tris (pH 9.0), thereby raising the pH from 7.0 to 8.1. An assay of enzyme activity showed that the thiolase regained 55% residual activity (from 14%) after a 1-h period. More importantly, acid precipitation of the protein at that point resulted in a dramatically lowered ^{14}C incorporation of 0.32 mol of ^{14}C label per mol of thiolase subunit (see Scheme 4). The third portion was isolated by gel filtration using 0.1 M KP_i (pH 7.0) as elutant. The recovered thiolase was 60% active and again contained only 0.32 mol of ^{14}C label per mol of subunit.



SCHEME 4. **Partition of bromoacetyl pan Analog 1a between enzyme-acylation and alkylation.**

TABLE III

Incorporation of ^{14}C eq per subunit into thiolase as a function of incubation time at pH 7

Time	^{14}C incorporation per subunit
	eq
2	0.24
5	0.25
15	0.57
30	0.83
150	1.08

These results clearly support the existence of an acid-stable but base-labile linkage, specifically a bromoacetyl-enzyme species which is responsible for the reversible component of thiolase inactivation. A linkage stable to both acid and base is also formed. Thus, the 0.32 eq of ^{14}C label obtained after acid precipitation of the pH 8.1 reaction (the above second treatment) and also from the gel filtration experiment reflect the degree of alkylation after 5 min (the third treatment). Acylation presumably accounts for the other 0.54 (Scheme 4) eq out of 0.86 eq of ^{14}C label originally present, thus explaining both the regain in enzyme activity and the loss of radioactivity upon base treatment or attempted gel filtration isolation. Assuming the bromoacetylation involves cysteine 89, the hydrolytic instability of bromoacetyl-enzyme during gel filtration isolation is not surprising considering our gel filtration experiments with [^{14}C]acetyl-enzyme (see above).

Another experiment was designed to indicate whether the irreversible component of inactivation was due to direct alkylation by bromoacetyl-thioester **1** or a secondary alkylation displacement from the bromoacetyl-enzyme species. Thus, five portions of thiolase (5 nmol subunit) were incubated at room temperature with BrAc-*S*-pan [^{14}C]pivalate analog **1b** at pH 7.0 for 2, 5, 15, 30, and 150 min, respectively. These reaction mixtures were then subjected to gel filtration with pH 6.8 KP_i buffer. Direct alkylation of thiolase with **1b** should be displayed by the incorporation of radioactivity into the inactivated protein (see Scheme 4), whereas the process of secondary alkylation should not increase the ^{14}C incorporation into the protein because this process would occur from the bromoacetyl-enzyme species. The ^{14}C eq incorporation per subunit in each experiment is tabulated below (Table III).

Table III shows that direct alkylation of **1b** is competing with enzyme acylation, and secondary alkylation of BrAc-*S*-enzyme appears to be occurring only to a minor extent, if at all, even though we have earlier obtained evidence that bromoacetyl-*S*-enzyme does accumulate significantly.

We have demonstrated that 1) upon incubation of the thiolase with bromoacetyl-*S*-pantetheine pivalate **1**, the two processes, alkylation and acylation, compete and irreversibly and reversibly inactivate the enzyme, respectively, and 2) base-labile bromoacetyl-enzyme formed by the latter process is hydrolyzed to generate enzyme which is subject to further attack of the inactivator.

Tryptic digestion of enzyme incubated with **1a** for 2 h at pH 7 (1 eq of ^{14}C per subunit) provided an HPLC profile containing several radioactive peptides, of which a major peptide (approximately 40% of total radioactivity) was, as expected, the one containing cysteine 89.

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Supplemental Material to
Biosynthetic Thiolase from Zoogloea ramigera.
2. Inactivation with Haloacetyl CoA Analogs

by

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Experimental Procedures

Materials

Thiolase was purified as described in reference (1) and samples with a specific activity of 400-420 U/mg were used throughout this work. [1-^{14}C]Ac-CoA was obtained from New England Nuclear, while the supplier of [1-^{14}C]bromoacetic acid and [^{14}C]$BaCO_3$ was Amersham. All organic reagents used in the synthesis of substrates and inhibitors were purchased from either Aldrich or Sigma.

Methods

General

The following operations were performed as described in (reference 1): enzyme assays of thiolase, ultrafiltration, UV measurements, and amino acid analysis of peptides.

Acid Precipitation of [^{14}C]Acetyl-S-Enzyme

Thiolase (8.5 μM) in 95 μl of 0.1 M KP_i (pH 7.0), 1 mM EDTA, and 1 mM ME was incubated with [1-^{14}C]Ac-CoA (0.47 mM, 11.96 nCi/nmol) for 5 min at 0°C.

The reaction mixture was diluted with 2 ml of chilled acetone-HCl (0.5% v/v), with 50 μg of bovine serum albumin added as a carrier. The precipitated enzyme was isolated by centrifugation, and the resultant pellet was loaded by pipet onto a glass fiber filter. The protein was washed with cold acetone (50 ml) and cold ethanol (5 x 10 ml). The protein was air-dried and the filter was then counted for radioactivity.

Gel Filtration of [1-^{14}C]Acetyl-S-Enzyme

Thiolase (9.67 μM) and [1-^{14}C]Ac-CoA (0.51 mM, 11.96 nCi/nmol) were incubated in a similar manner as described above. The reaction mixture was then applied to a 1 x 18 cm Bio-Rad P6 gel filtration column and eluted with 0.1 M KP_i (pH 6.8) at 0-4°C with a flow rate of 0.6 ml/min. Fractions were collected every minute and analyzed for radioactivity, protein concentration, and thiolysis activity.

Inactivation and Active-Site Labeling of Thiolase by Affinity Labels. Example: The Haloacetyl Oxoester, [2-^{14}C] Bromoacetyl-Pantetheine 11-Pivalate O-Ester, [[2-^{14}C]BrAc-O-Pan Analog 2a]

A variety of affinity-label type inactivators were used in this work (see the Synthesis Section for structures 1-7) to inactivate thiolase in the manner described in the accompanying paper (1) for [1-^{14}C]iodoacetamide inactivation. Typically, thiolase (2 nmol subunit) was incubated with [2-^{14}C]BrAc-O-Pan Analog 2a, (4-6 molar equiv, 5.0 nCi/nmol) in 0.2 M KP_i (pH 7.0) at room temperature. Enzyme activity was monitored, and when the activity was less than 5% of the control protein solution the reaction mixture was subjected to gel filtration and specific radioactivities of the isolated protein were determined.

For the isolation of peptides containing a labeled active site residue, thiolase (5 mg, 120 nmol) in 5 ml of stock buffer was diluted with 10 ml of 0.2 M Tris (pH 7.02) and incubated for 1 h at 25°C with 2a (1.2 μmol, 10 equiv). The radio-labeled protein was separated from unreacted 2a, denatured, carboxymethylated and finally digested with trypsin as described earlier (1). The labeled peptides were purified by reverse-phase HPLC for the determination of their amino acid compositions.

Inactivation of Thiolase by BrAc-S-Pan Analog 1 and Reactivation of Inactivated Thiolase in Basic Media

Thiolase (0.2 nmol subunit, dialyzed to remove ME) in 500 μl of 0.2 M KP_i (pH 7.0) at 25°C was incubated with Analog 1 (1.0 nmol, 5 equiv) and aliquots were assayed for enzyme activity. At various times after initiation of the reaction 30 μl aliquots were removed and diluted with equal volumes of 0.2 M Tris (pH 9.0) to bring the pH of the resulting solution to 8.1. Enzyme activity of these solutions was assayed over a 90 min period.

Measurement of Non-Enzymatic Hydrolysis of Analog 1

The kinetics of non-enzymatic hydrolysis of 1 at pH 7.0 and pH 8.1 were determined by using DTNB to titrate the thiol generated from hydrolysis.

A solution of DTNB (450 μM) in 0.2 M KP_i (pH 7.0) was made 90 μM in Analog 1 and the increase in absorbance at 412 nm was measured every 30 s over a 30 min period. A similar experiment was carried out at pH 8.1 (0.2 M KP_i) with a 24 μM solution of 1. An extinction coefficient of 13.6 mM^{-1} at 412 nm was used (9).

Kinetic constants for the pseudo-first-order hydrolysis reactions were determined from plots of ln[1] vs time from measurements obtained during the initial 6 min of reaction. After 10 min the hydrolysis reactions departed from first order kinetics, particularly at pH 8.1. This was presumably due to reaction of generated thiol with 1.

Stoichiometry of Thiolase Inactivated by [1-^{14}C]BrAc-S-Pan Analog 1a. Acid Precipitation and Gel Filtration Experiments

The mercaptoethanol and glycerol present in the stock buffer of thiolase were removed by repeated ultrafiltration with chilled 0.1 M KP_i, 1 mM EDTA buffer (5 x 2 ml). To the thiol-free enzyme (200 μl, 5.86 nmol) was added [1-^{14}C]BrAc-S-Pan analog 1a (60.6 nmol, 3.55 nCi/nmol) and the reaction mixture was incubated at room temperature for 5 min. Then the reaction was divided evenly into three portions, each of which was treated differently.

One portion (67 μl) was diluted with 67 μl of 0.1 M KP_i (pH 7.0) buffer and then with 2 ml of chilled (-20°C) acetone-HCl. Bovine serum albumin (25 μg) was added and the precipitated protein was isolated by centrifugation. The pellet was washed with cold acetone-HCl (25 ml) on a glass fiber filter and allowed to air dry before it was counted for radioactivity.

The second portion of the reaction mixture was diluted with 67 μl of 0.25 M Tris (pH 9.0) and the resultant solution was allowed to stand at room temperature for 1 h. Periodically aliquots were removed and assayed for thiolysis to monitor the restoration of enzyme activity. After 1 h the protein was precipitated with acetone-HCl treatment as described above for the first portion. The protein pellet obtained was counted for radioactivity.

The third portion of the reaction mixture (67 μl) was applied to a 1 x 15 cm Bio-Rad P6 gel column and elution with 0.1 M KP_i, 1 mM EDTA gave a protein fraction which was assayed for protein concentration, enzyme activity, and protein-bound radioactivity.

Gel Filtration of Thiolase Inactivated by Bromoacetyl-S-Pantetheine 11-[1-^{14}C]Pivalate (BrAc-S-Pan [1-^{14}C]Pivalate Analog 1b)

This experiment was carried out in the same manner as described above. Thus, thiolase (200 μg, 5 nmol) and 1b (40 nmol, 1.56 nCi/nmol) labeled with ^{14}C on the thiol portion of the haloacetyl thioester were incubated for 2 min at room temperature and the reaction mixture was subjected to gel filtration at 10-15°C.

The above experiment was repeated with incubation times of 5, 15, 30, and 150 min, before gel filtration and measurement of protein associated radioactivity.

In a separate run, protein that had been inactivated with 1b for 20 min was separated from small molecules and then allowed to stand overnight in 0.1 M KP_i (pH 7.0) at room temperature (so as to liberate the thiol leaving group by hydrolysis). Upon ultrafiltration, approximately 25% of the original protein-associated radioactivity was collected in the filtrates. The compound responsible for this radioactivity was identified as the disulfide of pantetheine 11-pivalate by TLC and cochromatography with the authentic disulfide.

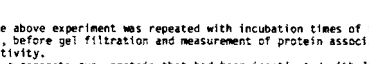
Release of Pantetheine 11-Pivalate upon Incubation of Thiolase with BrAc-S-Pan Analog 1

Thiolase (4.5 μM), obtained after ultrafiltration to remove residual ME from the storage buffer, was incubated with 48 μM DTNB and 18 μM Analog 1 in 0.2 M KP_i (pH 7.0) at room temperature. The absorbance at 412 nm generated over time was monitored and converted to thiol equiv (A_{412} = 13.6 mM^{-1}). The appropriate separate control experiments for enzyme alone and inhibitor alone were performed in a similar fashion.

Synthesis of Inactivators

Synthesis of BrAc-S-Pan Analog 1, and [1-^{14}C]BrAc-S-Pan Analog 1a, and BrAc-S-Pan [1-^{14}C]Pivalate Analog 1b

a. BrAc-S-Pan Analog 1



1

1a (* = ^{14}C)

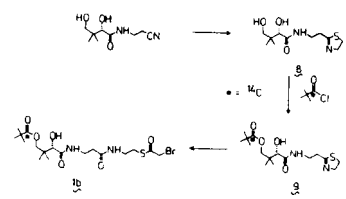
A solution of pantetheine 11-pivalate (reference 1) (53 mg, 0.14 mmol) and pyridine (0.34 ml, 0.42 mmol) in THF (2 ml) was cooled to -78°C. To this solution was added bromoacetyl bromide (12.2 ml, 0.14 mmol). The reaction mixture was warmed to -20°C over 1 h. The reaction was diluted with ethyl acetate (10 ml) and washed with saturated $CuSO_4$ solution (5 ml), H_2O (5 ml), and brine (10 ml). The organic phase was dried over $MgSO_4$, filtered and concentrated in vacuo. Chromatography (SiO_2, $CHCl_3$-MeOH 15:1) gave Analog 1 (46 mg, 68% as a colorless oil: UV (0.1 M KP_i) λ_{max}^{MeOH} = 242 nm (ε=3x 10^3); ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.19 (t, J=5.8 Hz, 1 H), 6.25 (t, J=5.5 Hz, 1 H), 4.17 (d, J=11.0 Hz, 1 H), 4.02 (s, 2 H), 3.79 (m, 2 H), 3.72 (d, J=11.0 Hz, 1 H), 3.38-3.58 (m, 4 H), 3.08 (t, J=6.4 Hz, 2 H), 2.40 (t, J=6.0 Hz, 2 H), 1.20 (s, 9 H), 1.04 (s, 3 H), 0.90 (s, 3 H).

b. [1-^{14}C] BrAc-S-Pan Analog 1a

To a solution of [1-^{14}C]bromoacetic acid (6.9 mg, 0.50 mmol, specific activity 6.0 mCi/mmol) in CH_2Cl_2 (0.5 ml) was added a solution of DCC (10.3 mg, 0.05 mmol) and pantetheine 11-pivalate (18.9 mg, 0.05 mmol) in CH_2Cl_2 (0.5 ml). The reaction mixture was allowed to stand at room temperature at 12 h. The reaction was cooled to -78°C and the precipitated dicyclohexylurea filtered. The filtrate was concentrated to give an oil which after chromatography (SiO_2, CH_2Cl_2-MeOH 20:1) gave 1a as a colorless oil. Both 250 MHz ^{1}H NMR and UV spectra were identical to unlabeled 1. The specific activity of 1a was 6.0 mCi/mmol.

c. [1-^{14}C]Pivaloyl Chloride (see Scheme 1)

Scheme I



To a mixture of [^{14}C]$BaCO_3$ (30 mg, 6.25 mCi/mmol) and "cold" $BaCO_3$ (90 mg) was added dropwise concentrated H_2SO_4 (1.4 ml) under vacuum (0.02 mm Hg). The carbon dioxide generated was condensed into a solution of t-butylmagnesium chloride (1 ml, 2.0 M in THF) frozen with liquid nitrogen. The reaction was warmed to room temperature and allowed to react for 30 min and then cooled to -78°C. This procedure was repeated three times and then the reaction was quenched by the addition of diluted H_2SO_4 and extracted with ether (3 x 25 ml). The ether extracts were dried and concentrated to give [1-^{14}C]pivalic acid in 100% yield as determined by radioactivity.

To a solution of [1-^{14}C]pivalic acid (51 mg, 0.50 mmol) in benzene (0.5 ml) was added oxalyl chloride (64 μl, 0.73 mmol) at room temperature under an argon atmosphere. The reaction was allowed to stir for 2 h until the bubbling had ceased. The reaction mixture was then heated at 50°C for 20 min. The mixture was cooled to room temperature and placed under low vacuum for 10 min. Then p-di-t-butylbenzene (50 mg) was added as an internal standard for NMR analysis, which showed an 83% yield of pivaloyl chloride.

d. Thiazoline 8

To a flame-dried round-bottom flask equipped with a condenser and an argon inlet was added MeOH (16 ml) and sodium metal (758 mg, 33 mmol). After the vigorous reaction had ceased the sodium methoxide solution was cannueled into a flask containing cysteamine hydrochloride (3.74 g, 33 mmol) in MeOH (15 ml) at 0°C. The resulting suspension was stirred at room temperature for 1 h.

The solution of the aminothiol was cannueled through a Schlenck funnel into a 100 ml flask containing pantothenonitrile (3.5 g, 17.6 mmol) in MeOH (15 ml). The reaction mixture was refluxed for 2 h and concentrated in vacuo. Chromatography of the residue (SiO_2, CH_2Cl_2-MeOH 10:1) gave a quantitative yield of the desired thiazoline 8 (4.5 g, 100%) as a colorless oil: ^{1}H NMR (250 MHz, $CDCl_3$) δ 4.21 (t, J=8.5 Hz, 2 H), 3.97 (s, 1 H), 3.68 (m, J=6.2 Hz, 2 H), 3.48 (s, 2 H), 3.31 (t, J=8.5 Hz, 2 H), 2.69 (t, J=6.1 Hz, 2 H), 1.02 (s, 3 H), 0.94 (s, 3 H).

e. Pantetheine 11-[1-^{14}C]Pivalate Analog 1b

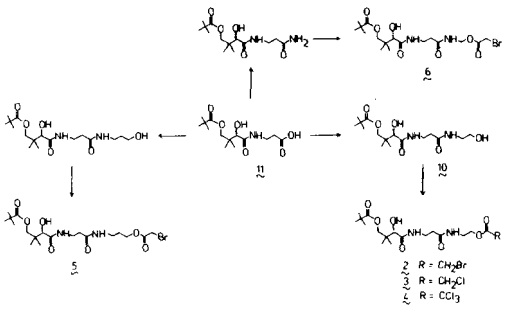
To a 0°C solution of 8 (126 mg, 0.37 mmol) in CH_2Cl_2 (2 ml) was added pyridine (316 µl, 3.9 mmol) followed by [1-^{14}C]pivaloyl chloride (52 mg, 0.43 mmol in benzene). After 3 h the reaction mixture was poured into a saturated $CuSO_4$ solution and extracted with ethyl acetate (3 x 5 ml). The extract was washed with dilute $NaHCO_3$ solution and the organic phase was dried over Na_2SO_4. Flash chromatography (SiO_2, EtOAc-hexane 1:1) gave the thiazoline [1-^{14}C]pivalate 9 as a colorless oil in 53% yield. The pivalate was used immediately in the next reaction.

The pivalate 9 (75 mg, 0.224 mmol) was dissolved in 5 ml of THF and to this solution was added 0.75 ml of 0.1 M oxalic acid. The reaction mixture was stirred overnight at room temperature under argon. The solvent was removed in vacuo and the residue was purified by flash chromatography (SiO_2, CH_2Cl_2-MeOH 20:1) to give pantetheine [1-^{14}C]pivalate as a viscous oil in 79% yield. The labeled pivalate was converted to BrAc-S-Pan Analog 1b in the manner described for 1a. Compound 1b was identical by UV and NMR to 1 and 1a. The resulting sample had a specific radioactivity of 1.56 mCi/mmol.

Synthesis of Haloacetyl Pantetheine Pivalate O-Esters (2,3,4) and the Corresponding Chain-Elongated (homo) and Chain-Shortened (nor) Compounds (5,6)

These affinity label compounds were prepared according to Scheme 2 shown below.

Scheme 2

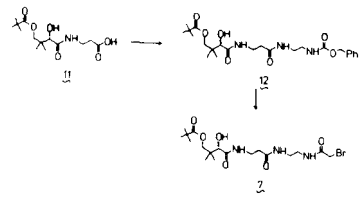


120 mg (95%) of 2: IR ($CHCl_3$ 1720, 1690); ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.16 (t, J=6.0 Hz, 1 H), 6.07 (t, J=5.0 Hz, 1 H), 4.21 (t, J=6.3 Hz, 2 H), 4.18 (d, J=11.0 Hz, 1 H), 3.84 (s, 2 H), 3.79 (d, J=5.5 Hz, 1 H), 3.71 (d, J=11.1 Hz, 1 H), 3.61 (d, J=5.6 Hz, 1 H), 3.55 (t, J=6.0 Hz, 2 H), 3.33 (m, 2 H), 2.42 (t, J=6.1 Hz, 2 H), 1.20 (s, 9 H), 1.05 (s, 3 H), 0.90 (2, 3 H); mass, 469 (M^+ + 2).

Synthesis of Bromoacetyl Pantetheine 11-Pivaloate Amide 7

The synthetic route is shown in Scheme 3.

Scheme 3



a. Benzyl Carbamate 12

To a solution of 8-pivaloyl pantothenic acid (11) (0.290 g, 0.956 mmol) and amine 13 (0.220 g, 1.15 mmol) (Scheme 3) in CH_2Cl_2 (10 ml) at room temperature was added DCC (0.240 g 1.15 mmol) and the reaction mixture stirred for 30 min. The reaction mixture was diluted with ethyl acetate (25 ml) and washed with dilute HCl (20 ml) and brine (20 ml). The organic phase was dried, filtered, and concentrated to give a residue which provided the benzyl carbamate 12 (0.340 g, 78%) upon chromatography (SiO_2, EtOAc-EtOH 5:1): ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.30 (bs, 1 H), 7.01 (bs, 1 H), 5.89 (bs, 1 H), 5.03 (d, J=3.5 Hz, 2 H), 4.08 (d, J=11 Hz, 1 H), 3.80 (s, 1 H), 3.74 (d, J=11 Hz, 1 H), 3.58-3.12 (m, 6 H), 2.31 (t, J=6 Hz, 2 H), 1.17 (s, 9 H), 1.00 (s, 3 H), 0.89 (s, H).

b. BrAc-N-Pan Analog 7

To a solution of benzyl carbamate 12 (0.270 g, 0.56 mmol) in ethanol (6 ml) was added 10% Pd on carbon (50 mg) and concentrated HCl (40 µl). This solution was hydrogenated at atmospheric pressure and the reaction monitored by tlc disappearance of 12. The catalyst was filtered and the filtrate concentrated in vacuo. The residue was evaporated from THF three times to remove residual ethanol and then dissolved in THF (10 ml). To the solution was added triethylamine (0.180 ml, 1.31 mmol) and the solution was cooled to 0°C. To the reaction mixture was added bromoacetyl bromide (0.0435 ml, 0.5 mmol) and the resultant mixture was stirred at 0°C for 1 h. The reaction mixture was concentrated in vacuo and dissolved in ethyl acetate. The triethylamine hydrobromide was filtered off and the filtrate was concentrated and chromatographed (SiO_2, EtOAc-EtOH 10:1.5) to give 7 (100 mg, 40%): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.26 (bs, 2 H), 6.77 (t, J=4.8 Hz, 1 H), 4.14 (d, J=11.0 Hz, 1 H), 3.83 (s, 2 H), 3.75 (d, J=11.0 Hz, 1 H), 3.70-3.23 (m, 6 H), 2.42 (t, J=4.9 Hz, 2 H), 1.89 (bs, 1 H), 1.29 (s, 9 H), 1.04 (s, 3 H), 0.90 (s, 3 H).