Studies on the Biosynthesis of β-Lactam Antibiotics. Part II.[1] Synthesis, and Incorporation into Penicillin G, of (2*RS*,2′*RS*,3*R*,3′*R*)-[3,3′-$^3H_2$]-Cystine and (2*RS*,2′*RS*,3*S*,3′*S*)-[3,3′-$^3H_2$]Cystine

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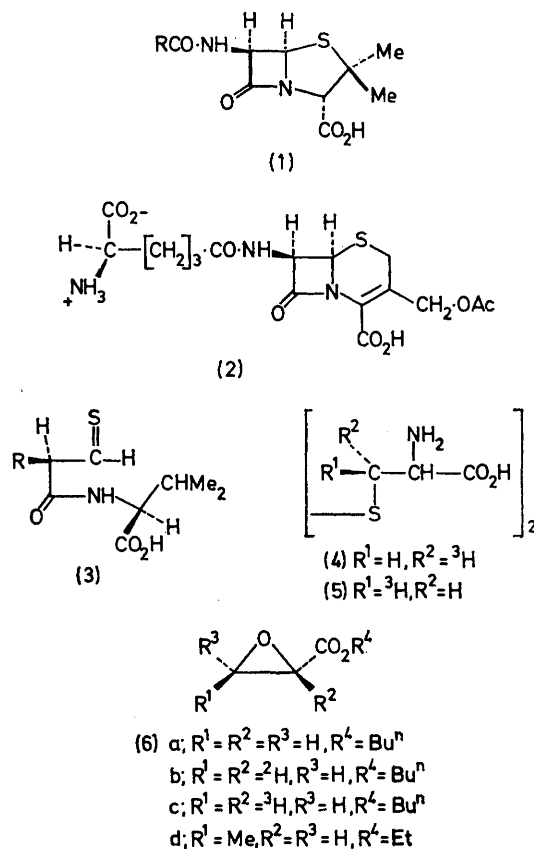
n-Butyl glycidate with sodium phenylmethanethiolate gave 3-benzylthio-2-hydroxypropanoic acid (70%). In contrast, ethyl 2,3-epoxybutyrate underwent α-attack by phenylmethanethiolate yielding, after methylation, methyl 2-benzylthio-3-hydroxybutyrate (25%) and ethyl (benzylthio)acetate (70%). n-Butyl[*cis*-2,3-$^2H_2$]acrylate (synthesized by reduction with deuterium of n-butyl 9,10-dihydro-9,10-ethenoanthracene-11-carboxylate, followed by thermolysis of the [$^2H_2$]adduct) was epoxidised stereospecifically with trifluoroperacetic acid to yield n-butyl[*cis*-2,3-$^2H_2$]glycidate, which with phenylmethanethiolate gave, stereospecifically, DL-3-benzylthio-2-hydroxy[*cis*-2,3-$^2H_2$]propanoic acid. The analogous tritium compound, obtained similarly, was resolved *via* the brucine and quinine salts to afford the (2*S*,3*S*)-acid (7c) and the (2*R*,3*R*)-enantiomer (14). These were converted into the title compounds by bromination, treatment with ammonia to give the corresponding *S*-benzylcysteines, equilibration of the α-tritium atoms with acetic anhydride–acetic acid followed by acidic hydrolysis, and finally reduction with sodium–liquid ammonia and oxidation in air of the resultant (3*R*)- and (3*S*)-[3-^{3}H]-cysteines.

The title compounds, mixed with [3,3′-^{14}C]cystine, were incubated with *Penicillium chrysogenum*, and the resultant benzylpenicillin was isolated as the *N*-ethylpiperidine salt. The ^{3}H : ^{14}C ratio of the salt indicated loss of the 3-*pro-S* and retention of the 3-*pro-R* hydrogen atom of cystine in the biosynthesis of penicillin. Thus the β-lactam ring is formed with retention of configuration at the β-carbon atom of cysteine.

THE β-lactam antibiotics penicillin (1) and cephalosporin C(2) are known to be biosynthesized from the amino-acids L-valine and L-cysteine.[2] Recent investigations in this laboratory and elsewhere [1,3] have shed light on the formation of the thiazolidine ring of (1), although numerous problems remain to be solved.

The formation of the β-lactam rings of (1) and (2) has been suggested [4] to occur *via* a thioaldehyde intermediate such as (3), postulated to accommodate the observations of Arnstein [4] that at least some of the tritium from [2-^{3}H]- or [3-^{3}H]-cystine is retained in the expected positions in the penicillin molecule. Thus a dehydrocysteine intermediate is excluded in the biosynthesis of (1) and presumably of (2). The observations of Arnstein *et al.* were recently confirmed by a double-label experiment.[5] In the formation of the β-lactam ring, whatever the exact structure of the intermediate, one of the β-hydrogen atoms of L-cysteine must be eliminated and one retained. It seemed likely that the hydrogen elimination, as a biochemical process, would be stereospecific, and we therefore undertook the determination of its stereochemistry. For this purpose, we developed a synthesis of (3*R*,3′*R*)-[3,3′-$^3H_2$]cystine (4) and its (3*S*,3′*S*)-isomer (5) (biochemically convertible into cysteine).

Our approach was based on a projected epoxide-opening reaction of a stereospecifically labelled glycidic ester, *e.g.* (6c), β-attack by phenylmethanethiolate anion upon which would yield, after hydrolysis, stereospecifically labelled 3-benzylthio-2-hydroxypropanoic acid (7c). Since compound (7a) had been resolved,[6] a route would



be available to both labelled cystines after some further appropriate transformations.

At the outset, however, no studies had been reported

[1] Part I, D. J. Aberhart and L. J. Lin, *J.C.S. Perkin I*, 1974, 2320.

[2] P. A. Lemke and D. R. Brannon, in 'Cephalosporins and Penicillins,' ed. E. H. Flynn, Academic Press, New York, 1972.

[3] (*a*) D. J. Aberhart, J. Y. R. Chu, N. Neuss, C. H. Nash, J. Occolowitz, L. L. Huckstep, and N. De La Higuera, *J.C.S. Chem. Comm.*, 1974, 625; (*b*) J. E. Baldwin, L. Löliger, W. Rastetter, N. Neuss, L. L. Huckstep, and N. De La Higuera, *J. Amer. Chem. Soc.*, 1973, **95**, 3796, 6511; (*c*) H. Kluender, C. H. Bradley, C. J. Sih, P. Fawcett, and E. P. Abraham, *ibid.*, p. 6149.

[4] H. R. V. Arnstein and J. C. Crawhill, *Biochem. J.*, 1957, **67**, 180.

[5] B. W. Bycroft, C. M. Wels, K. Corbett, and D. A. Lowe, *J.C.S. Chem. Comm.*, 1975, 123.

[6] D. B. Hope and M. Wälti, *J. Chem. Soc.* (*C*), 1970, 2475.

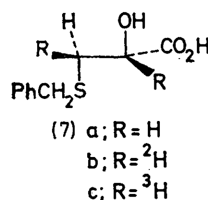
on the reactions of thiolate anions with glycidic esters,[7] and it was unclear whether the oxiran ring of (6) would be attacked at the electronically favoured α-position, or at the sterically favoured β-position. Therefore we first undertook a limited study of this reaction, examining the products formed from glycidic esters (6a and d) upon treatment with sodium phenylmethanethiolate.

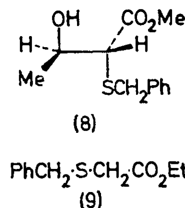
n-Butyl glycidate * (6a) (prepared from n-butyl acrylate with trifluoroperacetic acid[8]) with sodium phenylmethanethiolate (3 mol. equiv.) in tetrahydrofuran at reflux gave directly 3-benzylthio-2-hydroxypropanoic acid (7a) as the only detected product, identical with a sample obtained by treatment of β-chlorolactic acid with sodium phenylmethanethiolate. The ester cleavage accompanying the epoxide opening is in agreement with the findings of Sheehan[9] on the cleavage of phenacyl esters of penicillins and other acids by sodium benzenethiolate. The latter reagent, however, showed low reactivity towards alkyl esters.

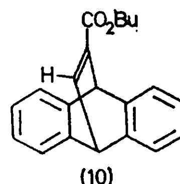
In contrast, the monomethyl analogue (6d) apparently underwent attack exclusively at the α-position yielding, after methylation with diazomethane, a mixture of methyl 2-benzylthio-3-hydroxybutyrate (8) (25%), distinguished from the 2-hydroxy-3-benzylthio-isomer by a downfield shift of 1.0 p.p.m. exhibited by the C-3 proton n.m.r. signal (originally at δ 4.20) on acetylation, and ethyl (benzylthio)acetate[10] (9) (70%), formed by retro-aldol fragmentation of an intermediate addition product. The ethyl ester portion of the starting material was retained in this product. This suggests that the epoxide functionality has an activating effect on the ester cleavage observed in the formation of (7a) and (8). The β-attack by phenylmethanethiolate anion on glycidic esters thus appears to be restricted to the C-3 unsubstituted cases, although further examples need to be examined.

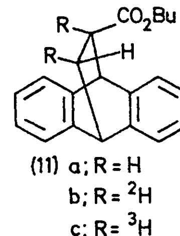
We next attempted the synthesis of the stereospecifically labelled glycidic ester (6c) required for the synthesis of labelled cystines (4) and (5). Although the steps to be used involved reactions whose stereochemistry had been established for other systems, we considered it necessary to establish the stereospecificity of such reactions for the present system. Furthermore, a cleanly stereospecific *trans* ring opening of (6) to (7), although expected, had not been established for any glycidic ester. Therefore, the process was first studied with use of deuterium labelling.

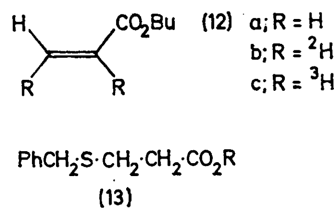
By the procedure developed by Hill[11] for the methyl ester, n-butyl propiolate was converted into the anthracene adduct (10), which with deuterium gas over palladium–calcium carbonate gave the [*cis*-$^2H_2$]-derivative (11b). Thermolysis of (11b) at 300 °C gave n-butyl [*cis*-2,3-$^3H_2$]acrylate (12b) in high overall yield. However, the n.m.r. spectrum of (12b) revealed the presence of *ca.* 5% ^1H at the C-2 and *trans* C-3 positions; thus the stereochemical homogeneity at the C-3 position was estimated to be *ca.* 95%.† Since the remaining steps in the synthesis could be carried out with apparently complete retention of stereochemical purity, we considered the above stereospecificity to be acceptable.

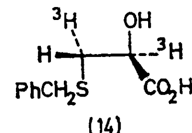


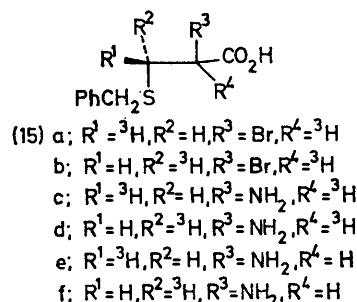












Butyl acrylate (12b) was stereospecifically converted

* The n-butyl ester (6a) was chosen in place of lower alkyl esters to reduce the volatility of intermediates, for future studies with tritium-labelled intermediates. An attempt to use *p*-t-butylphenyl esters failed on account of our inability to epoxidize *p*-t-butylphenyl acrylate in an acceptable yield.

† We have repeated the preparation of methyl [*cis*-2,3-^2H] acrylate according to the published procedure[11] and confirmed the higher stereospecificity of the synthesis in this case. Although a small amount of absorption could be detected in the n.m.r. at δ 5.8 and 6.2, the stereochemical purity was at least 98%. Presumably the lower rate of saturation of the butyl ester allows time for some hydrogen migration to occur.

[7] For other reactions of glycidic esters with sulphur-containing reagents, see J. M. Lalancette and A. Freche, *Canad. J. Chem.*, 1971, **49**, 4047; J. M. Lalancette, A. Freche, J. R. Brindle, and M. Laliberte, *Synthesis*, 1972, 526; N. F. Blau, J. W. Johnson, and C. G. Stuckwisch, *J. Amer. Chem. Soc.*, 1954, **76**, 5106; N. V. Stamicarbon, Belg. P. 643,752/1964 (*Chem. Abs.*, 1965, **63**, 8203); W. J. Sullivan and P. H. Williams, *J. Org. Chem.*, 1960, **25**, 2128; V. F. Martynov and N. A. Rozepina, *Zhur. obshchei Khim.*, 1952, **22**, 1577 (*Chem. Abs.*, 1953, **47**, 8016).

[8] W. D. Emmons and A. S. Pagano, *J. Amer. Chem. Soc.*, 1955, **77**, 89.

[9] J. C. Sheehan and G. D. Davies, *J. Org. Chem.*, 1964, **29**, 2006.

[10] J. G. Lombardino, W. M. Lamore, and G. D. Laubach, U.S. Pat. 3,042,671 (*Chem. Abs.*, 1962, **57**, 15124).

[11] R. K. Hill and G. R. Newkome, *J. Org. Chem.*, 1969, **34**, 740.

into (6b) by trifluoroperacetic acid.[9] The stereochemical purity of (6b) was established as equal to that of (12b) by the n.m.r. spectrum. Although the β-protons of unlabelled (6a) are magnetically equivalent, in the presence of $Eu(fod)_3$[12] (0.07 mol. equiv.), the α- and β-proton signals are well separated, appearing at δ 3.55 (α), 3.90, and 4.25. The corresponding spectrum of (6b) showed mainly a broadened singlet at δ 3.90, with minor absorption (*ca.* 10% total) at 3.55 and 4.25.

Treatment of (6b) with sodium phenylmethanethiolate gave a racemic mixture of (2*S*,3*S*)-3-benzylthio-2-hydroxy[2,3-$^2H_2$]propanoic acid (7b) and the (2*R*,3*R*)-enantiomer. The stereochemical homogeneity of this compound could be estimated from the 220 MHz n.m.r. spectrum of the corresponding methyl ester. The spectrum of (7a) methyl ester included well-separated doublets of doublets for the β-protons at δ 2.72 and 2.83. In the corresponding spectrum of (7b) methyl ester, a broadened singlet appeared at δ 2.83, with only minor absorption (*ca.* 5%) at δ 2.72. Thus, the epoxide-opening process (6b) ⟶ (7b) had proceeded in a clean, stereospecific manner, presumably *trans*.

For the syntheses of the cystines (4) and (5) (^{2}H instead of ^{3}H) it remained to carry out a resolution of the racemic (7b), convert the α-hydroxy-group into an amino-function, remove the α-deuterium atom, and convert the resultant *S*-benzylcysteine into cystine. Preliminary studies with unlabelled compounds indicated that although all the required reactions could be carried out, the overall yield was too low to be applied to the syntheses of asymmetrically deuteriated cystines.

The synthesis was therefore completed by using tritium-labelled intermediates. By a route analogous to the above sequence, the n-butyl propiolate–anthracene adduct (10) was reduced with tritium gas over palladium–calcium carbonate, and the product (11c) (after dilution with inactive carrier) was heated at 300 °C to yield n-butyl [*cis*-2,3-$^3H_2$]acrylate (12c). Epoxidation of (12c) gave a *ca.* 1 : 1 mixture of (12c) and n-butyl [*cis*-2,3-$^3H_2$]-glycidate (6c). To avoid working extensively with the highly radioactive, volatile glycidate (6c), this mixture was not separated by fractional distillation, but was treated directly with sodium phenylmethanethiolate in tetrahydrofuran. The product was a *ca.* 1 : 1 mixture of the desired DL-(7c), and 3-benzylthio-[2,3-$^3H_2$]propionic acid (13; R = H), the latter derived by addition of phenylmethanethiol to the unepoxidized butyl acrylate. The product mixture was easily separated by preparative t.l.c. after methylation with diazomethane. The desired (7c) was then obtained by saponification of the methyl ester under mild conditions. Under these conditions the specific rotation of an optically pure sample of (−)-L-hydroxy-acid (7a) was not changed, nor was the specific rotation changed by subjection of (−)-L-(7a) to the ring-opening conditions used for the (6c) ⟶ (7c) interconversion. Thus the relative configurations at C-2 and C-3 of (7c) must have been maintained.

Next, racemic (7c) was resolved by the published procedure,[6] the (−)-L-enantiomer {(2*S*,3*S*)-3-benzylthio-2-hydroxy-[2,3-$^3H_2$]propanoic acid (7c)} being obtained *via* the brucine salt, and the (+)-D-enantiomer [(2*R*,3*R*)] (14) *via* the quinine salt. The absolute configurations of the enantiomers were established in the published work [6] by conversion into the corresponding optically pure lactic acids.

The (−)-L-hydroxy-acid (7c) was then converted into the bromide (15a) with phosphorus tribromide, and thence into (2*RS*,3*S*)-*S*-benzyl[2,3-$^3H_2$]cysteine (15c) with aqueous ammonia at 80 °C in a pressure bottle. The process did not proceed stereospecifically under these conditions and the product was racemic. However, since the configuration at the critical C-3 position is unaffected by this process, the loss of *relative* configurational purity is inconsequential. The α-tritium atom of (15c) was then exchanged for hydrogen by treatment of (15c) with acetic anhydride–acetic acid, followed by hydrolysis of the acetate with aqueous hydrochloric acid.[13] A sample of *S*-benzyl[3-^{3}H]cysteine treated under the same conditions suffered no change in specific activity. Finally, the product (15e) was converted into (3*S*,3′*S*)-[3,3′-$^3H_2$]cystine (5) by hydrogenolysis of the *S*-benzyl group with sodium in liquid ammonia.[14] A radiochromatogram of the product indicated its high radiochemical purity.

In a similar manner, the (+)-D-hydroxy-acid (14) was converted *via* the bromide (15b) into (2*RS*,3*R*)-*S*-benzyl-[2,3-$^3H_2$]cysteine (15d), from which the α-tritium was removed by equilibration as before, yielding (15f) which was in turn converted into (3*R*,3′*R*)-[3,3′-$^3H_2$]cystine (4).

Incorporation of the Labelled Cystines (4) *and* (5) *into Penicillin G.*—The cystines (4) and (5) were mixed with DL-[3,3′-^{14}C]cystine, and the ^{3}H : ^{14}C ratio of the mixture was determined. Also a portion of the mixture was diluted with unlabelled cystine and recrystallized to constant specific activity and ^{3}H : ^{14}C ratio. The undiluted [^{3}H,^{14}C]cystines (*ca.* 5 μCi of ^{14}C) were then added to cultures of *Penicillium chrysogenum* Q 176 (ATCC 10002), additions being made 96 h after inoculation of the medium. At the same time, phenethylamine hydrochloride was added as a penicillin G side-chain precursor. After an additional 12 h incubation, penicillin G was isolated as the *N*-ethylpiperidine salt, and recrystallized to constant activity and ^{3}H : ^{14}C ratio. The incorporation of ^{14}C was variable, ranging from 0.1 to 1%. The results are shown in the Table and indicate that penicillin biosynthesis proceeds with loss of the 3-*pro-S*- and retention of the 3-*pro-R* hydrogen atom of cysteine. We did not consider it necessary to determine the location of the retained tritium atom in the biosynthetic penicillin, as Arnstein had established that

[12] For a report on the use of $Eu(dpm)_3$ in the simplification of the n.m.r. spectra of epoxides, see P. E. Manni, G. A. Howie, B. Katz, and J. M. Cassady, *J. Org. Chem.*, 1972, **37**, 2769.

[13] J. P. Greenstein and M. Winitz, 'Chemistry of the Amino-acids,' Wiley, New York, 1961, p. 1921.

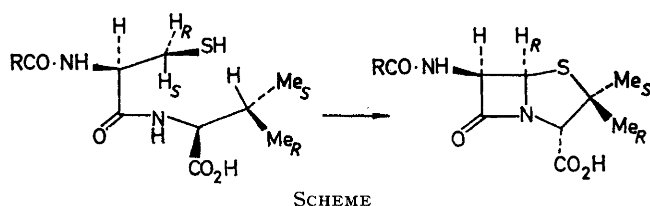
[14] J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, 1939, **130**, 109.

tritium from C-3 of cystine was located at C-5 of penicillin.[4] Some non-stereospecificity was observed in the biosynthetic results, part of which presumably arises from a minor amount of non-stereospecificity in the

Incorporation of [3,3′-$^3H_2$;3,3′-^{14}C]cystines into penicillin G

Precursor	^3H : ^{14}C ratio administered	^3H : ^{14}C ratio of isolated penicillin G *N*-ethylpiperidine salt	^3H retained (%)
(3*R*,3′*R*)	4.20 : 1	3.12 : 1	76
(3*S*,3′*S*)	5.16 : 1	1.25 : 1	21

synthetic route to the labelled cystines (4) and (5). Also, competing metabolic reactions in the mycelium may have resulted in some loss of stereochemical homogeneity in the labelled cystines, contributing to the observed results.[15] In any event, the observed ^3H : ^{14}C ratios of



SCHEME

the biosynthetic penicillins allow the conclusion that the formation of the β-lactam ring of penicillin G proceeds with *retention* of configuration at the β-carbon atom of cysteine as illustrated in the Scheme (and as also observed for the formation of the thiazolidine ring [1]). The same conclusion was recently reported by Morecombe and Young, who used tritiated cysteines prepared by an alternative route.[16]

If β-lactam ring formation proceeds *via* the postulated (but as yet undetected *in vivo*) thioaldehyde intermediate (3), then the oxidation of its cysteinyl precursor to the thioaldehyde proceeds with loss of the *opposite* hydrogen atom to that normally observed in the oxidation of primary alcohols to aldehydes by alcohol dehydrogenases [17] in which the *pro-R* hydrogen atom of the $-CH_2$·OH group is removed.

EXPERIMENTAL

I.r. spectra were taken with a Perkin-Elmer 337 spectrometer and n.m.r. spectra with a Varian A-60 spectrometer. The 220 MHz n.m.r. spectrum was provided by Dr. E. D. Becker, National Institutes of Health, Bethesda, Maryland. Mass spectra were taken with a Varian MAT-CH 5 spectrometer at 70 eV. Brinkmann silica gel HF 254 + 366 and Analtech MN 300 cellulose were used for t.l.c. Radiochromatogram scanning (Nuclear Chicago Actigraph III instrument) was used to establish radiochemical homogeneity of non-volatile compounds. Liquid scintillation counting was carried out by using a Packard Tri-Carb 314 E instrument. Water-insoluble samples were counted in 10 ml of solvent A [diphenyloxazole (PPO) (4 g) and *p*-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl POPOP) (100 mg) in toluene (1 l)]. Water-soluble samples were dissolved in 1N-ammonium hydroxide (0.25 ml) and diluted to 10 ml with solvent B [PPO (4 g), dimethyl POPOP (100 mg), and Beckman BBS-3 solubilizer (50 ml) in toluene (1 l)]. M.p.s were taken with a hot-stage apparatus and are corrected. Microanalyses were performed by Chemalytics Inc., Tempe, Arizona. The reduction of compound (10) with tritium was performed by New England Nuclear Co., Tritium Labelling Laboratory. DL-[3,3′-^{14}C]Cystine was obtained from Amersham-Searle Corp. The *Penicillium chrysogenum* culture was obtained from the American Type Culture Collection, Rockville, Maryland.

n-Butyl Glycidate (6a).—Trifluoroacetic anhydride (38.1 ml, 0.27 mol) was added over 10 min to 90% hydrogen peroxide (duPont; 6.2 ml, 0.225 mol) in ethylene chloride (25 ml) at 0°. After 15 min, the mixture was added over 30 min to a refluxing, stirred mixture of disodium hydrogen phosphate (102 g), ethylene chloride (125 ml), and n-butyl acrylate (13 g, 0.1 mol). Refluxing was continued for 30 min, then water (300 ml) was added. The ethylene chloride layer was separated, washed with 5% sodium hydrogen carbonate and water, dried ($MgSO_4$), and evaporated *in vacuo*. The resultant liquid was distilled to yield n-butyl glycidate (6.0 g, 42%), b.p. 62—65° at 4 mmHg; $\nu_{max.}$ ($CHCl_3$) 1 745, 1 425, 1 305, 1 261, 1 200, and 1 032 cm^{-1}; δ ($CDCl_3$) 1.0 (3 H, d, *J* 6 Hz), 1.2—1.8 (4 H, m), 2.94 (2 H, d, *J* 3 Hz), 3.43 (1 H, t, *J* 3 Hz), and 4.21 (2 H, t, *J* 6 Hz), *m/e* 144 (M^+, 1%), 101 (9), 87 (31), 73 (63), 71 (22), 57 (80), 56 (100), and 55 (69).

3-Benzylthio-2-hydroxypropanoic Acid (7a).—Phenylmethanethiol (8.5 g) in dry tetrahydrofuran (THF) (150 ml) was treated under nitrogen at 0 °C with an excess of solid sodium hydride. After hydrogen evolution had ceased, the white suspension was decanted from the excess of hydride. To the suspension was added n-butyl glycidate (6a) (3.6 g), and the mixture was refluxed for 2 h. Water (150 ml) was added and the pH was adjusted to 9.0. The solution was extracted several times with chloroform to remove excess of thiol, and then was acidified and extracted with ether. The extract was washed with saturated brine, dried (Na_2SO_4), and evaporated to a glass which crystallized. This was recrystallized from water to yield the acid (7a) (3.7 g, 70%), m.p. 97—98°, identical with an authentic sample.[6]

The methyl ester, prepared with diazomethane, was a gum which could not be crystallized or distilled. It gave a single spot on t.l.c. (25% ethyl acetate–hexane; R_F 0.3), and had $\nu_{max.}$ ($CHCl_3$) 3 530, 1 740, 1 448, 1 220, 1 092, 1 016, and 698 cm^{-1}; δ ($CDCl_3$; 220 MHz) 2.72 (1 H, dd, *J* 6 and 14 Hz), 2.83 (1 H, dd, *J* 4 and 14 Hz), 3.74 (3 H, s), 3.76 and 3.79 (2 H, ABq, *J* 13 Hz), 4.39 (1 H, dd, *J* 4 and 6 Hz), and 7.30 (5 H, m).

Methyl 2-Benzylthio-3-hydroxybutanoate (8).—Sodium phenylmethanethiolate was prepared as above from phenylmethanethiol (8.5 g) in THF (250 ml) and treated with ethyl 2,3-epoxybutyrate (6d) (3 g) at reflux for 90 min. Work-up as before gave a mixture which was methylated (CH_2N_2) and separated by preparative t.l.c. (ethyl acetate–hexane, 1 : 4) yielding the ester (8) (1.25 g, 25%), R_F 0.22, as a viscous liquid, b.p. 115—120° at 1 mmHg; $\nu_{max.}$ ($CHCl_3$) 3 480br, 1 725, and 1 604 cm^{-1}; δ ($CDCl_3$) 1.20, (3 H, d, *J* 6 Hz), 3.15 (1 H, m), 3.70 (3 H, s), 3.83 (2 H, s),

[15] For other reports on the unexpected exchange of α-hydrogen atoms of amino-acids in biosynthetic investigations, see N. Johns, G. W. Kirby, J. D. BuLock, and A. F. Ryles, *J.C.S. Perkin I*, 1975, 383.

[16] D. J. Morecombe and D. W. Young, *J.C.S. Chem. Comm.*, 1975, 198.

[17] R. Bentley, 'Molecular Asymmetry in Biology,' vol. II, Academic Press, New York, 1970, p. 14.

4.20 (1 H, m), and 7.33 (5 H, s), *m/e* 240 (M^+, 33%), 222 (20), 196 (100), 181 (64), 123 (77), 91 (95), and 77 (80).

Also isolated was ethyl (benzylthio)acetate (9) (3.40 g, 70%), identified by comparison of its n.m.r. spectrum [δ ($CDCl_3$) 1.20 (3 H, t, *J* 7 Hz), 3.01 (2 H, s), 3.70 (2 H, s), 4.14 (2 H, q, *J* 7 Hz), and 7.30 (5 H, s)] with that of an authentic sample.[10]

The acetate of (8), prepared with acetic anhydride–pyridine, was a viscous oil, $\nu_{max.}$ ($CHCl_3$) 1 740 cm^{-1}; δ ($CDCl_3$) 1.22 (3 H, d, *J* 6 Hz), 1.95 (3 H, s), 3.30 (1 H, d, *J* 8 Hz), 3.73 (3 H, s), 3.86 (2 H, s), 5.21 (1 H, m), and 7.40 (5 H, s).

n-Butyl Propiolate–Anthracene Adduct (*n-Butyl* 9,10-*Dihydro*-9,10-*ethenoanthracene*-11-*carboxylate*) (10).—Prepared (72%) from n-butyl propiolate (4) and anthracene by the procedure of Hill,[11] the *adduct* crystallized from methanol as prisms, m.p. 72—74°. A sample further purified by preparative t.l.c. (5% ethyl acetate–hexane) had m.p. 74—75°; $\nu_{max.}$ (KBr) 1 695, 1 450, 1 219, 1 070, 1 060, 758, 752, and 729 cm^{-1}; δ ($CDCl_3$) 0.8—1.75 (7 H, m), 4.15 (2 H, t, *J* 6 Hz), 5.25 (1 H, d, *J* 6 Hz), 5.70 (1 H, d, *J* 2 Hz), 6.9—7.6 (8 H, m), and 7.90 (1 H, dd, *J* 6.0 and 2.0 Hz), *m/e* 304 (M^+, 34%), 247 (9), 203 (100), 178 (2), and 57 (1.7) (Found: C, 82.85; H, 6.45. $C_{21}H_{20}O_2$ requires C, 82.9; H, 6.6%).

n-Butyl 9,10-*Dihydro*-9,10-*ethanoanthracene*-11-*carboxylate* (11a).—The *dihydro-adduct* (11a), prepared by the procedure of Hill,[11] crystallized from methanol as white prisms, m.p. 55—56°; $\nu_{max.}$ (KBr) 1 725, 1 460, 1 320, 1 200, 763, and 750 cm^{-1}, δ ($CDCl_3$) 0.7—1.6 (7 H, m), 1.95 (1 H, m), 2.15 (1 H, m), 2.8 (1 H, m), 3.96 (2 H, t, *J* 6 Hz), 4.30 (1 H, dd, *J* 4 and 4 Hz), 4.67 (1 H, d, *J* 3 Hz), and 6.9—7.4 (8 H, m) (Found: C, 82.15; H, 7.1. $C_{21}H_{22}O_2$ requires C, 82.3; H, 7.25%).

n-Butyl 9,10-*Dihydro*-[cis-11,12-$^2H_2$]-9,10-*ethanoanthracene*-11-*carboxylate* (11b).—The adduct (10) (5 g) and 10% Pd–$CaCO_3$ (300 mg) in ethyl acetate (40 ml) were stirred with deuterium gas at room temperature and atmospheric pressure until uptake ceased (18 h). The catalyst and solvent were removed, and the product was recrystallized from methanol to give prisms, m.p. 53—54°; $\nu_{max.}$ (KBr) 2 150, 1 725, 1 460, 1 258, 1 220, 760, and 745 cm^{-1}; δ ($CDCl_3$) 0.7—1.6 (7 H, m), 2.15 (1 H, d, *J* 4 Hz), 3.96 (2 H, t, *J* 6 Hz), 4.30 (1 H, d, *J* 4 Hz), 4.68 (1 H, s), and 6.9—7.4 (8 H, m) [a very small peak (*ca.* 0.05H) appeared at δ 2.8 (m, 11-H)]; *m/e* 308 (M^+, 3%), 235 (1), 207 (4), 206 (7), 205 (7), 204 (7), 203 (4), 178 (95), 177 (100), 152 (5), 130 (1), 129 (1), 128 (1), 57 (35), and 56 (15).

n-Butyl [cis-2,3-$^2H_2$]*Acrylate* (12b).—The deuteriated adduct (11b) (14 g) was heated under nitrogen at 290—330 °C, and the released product was collected in an ice-cold receiver. The product was redistilled giving n-butyl [*cis*-2,3-$^2H_2$]acrylate (5 g, 85%), b.p. 43—44° at 14 mmHg; $\nu_{max.}$ ($CHCl_3$) 1 715, 1 280, 1 230, 1 085 cm^{-1}; δ ($CDCl_3$) 0.85—1.80 (7 H, m), 4.20 (2 H, t, *J* 6 Hz), and 6.35 (1 H, t, *J* 2.5 Hz) [minor absorption (*ca.* 0.05 H) at δ 5.8 and 6.2]; *m/e* 130 (M^+, 0.01%), 115 (1), 87 (3), 75 (40), 57 (100), and 56 (90). A similar product was obtained from the deuteriated adduct (11b) [prepared by using 5% Pd–$BdSO_4$ or 10% Pd–C as catalyst]. However (tristriphenylphosphine)-rhodium chloride did not catalyse the reduction of (10) by deuterium gas.

n-Butyl [cis-2,3-$^2H_2$]*Glycidate* (6b).—Prepared as for n-butyl glycidate (6a) this had b.p. 62—65° at 4 mmHg; $\nu_{max.}$ ($CHCl_3$) 1 745, 1 308, 1 220, 1 040, and 945 cm^{-1}; δ ($CDCl_3$) 0.8—1.8 (7 H, m), 294 (1 H, s), and 4.21 (2 H, t, *J* 6 Hz) [minor absorption (0.05H) at δ 3.40]; *m/e* 146 (M^+, 1%), 101 (10), 89 (25), 73 (50), 71 (10), 57 (70), 56 (100), and 55 (40).

DL-3-*Benzylthio*-2-*hydroxy*[2,3-$^2H_2$]*propanoic Acid* (7b).—Prepared from (6b) as for the preparation of (7a), this had m.p. 98—99°; $\nu_{max.}$ (KBr) 3 420, 1 700, 1 255, 1 115, 765, and 695 cm^{-1}; δ [$(CD_3)_2SO$] 2.66br (1 H, s), 3.72 (2 H, s), and 7.25 (5 H, s) [weak absorption (<0.05H) at δ 4.15]. The methyl ester, prepared with diazomethane, was a gum, homogeneous on t.l.c., $\nu_{max.}$ ($CHCl_3$) 3 520, 1 740, 1 440, 1 218, and 1 090 cm^{-1}; δ ($CDCl_3$; 220 MHz) 2.83br (1 H, s), 3.77 (3 H, s), 3.79br (2 H, s), and 7.30 (5 H, m).

DL-3-*Benzylthio*-2-*hydroxy*[2,3-$^3H_2$]*propanoic Acid* (7c).—The adduct (10) (150 mg) in ethyl acetate (4 ml) containing 10% Pd–$CaCO_3$ (20 mg) was stirred with tritium gas (10 Ci) for 6 h at 20 °C. Labile tritium was removed *in vacuo* after adding benzene–ethanol (1 : 1; 20 ml). The solution was filtered and evaporated to dryness, yielding n-butyl 9,10-dihydro-[*cis*-11,12-$^3H_2$]-9,10-ethanoanthracene-11-carboxylate (11c) (8.6 Ci). A sample (18 mg; 1.0 Ci) of this material was mixed with unlabelled dihydro-adduct (11a) (4.8 g), and heated at 300 °C in a distillation flask (100 ml) with nitrogen inlet, the side-arm being directly inserted into an ice-cold receiver. The product was collected over 15 min, giving n-butyl [*cis*-2,3-$^3H_2$]acrylate (12c) (2.0 g; 800 mCi). This was then immediately epoxidized as follows.

Trifluoroacetic anhydride (14.5 ml), 1,2-dichloroethane (9 ml), and 90% hydrogen peroxide (2.3 ml) were mixed and kept in an ice-bath for 20 min. The solution was then added dropwise over 1 h to a refluxing stirred suspension of disodium hydrogen phosphate (37 g), dichloroethane (35 ml), and the product (12c) (2.0 g). Refluxing was continued for 90 min, then the mixture was cooled. Ether and water were added, and the ethereal layer was washed with 10% sodium hydrogen carbonate and saturated brine, dried (Na_2SO_4), and evaporated under low vacuum (40 °C) to an oil (3 g, *ca.* 700 mCi). An n.m.r. spectrum of the crude product indicated the presence of *ca.* 50% of starting material (12c) and 50% of n-butyl [*cis*-2,3-$^3H_2$]glycidate (6c) (as expected from studies with unlabelled materials; attempts to force the reaction to completion led to extensive formation of by-products and lower yields [18]), in addition to a small amount of dichloroethane. This mixture was not fractionated further but immediately used as follows.

THF (150 ml) and phenylmethanethiol (8.5 g) were treated under nitrogen with an excess of solid sodium hydride. After cessation of gas evolution, the white suspension was decanted into another flask which was then flushed with nitrogen. The crude n-butyl [*cis*-2,3-$^3H_2$]-glycidate (3 g) was added, and the mixture was refluxed for 2 h. After cooling, water (150 ml) was added, and the pH was adjusted to 9.0. The mixture was extracted with chloroform (3 × 100 ml). The aqueous phase was then acidified to pH 2 and extracted with ether. The extract was washed with saturated brine, dried (Na_2SO_4), and evaporated to a gum (2 g; 400 mCi). The product was converted into the methyl ester with diazomethane. A sample was examined by t.l.c. (ethyl acetate–hexane, 1 : 3). Radiochromatogram scanning indicated the presence of approximately equal amounts of two main products, DL-(7c) methyl ester and methyl 3-benzylthiopropionate (13; R = Me), with some minor radioactive material near the

[18] H. Y. Aboul-Enein, *Synth. Comm.*, 1974, **4**, 255.

origin. The major products were separated by preparative t.l.c., giving (13; R = Me) (150 mCi) and DL-(7c) (150 mCi). The latter (0.5 g) was treated with N-sodium hydroxide (5 ml) and methanol (10 ml) at 20 °C for 12 h. The resultant solution was extracted once with chloroform (very little activity extractable), then acidified and extracted with ether; the extract was dried (Na_2SO_4) and evaporated to yield (7c) (443 mg; 150 mCi) as a gum which crystallized. A small sample converted into the methyl ester with diazomethane, gave a single peak upon t.l.c. (radio scanning).

Resolution of DL-*3-Benzylthio-2-hydroxy*[2,3-$^3H_2$]*propanoic Acid* (7c).—DL-(7c) (350 mg) mixed with unlabelled hydroxy-acid (7a) (2.65 g) and anhydrous brucine (5.6 g; pre-dried for 48 h *in vacuo* at 110 °C) were dissolved in hot ethanol (30 ml; dried by refluxing for 48 h over Mg turnings followed by distillation), then kept at 20 °C for 48 h. The crystalline salt was filtered off and recrystallized from ethanol, yielding the optically pure brucine salt of (−)-L-acid (7c) (3.0 g; 40 mCi), m.p. 140—145°, $[\alpha]_D^{20}$ −21.5° (*c* 2 in EtOH) [lit.,[6] −22° (*c* 1 in EtOH)].

The brucine salt was dissolved in chloroform (50 ml) and washed with cold 2N-sodium hydroxide (3 × 25 ml). The combined aqueous washings were washed once with chloroform, then acidified with hydrochloric acid, and extracted with chloroform (6 × 20 ml). The extract was dried (Na_2SO_4) and evaporated to a glass which crystallized, and was recrystallized from carbon tetrachloride, giving (−)-L-acid (7c) (1.0 g) as white prisms, m.p. 69—70°, $[\alpha]_D^{20}$ −13° (*c* 2 in EtOH) [lit.,[6] −11.4° (*c* 1 in EtOH)].

The mother liquors from the first crystallization of the brucine salt were evaporated to dryness, and the residue was redissolved in chloroform (50 ml) and extracted with cold N-sodium hydroxide (3 × 50 ml). The extract was washed with chloroform, then acidified and extracted with chloroform (6 × 20 ml). This extract was dried (Na_2SO_4) and evaporated to yield a glass (1.64 g), which crystallized. Anhydrous quinine (2.52 g; dried for 24 h *in vacuo* at 140 °C) was added and the mixture was dissolved in hot absolute ethanol (18.9 ml; dried as above) and absolute ether (1.6 ml). The solution was kept at room temperature. After 24 h, the white needles were filtered off and recrystallized from ethanol–ether as before. As some difficulty was encountered in obtaining the optically pure salt, additional unlabelled, optically pure quinine salt (4 g) was added and the mixture was recrystallized twice more from ethanol–ether, yielding the pure quinine salt of the acid (14) (5.9 g; 40 mCi), m.p. 128—130°,* $[\alpha]_D^{20}$ −97° (*c* 2 in EtOH) [lit.,[6] −97° (*c* 1 in EtOH)].

The quinine salt was then separated into components as described for the brucine salt, and the (+)-D-acid (14) was recovered after crystallization from carbon tetrachloride (2.06 g; 35 mCi), m.p. 69—70°, $[\alpha]_D$ +11.6° (*c* 2 in EtOH) [lit.,[6] +11.4° (*c* 1 in EtOH)].

(2RS,2′RS,3S,3′S)-[3,3′-$^3H_2$]*Cystine* (5).—(2*S*,3*S*)-3-Benzylthio-2-hydroxy-[2,3-$^3H_2$]propanoic acid (7c) (1.0 g; 35 mCi) in chloroform (50 ml) was refluxed with phosphorus tribromide (1.1 ml) for 2 h. Then water (1.5 ml) was added, and refluxing was continued for a further ½ h. After cooling, more water (50 ml) was added and the chloroform phase was separated. The aqueous phase was washed with chloroform (2 × 50 ml), and the combined organic extracts were dried (Na_2SO_4) and evaporated to an oil.

The crude bromide (15a) was not purified but was treated directly with *ca.* 14M-ammonium hydroxide (25 ml) at 80 °C for 18 h in a pressure bottle. The oily bromide slowly dissolved during this time to give a homogeneous solution. After cooling, the aqueous ammonia was evaporated off *in vacuo* leaving a crystalline residue. T.l.c. [cellulose; butan-1-ol–acetic acid–water (4 : 1 : 5, upper phase)] with radioscanning showed the presence of a major radioactive impurity, R_F *ca.* 0.9 (50%), in addition to the *S*-benzylcysteine, R_F *ca.* 0.7 (50%). The majority of the impurity was removed by washing the crude product with ether, and the remainder by recrystallization of the washed crystals from boiling water (15 ml) to yield (2*RS*,3*S*)-S-benzyl-[2,3-$^3H_2$]cysteine (15c) (233 mg; 7 mCi). This product was then refluxed for 20 min in acetic acid (10 ml) and acetic anhydride (2 ml) under nitrogen. The resultant red solution was allowed to cool to room temperature, then evaporated to dryness *in vacuo*. The residue was refluxed for 2 h in 2N-hydrochloric acid (30 ml) under nitrogen, then the solution was evaporated to dryness, yielding a gummy residue, difficult to crystallize. To assist in crystallization, unlabelled DL-*S*-benzylcysteine (750 mg) was added, and the mixture was crystallized from hot water, with addition of ammonia to pH 6, yielding buff crystals of (2*RS*,3*S*)-*S*-benzyl-[3-^{3}H]cysteine (15e) (580 mg; 1.5 mCi), homogeneous on t.l.c. with radioscanning.

Finally compound (15e) (290 mg; 750 μCi) was suspended in liquid ammonia (30 ml) and treated with small pieces of clean sodium until a permanent blue colour persisted for 5 min. Then solid ammonium chloride was added to discharge the colour, and the ammonia was allowed to evaporate spontaneously. The residue was taken up in water (10 ml) and extracted with ether (15 ml). The aqueous layer was acidified to pH 8, a drop of 5% iron(III) chloride was added, and air was bubbled through the solution until it no longer exhibited a positive nitroprusside reaction. The pH was adjusted to 5.5 and the solution concentrated to yield crystalline (2*RS*,2′*RS*,3*S*,3′*S*)-[3,3′-$^3H_2$]cystine (5) (70 mg; 300 μCi), radiochromatographically pure.

(2RS,2′RS,3R,3′R)-[3,3′-$^3H_2$]*Cystine* (4).—In a manner similar to that described above, (2*R*,3*R*)-3-benzylthio-2-hydroxy[2,3-$^3H_2$]propanoic acid (14) (2.0 g; 35 mCi) was converted into the crude bromide (15b), and thence into (2*RS*,3*R*)-*S*-benzyl[2,3-$^3H_2$]cysteine (15d), crystallized from water (720 mg; 13 mCi). Equilibration of the α-tritium atom, which proceeded more cleanly in this case (no additional carrier was added), afforded (2*RS*,3*R*)-*S*-benzyl-[3-^{3}H]cysteine (15f) (350 mg; 3 mCi), which was then converted as before into the crystalline (2*RS*,2′*RS*,3*R*,3′*R*)-[3,3′-$^3H_2$]cystine (4) (100 mg; 1.3 mCi), radiochemically pure.

Incorporation of the Labelled Cystines (4) *and* (5) *into Penicillin G.—Penicillium chrysogenum* Q 176 (ATCC 10002) was grown at 30 °C for 7 days on Petri dishes prepared with Sabouraud dextrose agar, and colonies were then put into inoculum media (100 ml portions of the inoculum medium of Jarvis and Johnson,[19] in 250 ml Erlenmeyer flasks) and incubated at 26 °C on a gyrorotary shaker at 350 rev. min^{-1}. The growth medium consisted of (*a*) lactose (30 g l^{-1}) and glucose (10 g l^{-1}); (*b*) ammonium acetate (3.5 g l^{-1}) and ammonium lactate (5.5 g l^{-1}), plus other inorganic trace elements as described.[19] The inoculated media (100 ml) were incubated for 96 h before addition of radioactive precursors (yellow pigmentation appeared

* The optically pure quinine salt, recrystallised from acetone, in our hands consistently had m.p. 109—111° (lit.,[6] 124—126°); we apparently obtained a different crystalline modification.

[19] F. G. Jarvis and M. J. Johnson, *J. Amer. Chem. Soc.*, 1947 **69**, 3010.

after *ca.* 72 h). At this time, a sterile solution of phenethylamine hydrochloride (100 mg) in water (3 ml) was added, followed by the cystine (generally *ca.* 40 mg) dissolved in water (2 ml) with 1—2 drops of concentrated ammonia solution to aid dissolution. The cystine solution was added *via* a sterile 0.2 μ membrane filter in a syringe Swinney adaptor. Incubation was continued for an additional 12 h.

The mycelium was removed by vacuum filtration and washed with a little water. The filtrate was then saturated, as rapidly as possible, with an excess of solid ammonium sulphate, and refiltered. At this point, unlabelled benzylpenicillin (penicillin G) potassium salt (100 mg) in water (3 ml) was added to the filtrate, and the pH was adjusted to 2.5 with concentrated phosphoric acid. The solution was immediately extracted with ether (3 × 50 ml); each time the partially emulsified upper phase was centrifuged in a serum-capped narrow-neck centrifuge tube. The clear extract was dried (Na_2SO_4), and evaporated *in vacuo* below 30 °C to a gum (generally *ca.* 150—200 mg). After removal of volatile materials under high vacuum, acetone (5 ml) was added, followed by *N*-ethylpiperidine (added dropwise until a test drop gave an alkaline reaction on moistened pH paper). Crystallization commenced immediately, and the crystals (*ca.* 70—100 mg) were collected by centrifugation, washed several times with a little cold acetone, and then recrystallized from chloroform–acetone to constant specific activity and ^3H : ^{14}C ratio (see Table).

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