Biosynthesis of Tropolones in *Penicillium stipitatum*. VIII.[1] The Utilization of Polyketide Lactones for Tropolone Formation

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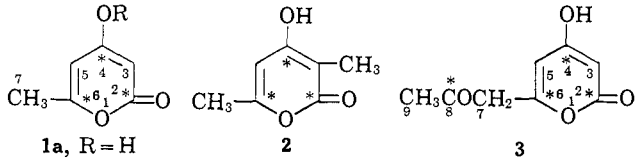
Abstract: Radioactive samples of triacetic lactone, methyl triacetic lactone, and tetraacetic lactone (6-(2-oxopropyl)-4-hydroxy-2-pyrone) were obtained by addition of acetate-1-C^{14} to cultures of *Penicillium stipitatum* grown in the presence of ethionine. In separate experiments, these lactones were added to normal cultures of *P. stipitatum;* incorporation of C^{14} into stipitatonic and stipitatic acids and other metabolites was determined. Of the three compounds, triacetic lactone was most efficiently used for tropolone synthesis. Degradation experiments, and the finding of radioactivity in ergosterol, suggested that the incorporation of activity was by breakdown to the acetate or acetoacetate level, rather than by a direct utilization of the lactones. The possible role of polyketides in tropolone biogenesis has been assessed.

In previous work[1,2] we have shown that addition of ethionine to cultures of *Penicillium stipitatum* NRRL 2104 and 1006 leads to inhibition of the usual tropolone biosynthesis. Under these conditions, triacetic lactone, **1a**, and tetraacetic lactone, **3** (6-(2-oxopropyl)-4-hydroxy-2-pyrone), accumulated as major nontropolone metabolites, along with smaller amounts of methyl triacetic lactone, **2**, orsellinic acid, and orcinol. Methyl triacetic lactone was isolated previously by Tanenbaum and his colleagues[3] from noninhibited cultures of *P. stipitatum* NRRL 1006.

Since these lactones accumulated under conditions where tropolone synthesis was inhibited, it was of interest to determine whether they represented intermediates in the pathway leading to the tropolones. For this purpose, acetate-1-C^{14} was administered to ethionine-inhibited cultures, and radioactive samples of triacetic, methyl triacetic, and tetraacetic lactones were obtained. These materials may be assumed to contain three equally labeled atoms for the triacetic compounds, and four for the tetraacetic lactone, distributed as shown below. Evidence that this was the



1a, R = H
b, R = CH_3

2

3

case was obtained by degrading a sample of the radioactive tetraacetic lactone with sodium hypoiodite; as required, the iodoform, representing the methyl group of the side chain, C_9, was without activity. A Kuhn–Roth oxidation, carried out on the methyl ether of the labeled triacetic lactone, **1b**, gave 1 mole of acetic acid ($C_7 + C_6$) containing 34.6% of the total activity; for three equally labeled atoms, the value would be 33.3%. Similarly, a sample of the triacetic lactone was converted to acetylacetone and CO_2 by treatment with acid. The CO_2, which is derived from C_2 of the pyrone ring, contained as required 33.4% of the total activity. The acetylacetone was further treated with sodium hypoiodite and the iodoform so obtained was without activity ($C_3 + C_7$).

Radioactive tri- and tetraacetic lactones were then added to standard *P. stipitatum* cultures and subsequently lactones and tropolones were isolated from the media. The results of these experiments are shown in Table I. In the case of the labeled triacetic lactone, at least 9.5% of the added radioactivity was recovered as unchanged lactone. The specific activity of this recovered material was within 1% of that originally added. Hence it may be concluded that there was no release of newly synthesized triacetic lactone in these "normal" cultures. About 45% of the added radioactivity was recovered in the combined tropolone acids. Despite this relatively high conversion to the tropolones, evidence was obtained that this was not a direct process but had taken place by a breakdown to acetate.

In the first place, the sterol component of the mycelium (ergosterol) had a specific activity of the same order of magnitude as the tropolones. Since the sterols are derived *via* acetate and mevalonate, this observation provides evidence that some breakdown of triacetic lactone to acetate (or acetoacetate) had occurred. Secondly, a resynthesis *via* acetate was suggested by a comparison of the specific radioactivity of the stipitatonic and stipitatic acids in the medium. Specific activities of 2.14×10^{-1} μcurie/mmole for stipitatic acid, and 2.85×10^{-1} μcurie/mmole for stipitatonic acid, give a ratio, stipitatic:stipitatonic, of 0.75. This is, in fact, the expected value if there are three labeled atoms in the stipitatic acid and four in the stipitatonic acid, as is the case for biosynthesis from acetate. A direct utilization of triacetic lactone, with three labeled atoms, would not give rise to four labeled atoms in stipitatonic acid (see also Biosynthetic Implications). In addition, chemical degradation of the isolated stipitatonic acid showed that the carbon dioxide released by treatment with boiling water contained 26.8% of the activity in the stipitatonic acid. This again agrees well with the value expected (25%) for biosynthesis from acetate.

(1) Part VII; R. Bentley and P. M. Zwitkowits, *J. Am. Chem. Soc.*, **89**, 676 (1967).
(2) R. Bentley, J. A. Ghaphery, and J. G. Keil, *Arch. Biochem. Biophys.*, **111**, 80 (1965).
(3) T. E. Acker, P. E. Brenneisen, and S. W. Tanenbaum, *J. Am. Chem. Soc.*, **88**, 834 (1966).

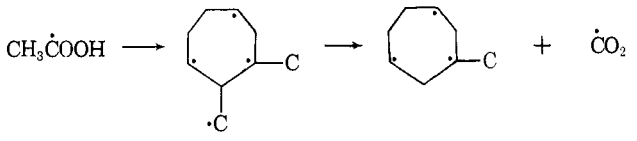
Table I. Utilization of Radioactive Lactones by *Penicillium stipitatum*

	Triacetic lactone			Tetraacetic lactone		
	Wt, g	Specific activity, dpm/mg	Total activity, μcuries	Wt, g	Specific activity, dpm/mg	Total activity, μcuries
Substrate added	0.4	36,570	6.5	0.22	67,780	6.7
Mycelium	6.3	472	1.35	6.7	161	0.49
(sterol)	(0.041)	(1,680)	(0.03)	(0.042)	(420)	(0.01)
Stipitatonic acid[a]	1.54	3,011	2.11	1.54	358	0.25
Stipitatic acid[a]	0.70	2,582	0.82	0.69	277	0.09
Triacetic lactone[b]	0.037	36,300	0.61	...	...	...
Methyltriacetic lactone[c]	0.033	5,500	0.08	...	...	...
Tetraacetic lactone[b]	...	...	...	0.1	67,500	3.07
Orsellinic acid	...	...	...	0.015	66,900	0.36
Activity recovered			4.97			4.26

[a] The amounts of the tropolones were determined as described by R. Bentley and C. P. Thiessen, *J. Biol. Chem.*, **238**, 1880 (1963). [b] The amounts of recovered lactones are based on the total radioactivity of the crude extracts and the specific activities of the purified lactone samples. More than 90% of the activity of the crude extracts was, in fact, associated with the lactones. These weights are certainly minimal owing to the losses in isolation. [c] The significance which can be attached to the results for methyl triacetic lactone is not clear. In the first place, none of this material could be detected in the culture to which tetraacetic lactone had been added. In the second place, the relatively low radioactivity (which persisted despite purification) obtained in this material when triacetic lactone was added tends to suggest that it was not derived by direct methylation of triacetic lactone.

In contrast to these results, tetraacetic lactone appeared metabolically more inert in normal cultures than the trimer. At least 45.8% of the added C^{14} was recovered as unchanged lactone and without change of specific activity (Table I). Only about 5% of the added radioactivity was recovered in the tropolones. Again, conversion to acetate had occurred as evidenced by the isolation of radioactive ergosterol from the mycelium.[4] The activity ratio, stipitatic:stipitatonic, for the acids isolated from the culture medium in this case was 0.678.[5] On chemical degradation, 26% of the stipitatonic acid activity was found in the carbon dioxide (see Table II). These results again suggest the presence of four labeled atoms in the stipitatonic acid. Since the orginal tetraacetic lactone also contains four labeled atoms, a direct conversion of lactone to tropolone is not wholly ruled out. This, however, appears unlikely in view of the finding of activity in ergosterol.

Table II. Chemical Degradation of Stipitatonic Acid



	Triacetic lactone expt		Tetraacetic lactone expt	
Compound	Specific activity, μcuries/mmole × 10^2	Activity in stipitatonic acid, %	Specific activity, μcuries/mmole × 10^2	Activity in stipitatonic acid, %
Stipitatonic acid	28.5	100	3.38	100
Stipitatic acid	20.8	73.2	2.21	65.4
CO_2	7.6	26.8	0.88	26.0

It may also be noted that in the experiment with the tetraacetic lactone, 5.4% of the added radioactivity was recovered as orsellinic acid. The specific activity of the latter was within 1% of that of the lactone, indicating that the orsellinic acid was essentially derived entirely from the added lactone and that no newly synthesized orsellinic acid had been excreted into the culture medium.

In a similar experiment, conducted on a much smaller scale with only 0.224 μcurie of radioactive methyl triacetic lactone, it was not possible to purify completely the recovered lactone. Nevertheless, the fractions which would have contained this material accounted for at least 40% of the added activity (see Table III). The tropolones contained 0.75% of the added activity. Since C^{14} was also present in the ergosterol fraction, it appears that, in this case also, the tropolone activity resulted from a breakdown of the lactone, rather than from a direct synthesis.

Table III. Utilization of Methyl Triacetic Lactone by *Penicillium stipitatum*

	Wt, mg	Specific activity, dpm/mg	Total activity, μcurie
Substrate added	14.4	34,000	0.224
Mycelium	300	128	0.018
(sterol)	(2.3)	(158)	(0.0002)
Stipitatonic acid	37.5	62	0.0011
Stipitatic acid	19.1	61	0.0006
Methyl triacetic lactone			
a. Ether extract, pH 5	10.0[a]	13,900	0.063
b. Acetone wash of stipitatic acid	28.6[b]	2,064	0.027

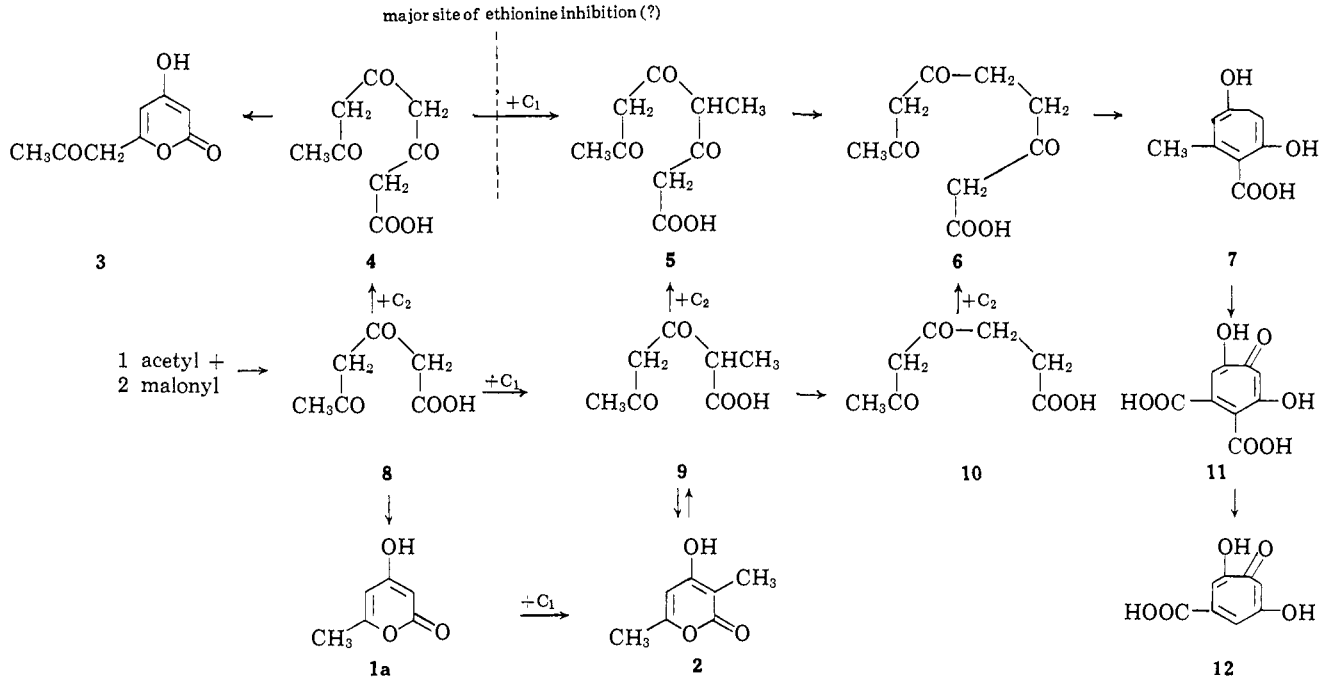
[a] This is equivalent to 4.1 mg of pure lactone at the original specific activity. [b] This is equivalent to 1.7 mg of pure lactone at the original specific activity.

Biosynthetic Implications. It has been shown that the over-all biosynthesis of stipitatonic and stipitatic acids (**11** and **12**, respectively) requires condensation of

(4) In this experiment, the ergosterol activity, as dpm/mg, was somewhat higher than that of the tropolones while with triacetic lactone the ergosterol had a lower activity than the tropolones.

(5) This value deviates somewhat from that expected for the presence of three and four labeled atoms in stipitatic and stipitatonic acids, respectively. Although the radioactivities of the samples were lower than in the case of the materials derived from triacetic lactone, they were sufficiently high so that no major error should have been involved in the counting procedure. The significance of the deviation, if real, remains unclear.

Scheme I



one acetate unit with three malonate units and that C_7 of the ring is introduced from the C_1 pool.[6] The possibility that aromatic compounds, in particular orsellinic acid, were acceptors for a C_1 unit was suggested at an early date.[7] Although this hypothesis could be fitted to the results of tracer studies, the evidence that orsellinic acid was an actual tropolone precursor has remained equivocal.[2,3,8] In considering a possible involvement of methyl triacetic lactone in tropolone biosynthesis, Acker, *et al.*,[3] postulated a further chain elongation of the corresponding acid, **9**, to form the next higher polyketide, **5**.[9] The latter was postulated to give rise to a tropolone precursor (such as **7** or a hydroxylated derivative) either by way of a cyclic intermediate and a ring expansion, or by the biologically more attractive migration, **5** → **6**. This migration may be compared to those involved in the transformation of methylmalonate to succinate and in the isomerization of glutamate to β-methylaspartate. Although there are no direct analogies for the biochemical migration of $COCH_2COOH$ (or $COCH_2COSCoA$), such a process does not seem inherently improbable; for example, migration of $CHNH_2COOH$ takes place in the glutamate mutase reaction.[10] (See Scheme I in which the compounds are written as free acids. It is, however, likely that they are present as activated forms, *e.g.*, with coenzyme A or acyl carrier protein.)

There are, of course, several possible variations on the original postulate, some of which are outlined below. The finding of the tetraacetic lactone in substantial amounts in inhibited cultures suggests that the methylation may occur at the C_8 level as indicated by **4** → **5**. (The lactone corresponding to **5** would be 5-methyl-6-(2-oxopropyl)-4-hydroxy-2-pyrone. Despite a careful examination of the extracts from inhibited cultures we have found no evidence for its presence. This would be in keeping with a key role for **5** in tropolone biosynthesis). Another possibility is the rearrangement **9** → **10**, followed by formation of **6** through chain elongation. This rearrangement is in fact more directly analogous to the methylmalonate → succinate conversion.

Reaction schemes of these types are also well suited for the construction of other tropolones found in molds. For example, a potential precursor for sepedonin[11] is 4-methyl-3,5,7,9-tetraoxodecanoic acid. In puberulonic acid, it is most likely that the labile carboxyl group is derived from a C_1 unit, rather than from an acetate carbon.[12] This can be accounted for by a further C_1 addition to one of the stipitatonic acid precursors at the carbon atom which was originally C_6 in 3,5,7-trioxooctanoic acid.

It is to be presumed that under normal conditions all of the polyketomethylene intermediates are bound to a multienzyme complex. In the presence of ethionine the methylation process is inhibited;[13] the lactones formed under conditions of ethionine inhibition, therefore, represent alternative and stabilized forms of the polyketomethylene intermediates. Our experiments suggest that, once removed from the enzyme complex, these stabilized polyketomethylene compounds can no longer reenter the normal pathway of tropolone

(6) For references see footnote 4 of part VII.

(7) R. Robinson, *Chem. Ind.* (London), 12 (1951); T. R. Seshadri, *J. Sci. Ind. Res.* (India), **14B**, 248 (1955).

(8) R. Bentley, *J. Biol. Chem.*, **238**, 1895 (1963).

(9) Although the lactone, **2**, and acid, **9**, were assumed to be in equilibrium, as shown in the scheme, this conversion would most likely require an enzyme owing to the anticipated stability of the lactone; see footnote 19.

(10) H. A. Barker, F. Suzuki, A. Iodice, and V. Rooze, *Ann. N. Y. Acad. Sci.*, **112**, 644 (1964).

(11) P. V. Divekar, H. Raistrick, T. A. Dobson, and L. C. Vining, *Can. J. Chem.*, **43**, 1835 (1965).

(12) The biosynthesis of puberulonic acid, and its decarboxylation product, puberulic acid, has been investigated by J. H. Richards and colleagues. For a discussion of the origin of the labile COOH of puberulonic acid, and the literature references, see footnote 8.

(13) The inhibition of methylation is not complete. Small amounts of tropolones and of methyl triacetic lactone are still formed. In addition, we have observed that the ethionine-inhibited mycelia contain a normal dihydroubiquinone 10, implying that the three methylation reactions required in its biosynthesis can still take place to some extent.

biosynthesis. In fact, when added to normal cultures they are in part recovered unchanged and in part appear to be broken down to acetate.

The conclusion regarding the breakdown *via* acetate is most secure in the case of triacetic lactone. In order to derive tropolones from this precursor by a direct process, a further addition of a C_2 unit is necessary at the carboxyl end of the 3,5-dioxohexanoic acid, **8**, corresponding to the lactone. Since this C_2 unit would not be labeled, the labile COOH group of stipitatonic acid (C_9, see **11**) would be without activity.[14] However, it was observed that this carbon atom contained almost exactly one-quarter of the radioactivity of the stipitatonic acid, precisely as required if this compound had been formed in the presence of labeled acetate. Since the tetraacetic lactone already contains four labeled carbon atoms, a similar conclusion cannot be made for the tropolones formed in the presence of this material. However, the fact that with both lactones, synthesis of labeled sterol had occurred strongly supports a utilization *via* acetate for the tetraacetic lactone. With methyl triacetic lactone, only 0.75% of the added activity was converted to tropolones; this low conversion and the finding of activity in the ergosterol again suggest utilization only after conversion to acetate.

This conclusion is in harmony with work carried out by Light, Harris, and Harris.[15] Working with synthetic carboxyl-labeled triacetic acid and lactone, they observed that these compounds were incorporated into aromatic metabolites in *Penicillium patulum* only after degradation to acetate. The results with *P. stipitatum* and *P. patulum* are similar to those obtained some years ago when the metabolism of triacetic acid and lactone were studied in animal tissues. Witter and Stotz[16] and Meister[17] demonstrated that in homogenates of rat liver and kidney, and beef and rabbit liver, triacetic acid, **8**, was broken down to 1 mole each of acetoacetic acid and acetic acid. A 100-fold purification of this enzyme was reported by Connors and Stotz.[18] For the utilization of triacetic lactone, conversion to the acid was necessary;[19] Meister[17] also showed that a separate lactonase enzyme was present in rat liver and more particularly in rat kidney. It is possible that a similar lactonase and hydrolyzing enzyme are involved in the utilization of the lactones studied in *P. stipitatum*. It is impossible to decide at present whether acetoacetic acid is used directly or only after a further conversion to acetate.

Experimental Section

A. Preparation of Radioactive Lactones. Three flasks, containing 700 ml of the usual growth medium plus ethionine, were inoculated with *P. stipitatum*. On the eighth and ninth day of growth, 83 μcuries of acetate-1-C^{14} was added to each flask, and on the fourteenth day, the combined media were worked up with the following modification of the method previously described.[1] The concentrated media were adjusted to pH 7 by addition of sodium bicarbonate, and were then extracted with ether. This ether extract was shown by paper chromatography to contain the bulk of the orcinol present in the medium. The residue (420 mg) obtained on evaporation was rechromatographed[21] on Celite (25 g). The orcinol-containing solutions (later chloroform and early CB-2 fractions) were evaporated and the material was sublimed at 100° (25 mm). The crystalline sublimate (22 mg) had mp 107–108°; 76,000 dpm/mg.[22]

Following this ether extraction, the pH of the media was adjusted to 5.0 and the subsequent extraction of the lactones was carried out as described earlier.[1] The yields were: 800 mg of triacetic lactone (36,570 dpm/mg); 460 mg of tetraacetic lactone (67,780 dpm/mg); 20 mg of methyl triacetic lactone (34,000 dpm/mg); 18 mg of orsellinic acid (62,000 dpm/mg). The total recovery of radioactivity in these products was 29 μcuries (5.8% of that added).

B. Chemical Degradations of Radioactive Lactones. To a solution of tetraacetic lactone (5 mg) in 2.5% NaOH (0.5 ml) was added a solution of iodine (KI, 0.2 g/ml; I_2, 0.1 g/ml) dropwise until a brown color persisted. The precipitated iodoform was removed after 15 min. In Bray's solution,[22] the count could not be distinguished from the background level.

The methyl ether of triacetic lactone (14.0 mg, 32,000 dpm/mg), prepared as described by Acker, *et al.*,[3] was refluxed with 5 ml of the Kuhn–Roth oxidizing mixture described by Eisenbraun, *et al.*,[23] for 1.5 hr (bath temperature, 130°). The acetic acid was removed by steam distillation, and the distillates were titrated with 0.1 *N* NaOH. The yield of acetic acid was 4.68 mg (78% of theory for 1 mole of lactone). The concentrated aqueous solution was counted in Bray's solution;[22] 23,279 dpm/mg as acetic acid.

For the decarboxylation of triacetic lactone, 13.5 mg (36,570 dpm/mg) was dissolved in 17 ml of water, and 1.3 ml of concentrated H_2SO_4 was added.[24] The apparatus used was the flask described by Stotz,[25] modified by the addition of a gas inlet tube. The solution was heated in an oil bath (150–180°) so that 8 ml of distillate was collected in 25 min; a stream of N_2 gas was passed through the apparatus and was finally led through two traps containing saturated barium hydroxide solution. The precipitated $BaCO_3$ (24.7 mg, 91% of theory) was counted in toluene suspension with Cab-O-Sil gel powder (Packard Instrument Co., Ill.); 7760 dpm/mg. The amount of acetylacetone in the distillate determined by reaction with *o*-phenylenediamine[24] was 9.13 mg (85% of theory). The bulk of the distillate (24 ml) was treated with sodium hypoiodite (2 ml of aqueous solution containing 0.2 g/ml of KI and 0.1 g/ml of I_2); the yield of iodoform was 27 mg. A portion dissolved in Bray's solution showed only the background level of counting.

C. Utilization of Lactones by Normal Cultures. To study the utilization of tri- and tetraacetic lactones, normal, 700-ml cultures not containing ethionine were prepared. Portions of the two lactones were added to separate cultures on the fifth to ninth day of growth (for details, see Table I). On the fourteenth day, the culture medium was removed and filtered. The medium was extracted with ethyl acetate, and these extracts were treated for recovery of the lactones as described earlier.[1] The culture fluid was then brought to pH 7 and worked up for the isolation of stipitatic and stipitatonic acids as previously described.[26] The crude stipitatic acid was washed with acetone prior to crystallization from water.

The mycelia obtained in these experiments were dried and the radioactivity was determined at infinite thickness. Ergosterol was then isolated from the mycelia following saponification.[27] The

(14) It is known that the "starter"acetate unit forms the C_8–C_4 unit of stipitatonic acid; the alternative, and in any case highly unlikely, addition of a C_2 unit to the methyl end of triacetic lactone is not considered.

(15) R. J. Light, T. M. Harris, and C. M. Harris, submitted for publication. The authors are grateful to Drs. Light and Harris for personal communications and a copy of this paper prior to publication.

(16) R. F. Witter and E. Stotz, *J. Biol. Chem.*, **176**, 501 (1948).

(17) A. Meister, *ibid.*, **178**, 577 (1949);

(18) W. M. Connors and E. Stotz, *ibid.*, **178**, 881 (1949).

(19) As Witter and Stotz[20] pointed out, triacetic lactone is remarkably stable; a 0.04 *M* solution of lactone is not affected by 1 *M* alkali at 30° for 16 hr, nor by 0.2 *M* alkali at 60° for 1 hr. There is no evidence that the lactone and free acid are readily interconvertible or in an equilibrium state in solution. The need for a lactonase is therefore readily apparent.

(20) R. F. Witter and E. Stotz, *J. Biol. Chem.*, **176**, 485 (1948).

(21) E. F. Phares, E. H. Mosbach, F. W. Dension, and S. F. Carson, *Anal. Chem.*, **24**, 660 (1952).

(22) Unless otherwise noted, all radioactivity determinations were carried out by liquid scintillation counting. This was performed either in toluene solution, or in the solution described by G. A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

(23) E. J. Eisenbraun, S. M. McElvain, and B. F. Aycock, *J. Am. Chem. Soc.*, **76**, 607 (1954).

(24) R. F. Witter, J. Snyder, and E. Stotz, *J. Biol. Chem.*, **176**, 493 (1948).

(25) E. Stotz, *ibid.*, **148**, 585 (1943).

(26) See Table I, footnote *a*.

(27) W. V. Lavate, J. R. Dyer, C. M. Springer, and R. Bentley, *J. Biol. Chem.*, **240**, 524 (1965).

radioactivities of the various materials are shown in Table I. Chemical decarboxylations of radioactive samples of stipitatonic acid were carried out as described previously by Bentley;[28] the results are shown in Table II.

To study the ultilization of methyl triacetic lactone, a *P. stipitatum* culture was grown on 25 ml of media contained in a 125-ml Erlenmeyer flask. Additions of radioactive methyl triacetic lactone were made on days 5 and 6, and the culture was filtered on the eleventh day of growth. Metabolites were isolated as in the prior experiments. The results are given in Table III.

Acknowledgment. This work was supported, in part, by Research Grants GM 08477 and AM 09311 from the National Institutes of Health, U. S. Public Health Service.

(28) R. Bentley, *J. Biol. Chem.*, **238**, 1889 (1963).

Synthesis of Secretin. I. The Protected Tetradecapeptide Corresponding to Sequence 14–27

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Contribution from The Squibb Institute for Medical Research, New Brunswick, New Jersey. *Received April 15, 1966*

Abstract: Synthesis of the protected tetradecapeptide, *t*-butyloxycarbonylnitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-leucyl-L-valinamide (XIV), is described. The chain was built up stepwise from its C-terminal moiety, L-valinamide. The nitrophenyl ester method was applied in all chain-lengthening steps.

The intestinal hormone (porcine) secretin was isolated in pure form by Jorpes and Mutt,[1] who, with their collaborators, also established the amino acid composition[2] and partial structure[3] of the hormone. Through a personal communication from Professor Jorpes and Dozent Mutt, we learned the tentative sequence of the 27 amino acids which constitute the single-chain molecule of secretin. The present paper reports the synthesis of the C-terminal half of the chain, a protected tetradecapeptide.

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    NO2 OBZL BZL     NO2       NH2 NO2           NH2
     |    |    |       |          |   |             |
BOC-Arg-Asp——Ser-Ala-Arg-Leu-Glu-Arg-Leu-Leu-Glu-
                                        Gly-Leu-Val-NH2
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The synthesis starts with the ammonolysis of benzyloxycarbonyl-L-valine *p*-nitrophenyl ester.[4] The resulting benzyloxycarbonyl-L-valinamide (I) was treated with hydrobromic acid in acetic acid to afford L-valinamide hydrobromide which was acylated, in the presence of triethylamine, with benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] to give benzyloxycarbonyl-L-leucyl-L-valinamide (II). The protecting group was removed from this dipeptide derivative in the same manner and the third amino acid residue, glycine, was attached. This stepwise chain lengthening by the nitrophenyl ester method[5] was continued, and the protected tri-, tetra-, penta-, and hexapeptide intermediates were all secured in excellent yield and in crystalline form. The next amino acid, arginine, was introduced as N^{α}-benzyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester.[6] This new active ester was used also in the preparation of the decapeptide intermediate X.

From the dodecapeptide stage on, the *t*-butyloxycarbonyl group rather than the benzyloxycarbonyl group was applied as amino protecting group to avoid partial O-acetylation of the serine residue during the removal of benzyloxycarbonyl groups with hydrobromic acid in acetic acid. Removal of the *t*-butyloxycarbonyl group with trifluoroacetic acid from the protected dodecapeptide XII and acylation with *t*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester[4] led to the desired tridecapeptide derivative XIII. The last moiety of the tetradecapeptide, L-arginine, was added in the form of N^{α}-*t*-butyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester which was prepared as described for the corresponding benzyloxycarbonyl derivative.[6]

The protected tetradecapeptide derivative N^{α}-*t*-butyloxycarbonyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminylnitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XIV) is a suitable intermediate both for the continuation of the synthesis by the stepwise approach and for the synthesis of the hormone by fragment condensation.

It may be interesting to note that the synthetic hexapeptide amide L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VIb) and the tridecapeptide, L-aspartyl-L-seryl-L-alanyl-L-arginyl-L-leucyl-L-glutaminyl-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide, are indistinguishable on paper chromatograms from the corresponding tryptic and thrombic fragments of porcine secretin.[7]

(1) J. E. Jorpes and V. Mutt, *Acta Chem. Scand.*, **15**, 1790 (1961).
(2) J. E. Jorpes, V. Mutt, S. Magnusson, and B. B. Steele, *Biochem. Biophys. Res. Commun.*, **9**, 275 (1962).
(3) V. Mutt, S. Magnusson, J. E. Jorpes, and E. Dahl, *Biochemistry*, **4**, 2358 (1965).
(4) This and all other active esters used in the synthesis of the tetradecapeptide were prepared according to the general procedure described in "Biochemical Preparations," Vol. 9, John Wiley and Sons, Inc., New York, N. Y., 1962, p 110.
(5) (a) M. Bodanszky, *Nature*, **175**, 685 (1955); (b) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959); (c) M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).
(6) M. Bodanszky and M. A. Ondetti, *Chem. Ind.* (London), 26 (1966)
(7) Personal communication from Dr. Mutt, *cf.* also ref 3.