The Controlled Synthesis of Peptides in Aqueous Medium. VIII. The Preparation and Use of Novel α-Amino Acid *N*-Carboxyanhydrides[1]

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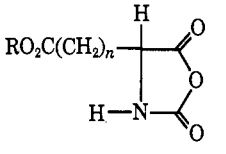
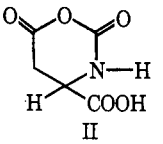
Abstract: The preparation of the crystalline α-amino acid *N*-carboxyanhydrides (Leuchs' anhydrides, NCA's) of the following amino acids is described: aspartic acid, asparagine, glutamine, *O*-trimethylsilylserine, *O*-trimethylsilylthreonine, *O*-trichloroacetylserine, histidine hydrobromide, ε-*N*-*tert*-butyloxycarbonyllysine, *O*-(2-tetrahydropyranyl)tyrosine. All of these NCA's, except those of histidine and of *O*-trichloroacetylserine, were shown to be useful in the controlled synthesis of peptides in aqueous medium. The advantages of the use in peptide chemistry of the NCA's of aspartic and glutamic acids, unprotected on the ω-carboxy group, are discussed. The novel, noncrystalline NCA of arginine hydrobromide was found useful in peptide synthesis. New procedures are given for the preparation of the known NCA's of serine, threonine, and *O*-acetyltyrosine. The process for the preparation of the NCA of ε-*N*-*tert*-butyloxycarbonyllysine should have general utility for the preparation of NCA's with acid-labile blocking groups. The mechanism of the hydrolytic rearrangement of the NCA's of serine, threonine, and histidine is discussed.

In a previous article[2] we described the use of known α-amino acid *N*-carboxyanhydrides (Leuchs' anhydrides, NCA's) in the synthesis of dipeptides and we discussed the mechanism and control of side reactions. In this paper we describe the preparation and use of novel NCA's[1] and we assess their usefulness in peptide synthesis.

NCA of Aspartic Acid. The preparation of the NCA of aspartic acid by the phosgenation of the unprotected amino acid was studied by Vajda and Bruckner.[3] The isolated reaction product was an amorphous solid which was formulated as a mixture of the desired Ib and II.



Ia, R = $C_6H_5CH_2$; $n = 1$
b, R = H; $n = 1$
c, R = H; $n = 2$



II

Treatment of this amorphous solid with aqueous ammonia had led to a mixture containing[3] significant amounts of asparagine in addition to isoasparagine and aspartic acid. The formation of asparagine was attributed[3] to the presence of II in the amorphous phosgenation product.

We reinvestigated the direct phosgenation of aspartic acid because Birkofer and Modic[4] had shown that β-amino acids do not readily form *N*-carboxyanhydrides on phosgenation. We succeeded in isolating the *crystalline* Leuchs' anhydride Ib *via* phosgenation of aspartic acid although the yield was low (20–25%). The development of a reproducible procedure for the isolation of this anhydride in crystalline form proved to be more difficult than with any of the other NCA's which we have prepared. The structural assignment is supported by the ir spectrum and elemental analysis. More importantly, treatment of the crystalline anhydride with aqueous ammonia under the conditions of Vajda and Bruckner[3] gave isoasparagine but no asparagine, and treatment of phenylalanylamide under our standard conditions[2] with this anhydride afforded only the α-linked aspartylphenylalanylamide, whereas the NCA of β-benzyl aspartate (Ia)[5] under our usual conditions[2] led to the loss of the benzyl ester and yielded a mixture of the α- and the β-aspartyl peptides. We have used the NCA of aspartic acid extensively in peptide synthesis and we employed it for the introduction of the four aspartic acid residues in the synthesis[6] of the S-protein of pancreatic ribonuclease.

The unprotected anhydride Ib offers several advantages over the commonly used reagents in which the third functionality of aspartic acid is protected. For example, Battersby and Robinson[7] have shown that saponification of peptides containing β-methyl

(1) A preliminary report dealing with certain aspects of this work has appeared: R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and R. Hirschmann, *J. Amer. Chem. Soc.*, **88**, 3163 (1966).

(2) R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schoenewaldt, H. Joshua, H. Barkemeyer, D. F. Veber, W. J. Paleveda, Jr., T. A. Jacob, T. E. Beesley, and R. G. Denkewalter, *J. Org. Chem.*, **32**, 3415 (1967).

(3) T. Vajda and V. Bruckner, *Acta Chim. Acad. Sci. Hung.*, **16**, 215 (1958).

(4) L. Birkofer and R. Modic, *Justus Liebigs Ann. Chem.*, **628**, 162 (1960).

(5) A. Berger and E. Katchalski, *J. Amer. Chem. Soc.*, **73**, 4084 (1951); M. Frankel and A. Berger, *Nature (London)*, **163**, 213 (1949); E. Katchalski in "Methods in Enzymology," Vol. 3, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, New York, N. Y., 1957.

(6) R. G. Strachan, W. J. Paleveda, Jr., R. F. Nutt, R. A. Vitali, D. F. Veber, M. J. Dickinson, V. Garsky, J. E. Deak, E. Walton, S. R. Jenkins, F. W. Holly, and R. Hirschmann, *J. Amer. Chem. Soc.*, **91**, 503 (1969); S. R. Jenkins, R. F. Nutt, R. S. Dewey, D. F. Veber, F. W. Holly, W. J. Paleveda, Jr., T. Lanza, R. G. Strachan, E. F. Schoenewaldt, H. Barkemeyer, M. J. Dickinson, J. Sondey, R. Hirschmann, and E. Walton, *ibid.*, **91**, 505 (1969); R. Hirschmann, R. F. Nutt, D. F. Veber, R. A. Vitali, S. L. Varga, T. A. Jacob, F. W. Holly, and R. G. Denkewalter, *ibid.*, **91**, 507 (1969), and other references cited therein.

(7) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).

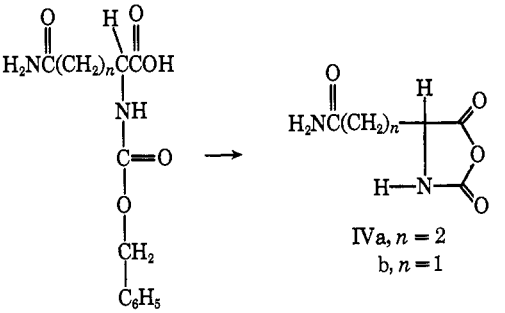
or ethyl esters of aspartic acid leads to the formation of β- as well as α-aspartyl peptides, doubtless *via* a cyclic imide intermediate. The same problem arises[8] with *β-benzyl* esters. Possibly the formation of α- and β-aspartyl peptides observed by us in the use of Ia occurs *via* such a rearrangement. Protection of the β-carboxy group as a *tert*-butyl ester is known to circumvent these problems to a large extent.[9] Nevertheless even the saponification of peptides containing *β-tert*-butyl aspartates may prove troublesome.[10] In the absence of a β-carboxy protecting group such rearrangements have been reported to occur in solution only on prolonged standing near neutrality[11a] or at high temperatures,[11b] and on heating anhydrous peptides *in vacuo*.[11c] In a recent discussion, Ondetti, *et al.*, pointed out[12] that peptides containing the aspartylglycyl sequence are unusually prone to form succinimido derivatives, even in the absence of base, when the β-carboxyl group is esterified.

The behavior of our fragments of S-protein toward enzymatic hydrolysis has been studied extensively[13] in these laboratories. In each case, complete enzymatic cleavage of the aspartyl peptide bond was observed under conditions which do not cleave β-aspartyl peptide bonds.[13] We have seen no evidence for an $\alpha \rightarrow \beta$ rearrangement under the conditions which we employ in the synthesis and purification of peptides.

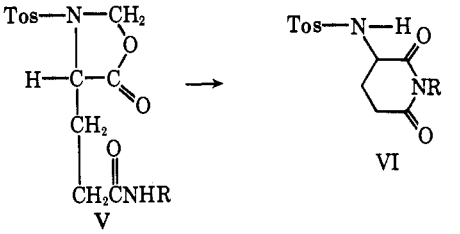
The absence of protection at the third functionality offers an advantage also in the preparation of hydrazides. Treatment of esters with hydrazine is not considered a generally applicable method for the preparation of more highly complex acylated polypeptide hydrazides.[14] The absence of ω-esters of the dibasic amino acids in our synthesis of S-protein may in part explain the successful preparation of hydrazides from polypeptide esters of high molecular weight.[6] Hydrazinolysis can lead to unwanted side products even when aspartic acid is present as the *β-tert*-butyl ester.[15]

NCA of Glutamic Acid. The NCA of glutamic acid (Ic) had been described by Kovacs and his associates.[16] These workers observed that the *N*-carboxy-L-glutamic 1,5-anhydride, an intermediate in the hydrogenolysis of *N*-benzyloxycarbonyl-L-glutamic anhydride, rearranges to give Ic. The latter was also obtained[16] from the catalytic hydrogenation of *N*-carboxy-L-glutamic anhydride γ-benzyl ester. In our hands attempts to isolate Ic by the first named procedure led to a further rearrangement product, pyroglutamic acid. Berger and his associates[17] prepared the NCA of glutamic acid by direct phosgenation of the amino acid, and this is the procedure which has been routinely employed in this laboratory. We have extensively used this anhydride in peptide synthesis and we have seen no evidence for the formation of γ-glutamyl peptides.

NCA's of Asparagine and Glutamine. The α-amino acid *N*-carboxyanhydrides of asparagine and glutamine had not been previously described. Because phosgene reacts with primary amide groups especially in the presence of bases to yield nitriles,[18] the NCA's of glutamine (IVa) and asparagine (IVb) cannot be prepared by the phosgenation of the amino acids. Indeed, Wilchek and his associates[19] have recently reported that the reaction of asparagine and glutamine with phosgene affords the anhydrides of β-cyano-*N*-carboxy-L-alanine and of γ-cyano-*N*-carboxy-α-amino-L-butyric acid, respectively. We prepared the NCA's of asparagine and glutamine by the Ben-Ishai–Katchalski modification[20] of Leuchs' method.[21] After the treatment of benzyloxycarbonyl-L-glutamine (IIIa) with PBr_3 in dioxane at room temperature the desired anhydride IVa could be isolated in crystalline form in



33% yield after silica gel chromatography. Use of this anhydride in peptide synthesis under the usual conditions[2] afforded the desired α-glutaminyl peptides without formation of any γ-glutaminyl peptides. The usefulness of this NCA is noteworthy in view of the possibility of intramolecular rearrangement. Thus it had been shown[22] that the structurally related oxazolidone amides V cyclized to the imides VI on recrystal-



(8) M. Goodman and F. Boardman, *J. Amer. Chem. Soc.*, **85**, 2483 (1963); R. W. Hanson and H. N. Rydon, *J. Chem. Soc.*, 836 (1964); M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *J. Amer. Chem. Soc.*, **89**, 6753 (1967).

(9) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).

(10) E. Wünsch and F. Drees, *Chem. Ber.*, **99**, 110 (1966); R. Roeske, *J. Org. Chem.*, **28**, 1251 (1963); K. L. Agarwal, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, 1384 (1968). These problems may occur also in solid-phase peptide synthesis [R. B. Merrifield in "Advances in Enzymology," Vol. 32, F. F. Nord, Ed., Interscience Publishers, New York, N. Y., 1969].

(11) (a) E. E. Haley, B. J. Corcoran, F. E. Dorer, and D. L. Buchanan, *Biochemistry*, **5**, 3229 (1966); (b) B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **47**, 2357 (1964); (c) D. L. Buchanan, E. E. Haley, F. E. Dorer, and B. J. Corcoran, *Biochemistry*, **5**, 3240 (1966).

(12) M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluscec, and O. Kocy, *ibid.*, **7**, 4069 (1968); M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *J. Amer. Chem. Soc.*, **89**, 6753 (1967).

(13) F. Holly, E. Walton, S. Jenkins, and R. Nutt, unpublished observations.

(14) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *J. Amer. Chem. Soc.*, **74**, 470 (1952).

(15) H. Zahn, H. Bremer, W. Sroka, and J. Meienhofer, *Z. Naturforsch., B*, **20**, 646 (1965).

(16) J. Kovacs, H. Nagy Kovacs, and R. Ballina, *J. Amer. Chem. Soc.*, **85**, 1839 (1963).

(17) A. Berger, J. Kurtz, T. Sadeh, A. Yaron, R. Arnon, and Y. Lapidoth, *Bull. Res. Counc. Isr. A*, **7**, 98 (1958).

(18) A. J. Speciale and L. R. Smith, *J. Org. Chem.*, **28**, 1805 (1963); S. Yanagida, H. Hayama, and S. Komori, *ibid.*, **34**, 4180 (1969).

(19) M. Wilchek, S. Ariely, and A. Patchornik, *ibid.*, **33**, 1258 (1968).

(20) D. Ben-Ishai and E. Katchalski, *J. Amer. Chem. Soc.*, **74**, 3688 (1952).

(21) H. Leuchs, *Ber.*, **39**, 857 (1906).

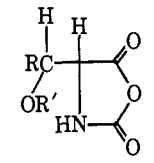
(22) J. Rudinger, *Rec. Chem. Progr.*, **23**, 3 (1962).

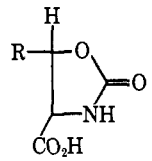
lization from polar solvents. The successful use of IVa in peptide synthesis is, however, consistent with the results obtained with the NCA of glutamic acid (see above). In the synthesis of S-protein,[6] the NCA of glutamine was used to introduce three glutamine residues.

Similarly benzyloxycarbonyl-L-asparagine (IIIb) was used to prepare the crystalline NCA IVb in about 35% yield. We have used this anhydride successfully in peptide synthesis but yields are generally too low to make the reagent satisfactory for use in *sequential* peptide synthesis without isolation of intermediates. For this reason, we have relied on *tert*-butyloxycarbonyl-L-asparagine hydroxysuccinimide ester for the introduction of this amino acid in the synthesis of S-protein.[6] Conventional methods for the introduction of asparagine and glutamine can, however, also lead to the formation of by-products.[23]

NCA's Derived from Serine and Threonine. The NCA of L-serine (VIIa) was first prepared by Fasman and Blout[24] by the phosgenation of L-serine in ethyl acetate. In our hands, the product was contaminated by the *O*-chlorocarbonylserine NCA (VIIb). There was evidence for the reaction at the alcoholic hydroxyl group even when only 1 equiv of phosgene was added to the amino acid as evidenced by a *broad* absorption maximum at 5.6 μ and a maximum at 8.6 μ in the ir spectrum. Because serine dissolved only slowly, the dissolved amino acid was, in effect, exposed to an excess of phosgene even under these conditions. In order to trap the HCl which was thought to catalyze the side reaction, we prepared the crystalline silver serinate and allowed it to react with a 2% excess of phosgene. This procedure afforded pure crystalline NCA VIIa in about 50% yield.[25] Treatment of amino acids or peptides with the NCA of serine under our usual conditions[2] did not lead to the formation of seryl peptides. This result was not surprising in view of the ease with which VIIa rearranges to the oxazolidone derivative VIIIa.[26] Furthermore, Fasman and Blout[24] had been unsuccessful in polymerizing VIIa.

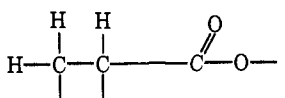
It is possible that the conversion of VIIa to VIIIa proceeds *via* the isocyanate IX. Inspection of molecular models shows that the observed catalysis of this ring opening reaction by the seryl hydroxyl group may be due to intramolecular abstraction of the NH proton by the hydroxyl oxygen or it may be attributed to intramolecular protonation of the C-5 carbonyl group by the hydroxyl hydrogen. An analogy for the former mechanism is the general base catalyzed formation of the isocyanates from NCA's, a side reaction[2] in peptide synthesis at pH 10. However, models seem to favor the alternate specific acid catalyzed pathway. Furthermore the amount of CO_2 evolved from the NCA of threonine (VIIf) or its *O*-trimethylsilyl derivative on





VIIa, R = R′ = H
b, R = H; R′ = OCCl
c, R = H; R′ = CCl_3C(=O)
d, R = H; R′ = CF_3C(=O)
e, R = H; R′ = Me_3Si
f, R = CH_3; R′ = H
g, R = CH_3; R′ = Me_3Si

VIIIa, R = H
b, R = CH_3



IX

treatment with sulfuric acid is only about half the theoretical amount. We attribute this result to a partial, acid-catalyzed, rearrangement of these NCA's to VIIIb. This is not true of the analogous NCA's of serine which evolve the theoretical amount of CO_2 under these conditions.

Since we wished to avoid saponification reactions in our scheme for the synthesis of polypeptides, use of the *O*-acetate ester of serine NCA[24] was not attractive. We prepared VIIc and VIId which contain more labile esters. Only the former could be obtained in pure crystalline form and neither reagent afforded seryl peptides in significant yield. We have successfully employed the known[27] NCA of *O*-benzylserine in peptide synthesis, but this reagent, too, requires removal of the protecting group as an extra step.

We therefore prepared the *O*-trimethylsilyl ether VIIe because this blocking group is known[28] to be removable with extraordinary ease. The NCA of serine (VIIa) could be converted into VIIe under anhydrous conditions with trimethylchlorosilane in THF using pyridine or 4-methylthiazole as the proton acceptor.[29] Similarly, we prepared the trimethylsilyl derivative (VIIg) of threonine NCA.

We have used the O-protected NCA's VIIe and VIIg extensively in peptide synthesis. The *O*-trimethylsilyl protecting group is removed under our standard[2] coupling conditions. The yields are, however, variable and generally lower than those obtained with NCA's lacking a third functionality. In the synthesis of bradykinin, a significant amount of the hydantoic acid by-product was formed.[13]

NCA of Histidine. To introduce histidine into peptide chains, we did not wish to use *im-N*-benzyl-

(23) D. T. Gish, T. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *J. Amer. Chem. Soc.*, 78, 5954 (1956); E. Schnabel, H. Klostermeyer, J. Dahlmans, and H. Zahn, *Justus Liebigs Ann. Chem.*, 707, 227 (1967); H. Takashima, V. du Vigneaud, and R. B. Merrifield, *J. Amer. Chem. Soc.*, 90, 1323 (1968); C. Meyers, R. T. Havran, I. L. Schwartz, and R. Walter, *Chem. Ind. (London)*, 136 (1969). See, however, R. Dewey, H. Barkemeyer, and R. Hirschmann, *ibid.*, 1632 (1969).

(24) G. D. Fasman and E. R. Blout, *J. Amer. Chem. Soc.*, 82, 2262 (1960).

(25) Y. Lapidot and E. Katchalski [*Isr. J. Chem.*, 6, 147 (1968)] studied the effect of the solvent used in the phosgenation step on the extent of esterification of the serine hydroxyl group. See also T. Kaneko, I. Takeuchi, and T. Inui, *Bull. Chim. Soc. Jap.*, 41, 974 (1968).

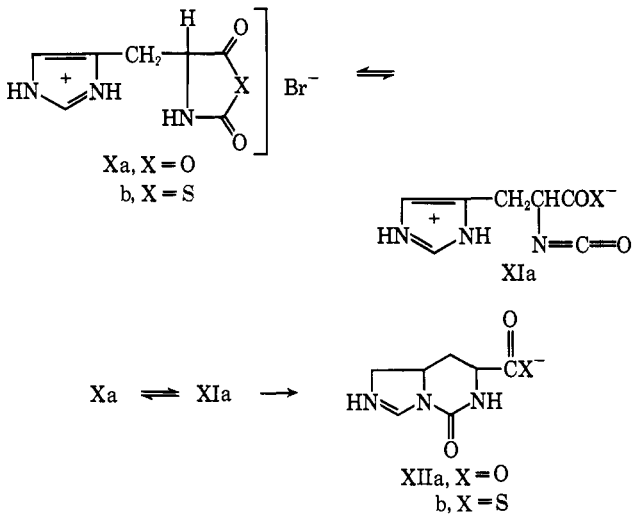
(26) T. Saito, *ibid.*, 37, 624 (1964).

(27) Z. Bohak and E. Katchalski, *Biochemistry*, 2, 228 (1963).

(28) L. Birkofer, W. Konkol, and A. Ritter, *Chem. Ber.*, 94, 1263 (1961); R. A. Boissonas in "Advances in Organic Chemistry," Vol. 3, R. A. Raphael, E. C. Taylor, and H. Wynberg, Ed., Interscience Publishers, New York, N. Y., 1963.

(29) Professor Scheraga has kindly informed us that the *O*-trimethylsilyl NCA of serine (VIIe) proved to be useful in trimethylamine-catalyzed polymerization reactions: H. Scheraga, R. H. Andreatta, and L. Hughes, private communication. The use of other novel NCA's described herein in polymerization reactions is now being studied in these laboratories.

histidine NCA,[30] because the removal of this imidazyl blocking group requires the use of sodium in liquid ammonia which can cause unwanted side reactions. We prepared the NCA of histidine as the crystalline hydrobromide salt Xa from α-*N*-benzyloxycarbonyl-L-histidine in THF with PBr_3. Attempts to use this reagent under our standard conditions of peptide synthesis did not lead to histidyl peptides. The major product from the reaction proved to be the tetrahydropyrimidin-5-one XIIa which was isolated as an amorphous solid. The same compound was obtained in addition to histidine in about 80% yield when Xa was hydrolyzed at pH 8. When the hydrolysis of the NCA was carried out at pH 6.5, histidine was obtained as the major reaction product. At an intermediate pH (7.5) histidine and XIIa were formed in about equal yield. Hydrolysis of XIIa at pH 5.3 in citrate buffer at 100° for 15 min afforded histidine, but XIIa was essentially stable at pH 10 and in 2.5 *N* HCl at 100° for 15 min. Partial decomposition occurred after 15 min at 100° at pH 7. The structure assignment is supported by the ir spectrum of XIIa (ν_{max}^{Nj} 1700 cm^{-1}) and by the peaks in the nmr spectrum at 483 and 405 cps. The structure assigned to the rearrangement product is also supported by the fact that the *N*-thiocarboxyanhydride[31,32] (the 2,5-thiazolidinedione) of histidine hydrobromide (Xb) rearranges at pH 10 to afford a product (XIIb) which, in contrast to XIIa, could be isolated as a crystalline solid[31,32] and therefore characterized also by elemental analysis. We believe that the imidazotetrahydropyrimidine XIIa is formed *via* the isocyanate XIa because a molecular model indicates that nitrogen-1 of the imidazole ring is favorably located for proton abstraction from the nitrogen of the anhydride ring. Further support for this mechanism is provided by the fact that the ratio of the amounts of XIIa and histidine formed changes sharply at about pH 7, close to the p*K* of imidazole. A possible explanation for the fact that Xb but not Xa is useful in peptide synthesis has been proposed.[31]

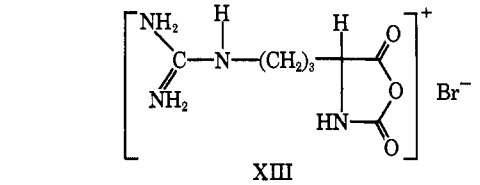


In a recent paper[33] Frensdorff, Wilchek, and Sela reported the preparation of an amorphous NCA of histidine hydrochloride. Treatment of ribonuclease at 0–4° with this anhydride for 16 hr at an initial pH of 7.0 and a final pH of 3.8 or at a constant pH of 7.0 led to a derivative enriched with up to 22 histidine residues per ribonuclease molecule.[33] The successful use of the NCA of histidine by these workers to form polyhistidyl peptides may be due to the fact that the rearrangement of the NCA to XIIa is minimized at the lower pH permissible in the uncontrolled introduction of polyhistidyl residues. It is also possible that XIIa served to introduce histidyl residues.[32]

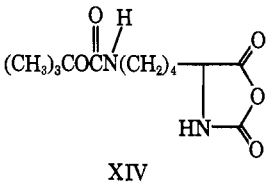
Derivatives of Tyrosine NCA. In a previous communication[2] we reported the use of the NCA of tyrosine to prepare five tyrosyl dipeptides. This NCA suffers from two limitations. Because the NCA reactions are carried out at a pH at which the phenolic hydroxyl is about half-ionized, the phenolate anion can compete with amino groups as a nucleophile in coupling reactions. In addition, this anhydride, like the NCA of tryptophan, is particularly insoluble in the reaction mixture and as a result significant amounts of the unreacted Leuchs' anhydride are often recovered even after a reaction has been carried out for 5 min in a Waring Blendor. We therefore studied the use of the *O*-acetyltyrosine NCA in peptide synthesis in aqueous medium. This reagent was first prepared by Bailey[34] from *O*-acetyl-*N*-benzyloxycarbonyl-L-tyrosine and later by Schlögl[35] by the direct phosgenation of the O-acetylated amino acid. This reagent has been employed both in controlled peptide synthesis under anhydrous conditions[34,36] and in polymerization reactions.[35,37] We were able to prepare this reagent by the acetylation of tyrosine NCA with acetyl chloride in THF in the presence of pyridine. We have not, however, used it extensively in peptide synthesis in aqueous medium because the tyrosyl acetate ester is itself an active ester capable of undergoing O → N rearrangement reactions of the acetyl group. We prepared the *O*-tetrahydropyranyl ether XVa by treatment of tyrosine NCA with dihydropyran in the presence of *p*-toluenesulfonyl chloride. The reaction afforded two diastereoisomeric pyranyl ethers having $[\alpha]^{25}_{589}$ −89.3 and −177°, respectively. The pyranyl ether $[\alpha]_{589}$ −89.3° proved to be far more soluble in the aqueous reaction mixture than tyrosine NCA, and it generally afforded tyrosyl peptides in higher yields than the unprotected NCA. The pyranyl ether protecting group is lost during the decarboxylation steps and thus the reaction product contains tyrosine as the free phenol. The other isomer ($[\alpha]^{25}_{589}$ −177°) proved to be very insoluble and was, therefore, not used for controlled peptide synthesis in aqueous medium.

NCA of Arginine Hydrobromide. We have prepared the NCA of arginine hydrobromide (XIII) as an amorphous solid from L-α-*N*-benzyloxycarbonylarginine and

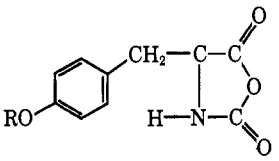
(30) A. Patchornik, A. Berger, and E. Katchalski, *J. Amer. Chem. Soc.*, 79, 5227 (1957).
(31) R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, Jr., H. Schwam, H. Barkemeyer, B. H. Arison, D. F. Veber, R. G. Denkewalter, and R. Hirschmann, *ibid.*, 90, 3254 (1968).
(32) R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, Jr., H. Schwam, H. Barkemeyer, B. H. Arison, D. F. Veber, R. G. Strachan, J. Milkowski, R. G. Denkewalter, and R. Hirschmann, *J. Org. Chem.*, 36, 49 (1971).
(33) A. Frensdorff, M. Wilchek, and M. Sela, *Eur. J. Biochem.*, 1, 281 (1967).
(34) J. L. Bailey, *J. Chem. Soc.*, 3461 (1950); J. L. Bailey, *Nature (London)*, 889 (1949).
(35) K. Schlögl, F. Wessely, and E. Wawersich, *Monatsh. Chem.*, 84, 705 (1953).
(36) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, 20, 1190 (1955).
(37) B. G. Overell and V. Petrow, *J. Chem. Soc.*, 232 (1955).



XIII



XIV



XVa, R =

b, R = CH_3CO-

PBr_3 in the THF.[38] The product showed ir maxima at 5.44 and 5.64 μ and was generally found to contain about 50% of the desired anhydride by measuring the amount of CO_2 evolved on treatment of the amorphous solid with H_2SO_4. The approximate concentration of the NCA in the product could also be determined by the yield of arginylleucine obtained with a given sample. The impurities are thought to be largely derived from benzyl bromide and PBr_3. Although the compound was not obtained in crystalline form, it is useful because it represents one of the few derivatives of arginine, protected at the guanidino group only by a proton, which is available for the introduction of arginine into peptides.

NCA of ε-Boc-Lysine. The NCA of ε-*tert*-butyloxycarbonyllysine (XIV) was prepared by allowing the ε-N-protected amino acid to react with silver cyanide prior to the phosgenation step. In this manner the acid-labile ε-amino blocking group was not exposed to the HCl which is formed when an amino acid is phosgenated under standard conditions.

Experimental Section

General Procedure for the Preparation of Leuchs' Anhydrides. The following procedure is applicable to the preparation of the NCA's of Ala, Val, Leu, Ile, Phe, Met, Trp, Tyr, S-benzyl-Cys, and ε-benzyloxycarbonyl-Lys. Phosgenation reactions may generally be carried out on a 200-g scale. The amino acids were used as supplied except that tyrosine was vacuum dried at 100°. All amino acids were finely ground in a mortar prior to use. Either tetrahydrofuran or dioxane was used as the solvent for the phosgenation step. Dioxane was purified as described by Fieser and Fieser. The THF (MC&B, reagent) was used without purification providing it was essentially free of peroxide. The latter solvent generally permitted shorter reaction times and frequently gave higher yields. In the preparation of the NCA of Glu, phosgenation in THF (7 hr at 40–50° followed by standing for 14 hr at 25°) doubled the yield and gave a product of only slightly lower quality (see below). Similarly, in the preparation of the NCA of Ala, tetrahydrofuran was the solvent of choice. The ethyl acetate was dried by storage over molecular sieves. Hexane was used as supplied. We have successfully used a water aspirator to evaporate THF from reaction mixtures, using two Dry Ice traps and a safety flask in series.

Phosgene was passed through a safety trap and introduced into the stirred suspension of amino acid and solvent. It was generally used in large excess. To minimize variables a flowmeter was used. Heat was supplied with an oil bath or with a heating mantle. In general phosgene was introduced until the solution became completely clear. In some instances, however, complete solution may not be obtained. Nitrogen was passed through the reaction mixture for 30 min after the reaction was completed to remove some of the excess phosgene. The solutions were generally filtered prior to the concentration step.

During the subsequent operations, moisture was carefully excluded at all stages. All transfers were made under a dry nitrogen flow or a nitrogen blanket, and the products were filtered under dry nitrogen pressure. Transfers of solid products were generally carried out in a drybox. The product was subdivided into smaller samples to avoid the repeated warming and opening of a given sample. The NCA's were kept in larger bottles or plastic bags containing Drierite and stored in a Dry Ice chest. The samples were allowed to come to room temperature before the containers were opened. Materials stored in this manner were generally found to be stable for months except for the anhydride of *glycine* which should only be made immediately prior to use. The NCA of proline was also found to be relatively unstable.

Our general procedure for the preparation of NCA's, typified by the preparation of the NCA of valine, is given below.

CO_2 Assay of the Leuchs' Anhydrides. The gravimetric method developed in these laboratories is a modification of the titrimetric procedure of Patchornik and Shalitin.[39] A sample equivalent to 4–8 mg of CO_2 was treated with 5 ml of 2 *N* H_2SO_4 and the reaction mixture was heated in a boiling water bath for 15 min. The evolved CO_2 was absorbed on Ascarite and measured gravimetrically.

NCA of L-Valine. In a hood with good draft, a 1-l., three-necked round-bottomed flask fitted with a mechanical stirrer having a nitrogen inlet around the stirrer shaft, a phosgene inlet (subsurface), an adapter supporting a thermometer, and reflux condenser (fitted with a $CaCl_2$ drying tube) was flushed with dry, high-purity nitrogen for 1 hr before use. THF (300 ml) and finely ground L-valine (30 g, 0.256 mol) were charged to the flask, phosgene addition was begun at a moderate rate, and gentle external heating was applied to bring the stirred reaction mixture to 45–50°. Phosgenation and stirring were continued at 45–50° until all solids dissolved (45–60 min) and for 15 min thereafter. Phosgene addition and heating were stopped, and nitrogen was connected to the subsurface inlet and was bubbled through the solution as it cooled to room temperature. Flushing was discontinued and the stirred reaction mixture was evaporated to dryness (aspirator, safety trap, Dry Ice trap, and Dry-Iced receiver) using a water bath at a temperature not exceeding 40°. The flask was vented with dry nitrogen and all subsequent operations were carried out under nitrogen.

The white solid residue was dissolved in 55 ml of ethyl acetate and filtered. Hexane was added to the magnetically stirred solution to turbidity (150 ml) and crystallization was induced by stirring, scratching, or seeding. The slurry of crystals was stirred for 30 min to 1 hr, more hexane (65 ml) was added, and the slurry was stirred for another 30 min. The product was filtered under N_2 pressure and washed with about 60 ml of 9:1 hexane–ethyl acetate. The product was dried on the funnel under continued nitrogen flow. It was weighed, and subdivided in a drybox. The yield was 20.55 g (56.8%); dec pt 67–69° (lit.[40b] dec pt 65°); $[\alpha]^{27}_{589}$ −44.4° (*c* 4.476, acetone). *Anal.* Calcd for $C_6H_9NO_3$: C, 50.35; H, 6.34; N, 9.79; CO_2, 30.7. Found: C, 50.61; H, 6.46; N, 9.74; CO_2, 30.5.

L-2,5-Dioxo-4-oxazolidineacetic Acid. (NCA of L-Aspartic Acid) (Ib). A 1-l., three-necked flask was fitted with a mechanical stirrer, a thermometer, a drying tube vented to the rear of the hood, and with a source of phosgene. Phosgene was passed into a suspension of 10 g of L-aspartic acid in 400 ml of dry THF (MCB Industrial Grade, Karl Fischer analysis 0.03 mg of H_2O/ml, peroxide content 0.001%) for 30 min at room temperature at a flow rate of about 1 g/min. After stirring for an additional 20 min, the undissolved aspartic acid was removed by filtration. The filtrate was diluted with 400 ml of dry ethyl acetate and the solution was concentrated *in vacuo* at room temperature to a volume of 100 ml. An additional 100 ml of ethyl acetate was added, followed by the dropwise addition of hexane. As soon as turbidity persisted, the unwashed insolubles were removed by filtration as rapidly as pos-

(38) Unsuccessful attempts to utilize N^{ω}-*p*-nitrobenzyloxycarbonyl-L-arginine-*N*-carboxyanhydride hydrochloride in peptide synthesis have been described: D. T. Gish and F. H. Carpenter, *J. Amer. Chem. Soc.*, **75**, 5872 (1953).

(39) A. Patchornik and Y. Shalitin, *Anal. Chem.*, **33**, 1887 (1961). We are greatly indebted to Mr. R. N. Boos for developing a modification of this analytical technique for the routine CO_2 analysis of Leuchs' anhydrides.

(40) (a) W. T. Astbury, C. E. Dalgliesh, S. E. Darmon, and G. B. B. M. Sutherland, *Nature* (*London*), **162**, 596 (1948); (b) S. M. Bloom, G. D. Fasman, C. de Lozé, and E. R. Blout, *J. Amer. Chem. Soc.*, **84**, 458 (1962); L. Bichowsky-Skomnicki, A. Berger, J. Kurtz, and E. Katchalski, *Arch. Biochem. Biophys.*, **65**, 400 (1956).

sible. Addition of hexane followed by rapid removal of insolubles was repeated until a more flocculent crystalline product was obtained. The latter material (2.5–3.0 g, 21–25% yield) was dried *in vacuo* and stored over Drierite in a Dry Ice chest. The crystalline anhydride decomposed on heating and it was not sufficiently stable in solution to permit the determination of its specific rotation. *Anal.* Calcd for $C_5H_5O_5N$: C, 37.74; H, 3.17; N, 8.80; CO_2, 27.6. Found: C, 37.94; H, 3.38; N, 9.07; CO_2, 27.2.

Reaction of the NCA of Aspartic Acid with Ammonia. A 100-mg sample of the crystalline NCA of aspartic acid, prepared as described above, was treated with 1 ml of an aqueous solution of NH_4OH at 0° as described by Vajda and Bruckner.[3] A 5-μl aliquot of this solution was spotted on Whatman No. 3 MM paper. After electrophoresis at pH 2 (500 V, 6 hr) reference samples of aspartic acid, asparagine, and isoasparagine had moved $2^3/_4$, $4^1/_2$, and $6^3/_8$ in., respectively. The reaction mixture showed isoasparagine as the major product. No asparagine was detected. Under these conditions the formation of 5% asparagine would have given a strong ninhydrin response.

Aspartylphenylalanylamide. A solution of 3.02 mmol of [^{14}C]-L-phenylalaninamide (0.16 mCi) was allowed to react with 525 mg (3.3 mmol) of the NCA of L-aspartic acid in the usual manner.[2] The pH was adjusted to 7 with concentrated aqueous hydrochloric acid. An aliquot (0.14 mCi) was diluted to 50 ml and purified by passage through 350 g of Sephadex G-25. The column was developed with water and the effluents were evaluated by circular paper chromatography. The fractions containing only product were combined (0.076 mCi, 55% isolated yield). The product was homogeneous by circular and descending paper chromatography (*sec*-butyl alcohol–water–acetic acid (6:3:1) and a radioscan revealed one symmetrical peak. After acid hydrolysis, an amino acid analysis gave $Asp_{1.00}Phe_{1.01}$.

L-2,5-Dioxo-4-oxazolidinepropionic Acid (NCA of L-Glutamic Acid) (Ic). A suspension of 35 g of finely divided L-glutamic acid in 1.1 l. of dry spectral grade THF was treated with phosgene at 50° for 7 hr under the usual conditions. The mixture was maintained at 50° for 1 additional hr and stirring was continued overnight without further heating. During that time, nearly all of the amino acid dissolved. Insolubles were removed by filtration, and the filtrate was concentrated *in vacuo* at a bath temperature below 40°. The resulting semisolid residue was dissolved in 175 ml of ethyl acetate, the solution was filtered to remove insolubles, and the latter were washed twice more with 50-ml portions of ethyl acetate. The filtrate and washings were combined and treated with 150 ml of *n*-hexane over a period of 1.5 hr. After the addition of the hexane was completed, the mixture was stirred for 2 more hr and the product was removed with filtration in a nitrogen atmosphere. The anhydride was redissolved in 250 ml of ethyl acetate and again precipitated by the careful addition of 150 ml of hexane. The yield of the dry anhydride was 23.2 g (56.5% yield): dec pt 83° (lit.[17] dec pt 83°); $[\alpha]^{27}_{589}$ −21.2° (*c* 3.05, acetone), $[\alpha]^{27}_{589}$ −24.6° (*c* 2.53, dioxane) (lit.[16] $[\alpha]^{26}D$ −29.52° (*c* 1.05, dioxane). *Anal.* Calcd for $C_6H_7NO_5$: C, 41.65; H, 4.05; N, 8.08; CO_2, 25.4. Found: C, 41.79; H, 4.31; N, 7.97; CO_2, 24.2. When the phosgenation was carried out in dioxane, the product (29% yield) gave a CO_2 value of 25.1.

Reaction of the NCA of Glutamic Acid with Ammonia. A solution of the above NCA (173 mg, 1.0 mmol) in 2 ml of dioxane (freshly distilled from sodium) was added dropwise to 10 ml of a cold, stirred, saturated solution of anhydrous ammonia in dioxane. An amorphous precipitate separated immediately. The solvent was removed *in vacuo*. Tlc on silica gel in *n*-butyl alcohol–acetic acid–water (10:1:3) revealed no glutamine (R_f 0.075) and showed the product to be nearly pure isoglutamine (R_f 0.13). The reaction product was dissolved in the minimum amount of aqueous acetic acid (1:1) and crystallized by the addition of ethanol. The product (75 mg, 51% yield) decomposed at 187° (lit.[41] dec pt 186°). *Anal.* Calcd for $C_5H_{10}N_2O_3$: C, 41.09; H, 6.90; N, 19.17. Found: C, 41.01; H, 7.01; N, 18.92.

Glutamylleucine. A solution of 296 mg (2.26 mmol) of L-leucine in 20 ml of 0.45 *M* sodium borate buffer was allowed to react in a Waring Blendor with 430 mg (2.49 mmol) of the NCA of glutamic acid in the usual manner.[2] Circular paper chromatography (pyridine–ethyl acetate–acetic acid–water (5:5:1:3)) showed the product to be the α-peptide (R_f 0.55) and demonstrated the absence of the γ isomer (R_f 0.41). Tlc in *n*-butyl alcohol–acetic acid–water (10:1:3) also showed the desired isomer (R_f 0.27) and none of the γ isomer (R_f 0.14). Tlc in the isopropyl alcohol–water–concentrated ammonia system (7:2:1) gave a disappearance yield[2] of 95%.

The mixture was filtered and the pH of the filtrate was adjusted to 3.8 with 50% sulfuric acid to give 325 mg of crystalline dipeptide (55% yield). An analytical specimen, $[\alpha]^{25}_{589}$ +7.14° (*c* 1.6, 0.5 *N* HCl), was prepared by acidification of an aqueous alkaline solution of the peptide with acetic acid. The reported[42] rotation is $[\alpha]^{18}D$ +8.6° (*c* 1.75, in water containing 1 mol of HCl) for the α-isomer and $[\alpha]^{19}D$ −13.5° [43a] (*c* 2.3, H_2O); $[\alpha]^{26}D$ −17.6° [43b] (*c* 2, H_2O); $[\alpha]^{25}D$ −14.0° [43c] (*c* 2, H_2O) for the γ isomer. *Anal.* Calcd for $C_{11}H_{20}N_2O_5$: C, 50.70; H, 7.69; N, 10.80. Found: C, 50.99; H, 7.67; N, 11.07. After acid hydrolysis, a Spinco amino acid analysis gave $Glu_{0.98}Leu_{1.00}$.

NCA of L-Serine and Its Trimethylsilyl Ether. (A) Silver L-Serinate. All operations should be carried out with shielding from light. To a stirred solution of L-serine (105 g) in 2.5 l. of water was added freshly prepared silver oxide (116 g) over a 5-min period. Stirring was continued for 1 additional hr, Merck activated carbon (50 g) was added, and the mixture was stirred for another 20 min. The slurry was filtered through Celite and the filtrate, with external ice cooling and mechanical stirring, was treated with 750 ml of methanol, added over a 2-hr period, followed by 2 l. of methanol added dropwise overnight at 0–5° The product was removed by filtration, washed with methanol, and dried *in vacuo* to give 126 g (39.5% yield) of silver L-serinate as an off-white crystalline solid.

(B) NCA of L-Serine (VIIa).[44] The calculated amount of phosgene was introduced into a stirred suspension of silver L-serinate (42.4 g) in 500 ml of anhydrous dioxane which was protected from light. The reaction mixture was stirred for another hour, insolubles were removed by filtration, and the filtrate was freeze-dried. The residue was dissolved in the minimum volume of ethyl acetate and petroleum ether (30–60°) was added to the cloud point. The mixture was stirred and chilled in an ice bath to induce crystallization. When the supernatant had clarified, petroleum ether was again added to the cloud point and stirring continued until the supernatant was clear. This latter addition was repeated twice more. The product was removed by filtration, washed with petroleum ether, and vacuum dried to give 11 g (42% yield) of L-serine NCA: dec pt 82° (lit.[24] dec pt 115°); ir (Nujol) 2.78, 3.03, 5.35, 5.57 μ. *Anal.* Calcd for $C_4H_5NO_4$: CO_2, 33.6. Found: CO_2, 34.7.

(C) *O*-Trimethylsilyl-L-serine NCA (VIIe). In a dried flask flushed with nitrogen and protected from atmospheric moisture, the above L-serine NCA (3.75 g, 28.6 mmol) was dissolved in 65 ml of fresh anhydrous THF. The solution was chilled to 0° and trimethylchlorosilane (3.68 ml, 28.6 mmol) was added. To the chilled stirred solution was added dropwise from a hypodermic syringe 2.51 ml (28.6 mmol) of 4-methylthiazole. The cooling bath was removed and the reaction mixture was allowed to warm to 25° and was stirred at that temperature for 2 hr. The precipitated 4-methylthiazole hydrochloride was removed by filtration and the colorless filtrate was concentrated *in vacuo* to give an oil. The oil was dissolved in 30 ml of ethyl acetate and 100 ml of *n*-hexane was added. An oil precipitated and the flask was allowed to stand for 2 hr until the supernatant had clarified. The supernatant was decanted and evaporated to dryness *in vacuo*. The colorless crystalline residue was triturated with 50 ml of *n*-hexane, filtered, washed twice with *n*-hexane, and dried to constant weight in a stream of nitrogen to give 2.84 g (49% yield) of a crystalline product which decomposed on heating: $[\alpha]^{25}_{589}$ −46.7° (*c* 1.13, $CHCl_3$); ir (Nujol) 3.07, 5.49, 5.70 μ.

Anal. Calcd for $C_7H_{13}NO_4Si$: C, 41.36; H, 6.44; N, 6.89; CO_2, 21.6. Found: C, 41.18; H, 6.51; N, 7.03; CO_2, 22.1.

NCA of *O*-Trifluoroacetylserine (VIId). A solution of 3.93 g of the above NCA of L-serine in 200 ml of freshly purified dioxane was treated with 5 ml of trifluoroacetic anhydride. After standing at room temperature for 100 min the solution was concentrated to

(41) J. M. Swan and V. du Vigneaud, *J. Amer. Chem. Soc.*, 76, 3110 (1954).

(42) W. J. Le Quesne and G. T. Young, *J. Chem. Soc.*, 1954 (1950).

(43) (a) D. A. Rowlands and G. T. Young, *ibid.*, 3937 (1952); (b) P. J. Fodor, A. Miller, A. Neidle, and H. Waelsch, *J. Biol. Chem.*, 203, 991 (1953); (c) G. Amiard, R. Heymès, and L. Velluz, *Bull. Soc. Chim. Fr.*, 97 (1956).

(44) In the preparation of the NCA of L-serine from silver serinate, in one experiment the mother liquors on standing deposited crystals which gave a strong Beilstein test and which had an ir spectrum consistent with VIIb. The compound **L-*O*-chlorocarbonylserine** NCA gave the expected elemental analysis. It evolved less than 2 equiv of CO_2 on treatment with sulfuric acid. The DL analog has been prepared,[25] but the amount of active CO_2 was not reported. *Anal.* Calcd for $C_5H_4NClO_5$: C, 31.31; H, 2.08; N, 7.24; CO_2, 45.5. Found: C, 31.17; H, 2.18; N, 7.33; CO_2, 35.4.

dryness *in vacuo*. The resulting oil was redissolved in dioxane and again taken to dryness. Attempts to crystallize this material were unsuccessful. The product showed ir maxima (Nujol) at 5.35 and 5.7 μ (anhydride and amide), 8.15 μ (OC), and 8.6 μ (CF). *Anal.* Calcd for $C_6H_4O_5NF_3$: C, 31.70; H, 1.75; N, 6.17; F, 25.10. Found: C, 31.45; H, 2.26; N, 6.88; F, 24.30.

NCA of *O*-Trichloroacetylserine (VIIc). The trichloroacetyl derivative of serine NCA was prepared from 3.93 g of L-serine NCA and 4 ml of trichloroacetyl chloride in dioxane essentially as described above for the fluoro analog. After standing at room temperature for 15 hr, the mixture was filtered to remove insolubles and the filtrate was taken to dryness *in vacuo*. The resulting oil was redissolved in dioxane and again taken to dryness to give an oil which crystallized on standing. In Nujol VIIc showed maxima at 5.34 μ (m), 5.43 μ (s), 5.58 μ (s), and 8.16 μ (m). *Anal.* Calcd for $C_6H_4O_5NCl_3$: C, 26.05; H, 1.46; N, 5.06; Cl, 38.35; CO_2, 15.90. Found: C, 25.92; H, 1.73; N, 4.98; Cl, 38.00; CO_2, 15.80.

Serylphenylalanylleucine. L-Phenylalanyl-L-leucine[2] (0.556 g, 2.0 mmol) in 20 ml of a 0.45 *M* solution of borate buffer was allowed to react in a Waring Blendor with *O*-trimethylsilylserine NCA (0.447 g, 2.2 mmol) in the usual manner.[2] Tlc using the *n*-butyl alcohol–acetic acid–water system (10:1:3) gave an 80% disappearance yield.[2] The pH of the reaction mixture was adjusted to 5.2 with 50% H_2SO_4 and the solution was freeze-dried. The residue was extracted with 100 ml of ethanol and the solvent was removed *in vacuo*. Trituration with 14 ml of 45% aqueous methanol yielded 220 mg (30% yield) of the crystalline tripeptide. Recrystallization from hot 50% aqueous methanol provided an analytical specimen, which moved as a single component (R_f 0.39) in the above system. *Anal.* Calcd for $C_{18}H_{27}N_3O_5$: C, 59.16; H, 7.45; N, 11.50; equiv wt, 365. Found: C, 59.26; H, 7.75; N, 11.17; equiv wt, 357. After acid hydrolysis, an amino acid analysis gave Ser, 1.04, Phe, 0.99, Leu, 1.00.

NCA of L-Threonine. (A) Silver L-Threoninate. Using essentially the procedure described for the preparation of the serine salt, 11.6 g of freshly prepared silver oxide was added to a solution of 11.9 g of L-threonine in 250 ml of water. The mixture was stirred for 1 hr, 3.0 g of Merck activated carbon was added, and stirring was continued for 30 min. The reaction mixture was filtered and 270 ml of methanol was added over a 90-min interval with external cooling. Crystallization was induced by scratching. After aging for 30 min, the product was removed by filtration, washed with methanol, and dried *in vacuo* to give 9.5 g (42% yield) of silver L-threoninate.

(B) NCA of L-Threonine (VIIb). The above silver salt, suspended in 100 ml of anhydrous purified dioxane, was treated with phosgene for 1 hr essentially as described above. The mixture was filtered and the filtrate was concentrated to an oil *in vacuo*. The residue was dissolved in 30 ml of ethyl acetate, and crystallized by the slow addition of 65 ml of hexane. The mixture was stirred for 1 hr in the cold and the product was removed by filtration under nitrogen, washed with a mixture of hexane–ethyl acetate (2:1), and dried to constant weight under a stream of nitrogen to give 3.0 g (49% yield) of L-threonine NCA: dec pt 94–98° (lit.[26] dec pt 111°), $[\alpha]^{27}_{589}$ −62.7° (*c* 3.450, acetone). The ir (Nujol) showed a maximum at 3540 cm^{-1} (OH). *Anal.* Calcd for $C_5H_7NO_4$: C, 41.38; H, 4.86; N, 9.65; CO_2, 30.3. Found: C, 41.15; H, 4.78; N, 9.54; CO_2, 18.4.

(C) *O*-Trimethylsilyl-L-threonine NCA (VIIg). A solution of 5.8 g (40 mmol) of L-threonine NCA was dissolved in 73 ml of THF. The solution was chilled in an ice bath as 5.14 ml (40 mmol) of trimethylchlorosilane was added. A solution of 3.24 ml (40 mmol) of anhydrous pyridine in 40 ml of THF was added dropwise with stirring and cooling. The reaction mixture was allowed to warm to room temperature, the precipitated pyridine hydrochloride was removed by filtration under nitrogen, and the filtrate was concentrated to an oil *in vacuo*. The oil was taken up in 35 ml of ethyl acetate and 110 ml of *n*-hexane was added. The mixture was turbid at first but clarified on standing for 30 min at room temperature followed by 30 min in the refrigerator. The supernatant was decanted and concentrated *in vacuo* to give a crude semicrystalline product which was triturated twice with 50-ml portions of *n*-hexane and dried to constant weight under a stream of nitrogen to give 5.60 g (64.7% yield) of *O*-trimethylsilyl-L-threonine NCA: dec pt 76°, $[\alpha]^{27}_{589}$ −50.5° (*c* 1.886, ethyl acetate); ir (Nujol) 2.95, 3.36, 5.35, 5.62 μ. *Anal.* Calcd for $C_8H_{15}NO_3Si$: C, 44.22; H, 6.96; N, 6.44; CO_2, 20.2. Found: C, 44.58; H, 7.06; N, 6.77; CO_2, 12.6.

L-Threonyl-L-phenylalanyl-L-leucine. A solution of 0.556 g (2.0 mmol) of L-phenylalanyl-L-leucine[2] in 20 ml of 0.45 *M* sodium borate buffer was treated in the usual manner[2] with 0.552 g (2.4 mmol) of *O*-trimethylsilyl-L-threonine NCA. The reaction mixture was filtered and the pH was adjusted to 5.6 with 50% aqueous sulfuric acid. The crystalline product was removed by filtration, washed with water, and air-dried to constant weight to give 0.375 g (49% yield) of the desired tripeptide which was shown to be homogeneous by tlc. An analytical specimen, $[\alpha]^{25}_{589}$ −12.2° (*c* 0.94, acetic acid), was prepared by recrystallization from water. After acid hydrolysis an amino acid analysis gave $Thr_{1.00}Phe_{0.92}Leu_{1.00}$. *Anal.* Calcd for $C_{19}H_{29}N_3O_5$: C, 60.14; H, 7.70; N, 11.07. Found: C, 60.01; H, 7.99; N, 11.21.

NCA of ε-*N*-Butyloxycarbonyl-L-lysine (XIV). A suspension of 1.23 g of ε-*N*-Boc-lysine in 50 ml of dry acetonitrile was treated with 1.5 g of silver cyanide in a 100-ml round-bottomed flask with magnetic stirring and exclusion of moisture. When the mixed solids began to disperse, 6 ml of a solution containing 1 mmol of phosgene/ml of dioxane was added. Stirring at room temperature was continued for 1.25 hr. The resulting fine suspension was filtered and the solvent was removed *in vacuo*. The residue was stored in a Dry Ice chest overnight and then treated with 25 ml of dry ethyl acetate. The resulting slightly hazy solution was treated with *n*-hexane until turbidity was observed. The solution began to crystallize and was treated with 75 ml of hexane. After standing for 1 hr, the product was removed by filtration, washed with hexane, and dried at room temperature to give 743 mg (54.6% yield) of the desired anhydride. The product [dec pt 123°; $[\alpha]^{27}_{589}$ −17.3° (*c* 1.65, acetone); $[\alpha]^{25}_{589}$ −34.7° (*c* 1.103, CH_2Cl_2)] showed ir maxima at 5.49, 5.78, and 5.91 μ, and a shoulder at 5.24 μ. *Anal.* Calcd for $C_{12}H_{20}O_5N_2$: C, 52.93; H, 7.40; N, 10.29; CO_2, 32.3. Found: C, 52.53; H, 7.08; N, 10.56; CO_2, 30.9.

ε-*N*-Butyloxycarbonyl-L-lysyl-L-leucine. A solution of 2 mmol of L-leucine in 20 ml of borate buffer was treated with a 5% excess of the above NCA in the usual manner.[2] The reaction was acidified and the crude reaction product was removed by filtration. The precipitate was shown to be impure and recombined with the freeze-dried filtrate. The residue was dissolved in $CHCl_3$–MeOH–NH_3 (50:40:10) and purified by dry column chromatography[2] on silica gel H using the same solvent system. Crystallization from hot methanol afforded 282 mg (45% yield) of product which was shown to be a single component by tlc. An analytical specimen was prepared by one additional crystallization from methanol. After acid hydrolysis, an amino acid analysis gave $Leu_{1.00}Lys_{1.03}$.

L-2,5-Dioxy-4-oxazolidinepropionamide (NCA of L-Glutamine) (IVa). A suspension of 30 g of benzyloxycarbonyl-L-glutamine (IIIa) in 300 ml of dioxane was stirred for 5 min in a nitrogen atmosphere. The resulting solution was treated over a 1 min interval with a solution of 4.0 ml of PBr_3 in 5 ml of dioxane added from a dropping funnel. All of the starting material dissolved during the addition of PBr_3. The mixture was stirred for an additional 2 hr during which a solid separated. A silica gel column was prepared in methylene chloride. The solvent was displaced with dioxane. An exothermic reaction resulted. The column was then washed with methylene chloride to resettle the silica gel column. The crude reaction mixture was added to the column which had been allowed to stand overnight and the flask was rinsed with 60 ml of dioxane. The column was developed with 6 l. of acetone–methylene chloride (1:1) followed by 6 l. of acetone–methylene chloride (3:1). The next 2–3 l. of eluent contained the lacrimator benzyl bromide. Then 250-ml fractions were collected. It was convenient to follow the chromatography by concentrating 5-ml aliquots to dryness and triturating the residue with a few drops of dioxane. Fractions containing crystallizable product were combined, and concentrated *in vacuo* to a volume of 200 ml. The resulting solution was treated at room temperature by the slow addition of *n*-hexane. The product was collected after the addition of 50 ml of hexane by filtration in a dry atmosphere and dried to constant weight at room temperature to give a total of 6.1 g (33% yield). This material was about 95% pure as indicated by CO_2 analysis (found: 24.1). If necessary, a sample (1.0 g) of slightly impure NCA may be purified by dissolution in 80 ml of acetone followed by filtration and crystallization by the addition of *n*-hexane. An analytical specimen, dec pt 270°, $[\alpha]^{25}_{589}$ −9.3° (*c* 1, acetone), did not undergo a change in rotation for 1 hr. *Anal.* Calcd for $C_6H_5O_4N_2$: C, 41.86; H, 4.86; N, 16.27; CO_2, 26.0. Found: C, 42.12; H, 4.75; N, 16.18; CO_2, 25.4.

L-Glutaminyl-L-leucine. A solution of 1 mmol (157.2 mg) of ^{14}C-labeled L-leucine was dissolved in 10 ml of potassium borate buffer and treated at 0° with a 5% excess of the above NCA at pH 10 with mechanical stirring. The pH was maintained by the addition of alkali, and after 2 min the pH was adjusted to 3.0 with sul-

furic acid. The disappearance yield[2] was about 92%. Tlc revealed the presence of leucine, product, glutamine, but no glutamic acid. The radioscan indicated in addition the presence of the hydantoic acid[2] as a minor component. A crystalline solid which had separated from the reaction mixture on standing was removed by filtration and shown to be pure glutaminylleucine: mp 206–208° (103 mg, 40% yield); $[\alpha]^{24}_{589}$ +4.4° (*c* 2, 1 *N* HCl) [lit.[45] $[\alpha]_D$ +4.5° (*c* 2, 1 *N* HCl)] [lit.[46] $[\alpha]^{22}_D$ +11.5° (*c* 1, 1 *N* HCl)]. After acid hydrolysis, amino acid analysis gave $Glu_{1.00}$ and $Leu_{0.96}$. *Anal.* Calcd for $C_{11}H_{21}N_3O_4$: C, 50.95; H, 8.20; N, 16.20. Found: C, 51.14; H, 8.61; N, 16.02. The filtrate was desalted on Dowex 50, the eluents containing peptide were taken to dryness, and the residue was extracted with butanol and ethyl acetate. The residue (120 mg) was dissolved in 1.9 ml of 90% ethyl alcohol containing 0.2 ml of 1 *N* HCl. The product was crystallized by the cautious addition of dlute sodium hydroxide in the cold to afford an additional 74 mg of dipeptide which was identical with the material described above, bringing the overall yield to 69%. Enzymatic hydrolysis of the dipeptide with leucine aminopeptidase gave only glutamine and leucine.

L-2,5-Dioxy-4-oxazolidineacetamide (NCA of Asparagine) (IVb). A suspension of 10 g of benzyloxycarbonyl-L-asparagine (IIIb) in 200 ml of dioxane was stirred for 5 min in a nitrogen atmosphere. During the addition of a solution of 1.2 ml of PBr_3 in 5 ml of dioxane from a dropping funnel over a 1-min interval, the suspension turned to pale yellow and about 10 min later all of the starting material had dissolved. After stirring for 1 additional hr, the solution was transferred to a 500-ml column of silica gel prepared essentially as described for IVa. The residual reaction mixture was transferred to the column with 25 ml of dioxane and the column was then developed with 2 l. of acetone–methylene chloride (1:1) followed by 2 l. of acetone–methylene chloride (3:1). Aliquots of the effluents were concentrated and triturated as described above for IVa. The fractions which crystallized readily were combined and concentrated to a volume of about 45 ml. The resulting solution was diluted with 100 ml of methyl ethyl ketone–acetone (80:20), immersed in an ice bath, and treated dropwise with 400 ml of *n*-hexane. The crystallized product was removed by filtration and dried at room temperature taking the usual precautions. The yield of crystalline NCA was 2.11 g (36% yield). This material was shown to be of high purity by CO_2 analysis (found, 28.0). NCA of lower purity (2 g) may be purified by adding 200 ml of hot ethyl acetate, stirring the mixture for 1 min, and removing insolubles by filtration. The filtrate is then allowed to cool to room temperature and the NCA crystallized by the gradual addition of about 200 ml of *n*-hexane. An analytical specimen, $[\alpha]^{25}_{589}$ −47.8° (*c* 1.0, acetone), gave the correct analysis. *Anal.* Calcd for $C_5H_6O_4N_2$: C, 38.00; H, 3.82; N, 17.70; CO_2, 27.8. Found: C, 37.90; H, 3.64; N, 17.42; CO_2, 28.0.

NCA of Arginine Hydrobromide (XIII). To a suspension of 10 g (31.5 mmol) of α-*N*-benzyloxycarbonyl-L-arginine in 200 ml of THF was added rapidly (10 sec) with vigorous stirring at room temperature a solution of 10 ml of PBr_3 in 40 ml of THF which had been cooled to *ca.* 5°. After stirring for 3 hr at room temperature, the THF was decanted from the product which had deposited as a heavy oil. The oil was washed by decantation with 200-ml portions of THF and dried *in vacuo* to give a light yellow tar (7 g) which showed bands at 5.44 and 5.64 μ in the ir, indicative of NCA formation. This crude product is useful for the preparation of arginine containing peptides. It generally contains about 50% by weight of the hydrobromide salt of arginine NCA, the impurities being largely derived from benzyl bromide and PBr_3. The product was dissolved in 25 ml of DMF and used to prepare L-arginyl-L-leucine as described below. The concentration of NCA may be determined by CO_2 analysis, or by determining the amount of DMF solution which is required to give optimal yields of Arg-Leu on treatment of leucine with the NCA under the usual conditions (see below).

L-Arginyl-L-leucine Acetate. L-Leucine (0.655 g, 5 mmol) was dissolved in 50 ml of 1 *M* potassium borate buffer at pH 10.2 and treated with 10 ml of the above solution of arginine NCA. Tlc in butanol–acetic acid–water (10:2.3:6) showed an 80–85% disappearance yield[2] of leucine and revealed Arg-Leu as the major new component. Other reactions with leucine have given disappearance yields as high as 95%. No D-Arg-L-Leu could be detected in the crude reaction product by tlc in this system, which separates the D,L from the L,L isomer. After adjustment of the pH to 5.5, the mixture was filtered, evaporated to dryness, and flushed several times with methanol to remove borate as methyl borate. The crude peptide was dissolved in 60 ml of water and 2.5 g of lead subacetate was added; the pH was maintained at 5.7 with acetic acid to precipitate bromide and sulfate, thus converting the dipeptide to its acetate salt. After standing for 2 hr to allow complete precipitation, the mixture was filtered and treated with H_2S to remove excess lead. After evaporation to dryness, the product was crystallized from water–acetone to give 1.04 g (60% yield) of dipeptide containing a small amount (<5%) of arginine. Crystallization from water–acetone afforded an analytical specimen (0.64 g), single spot by tlc, $[\alpha]^{25}_{589}$ +9.8° (*c* 1.0, H_2O) (lit.[47] $[\alpha]^{28}_D$ +9.6° (*c* 1.35, H_2O)). The crystallization must be carried out rapidly to avoid the formation of a Schiff's base with acetone. *Anal.* Calcd for $C_{14}H_{29}N_5O_5$: C, 48.39; H, 8.41; N, 20.15. Found: C, 48.16; H, 8.50; N, 20.34.

NCA of Histidine Hydrobromide (Xa). Finely ground α-*N*-benzyloxycarbonyl-L-histidine (1.734 g, 6.0 mmol) was suspended in 30 ml of THF at 25° and PBr_3 (3.34 g, 6 mmol) was added rapidly with vigorous stirring. The reaction mixture was stirred for 2 hr at 25° and the crude NCA was isolated by filtration under dry N_2 and washed with THF. The crude NCA (1.33 g) was dissolved in 8 ml of methyl Carbitol, insolubles were removed by centrifugation, and the product was crystallized by the addition of 15 ml of ethyl acetate. After standing for 2 hr, the product was isolated by filtration, washed with ethyl acetate, and dried *in vacuo* at 25° to give 0.78 g (50% yield), $[\alpha]^{25}_{589}$ −7.4° (*c* 1.1, methyl Carbitol). The ir spectrum showed maxima at 5.38 and 5.57 μ. *Anal.* Calcd for $C_7H_8N_3O_3Br$: C, 32.1; H, 3.08; N, 16.02; Br, 30.5. Found: C, 32.30; H, 3.04; N, 16.02; Br, 29.2, 29.7. The hydrochloride salt could also be prepared using $SOCl_2$ in place of PBr_3 but the crystalline product, dec pt 225–230°, was not obtained in pure form.

Treatment of L-Alanine with the NCA of L-Histidine Hydrobromide. When L-alanine was treated with the above NCA of histidine at pH 9.5 at 0°, less than 5% of histidylalanine was obtained. The major product was the rearrangement product XIIa. In the absence of alanine at pH 8, the NCA gave an 80% yield of XIIa, as well as histidine. At a pH below 6.5, histidine was obtained as the major reaction product (90% yield). Approximately equal amounts of histidine and XIIa are formed at pH 7.5. The following experiment typifies the formation and isolation of the rearrangement product XIIa. A solution of 3 mmol of the above NCA in 105 ml of 0.1 *M* sodium borate (pH 10.0) buffer was maintained at 0° for 10 min. The product was adsorbed on 60 ml of Pittsburgh OL granular carbon, and salts were eluted with 400 ml of H_2O. The product was then eluted with 60 ml of a 20% solution of aqueous pyridine, and the solution was evaporated to dryness to give an oil which was precipitated from methanol by the addition of ether. An analytically pure sample of 7-carboxy(imidazo[1,5-*c*]-tetrahydropyrimidin)-5-one was not obtained. Nmr in D_2O showed single peaks at 483 and 405 cps (aromatic protons) in addition to a complex aliphatic multiplet. Ir showed ν_{max}^{Nj} 1700 cm^{-1}. These physical constants are in good agreement with those reported for related structures.[48]

Hydrolysis of Xa at pH 5.3 in citrate buffer (100°, 15 min) afforded histidine. However, XIIa is essentially stable at pH 10 and in 2.5 *N* HCl for 15 min at 100°. Partial hydrolysis occurred after 15 min at 100° at pH 7.

NCA of *O*-Tetrahydropyranyl Ethers of L-Tyrosine (XVa). **(A) NCA of L-Tyrosine.**[40a] A suspension of 30 g of finely divided L-tyrosine in 300 ml of dry, peroxide-free THF was treated with phosgene at 40–45° with good stirring. The slurry thinned and then thickened after about 15 min. The addition of phosgene was continued for a total of 1.25 hr, the mixture was then stirred for an additional 2 hr and cooled to 10°, and the product was removed by filtration. After washing with two portions of THF the anhydride was dried *in vacuo* to give 27 g (78.6% yield). *Anal.* Calcd for: $C_{10}H_9NO_4$: CO_2, 21.2. Found: 20.9.

(B) Conversion to the Tetrahydropyranyl Ether. A suspension of 10 g of the above tyrosine NCA and 0.5 g of *p*-toluenesulfonyl chloride in 200 ml of dihydropyran, freshly distilled from sodium hydroxide, was stirred for 65 hr at room temperature. The reaction mixture was filtered and the filtrate was treated with 300 ml of

(45) G. Amiard and R. Heymes, *Bull. Soc. Chim. Fr.*, 1373 (1957).
(46) E. Sondheimer and R. W. Holley, *J. Amer. Chem. Soc.*, 76, 2816 (1954).
(47) K. Hofmann, W. D. Peckham, and A. Rheiner, *ibid.*, 78, 238 (1956).
(48) R. Mechoulam, F. Sondheimer, A. Melera, and F. A. Kind, *ibid.*, 83, 2022 (1961); J. Derkosch, K. Schlögl, and W. Woidich, *Monatsh. Chem.*, 83, 35 (1957).

petroleum ether. The resulting product was removed by filtration, washed twice with 30-ml portions of petroleum ether, and dried *in vacuo* at room temperature to afford 9.2 g (65% yield) of product, $[\alpha]^{25}_{589}$ −89.3° (*c* 1.00, CH_2Cl_2). The rotation remained essentially unchanged (−89.7°) on recrystallization from the same solvent system. *Anal.* Calcd for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81; CO_2, 15.1. Found: C, 61.62; H, 5.89; N, 4.56; CO_2, 15.0.

Treatment of the above mother liquor with an additional 200 ml of petroleum ether gave 3.1 g (22% yield) of the diastereoisomer as a crystalline product, $[\alpha]^{25}_{589}$ −177.0° (*c* 1.00, CH_2Cl_2); one further recrystallization from methylene chloride–ether brought the rotation to −178.2° *Anal.* Calcd for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81; CO_2, 15.1. Found: C, 61.92; H, 5.71; N, 5.27; CO_2, 15.00.

O-Acetyl-L-tyrosine NCA (XVb). To a suspension of 2.072 g of L-tyrosine NCA, prepared as described above, in 35 ml of freshly distilled THF which had been cooled in an ice bath was added from a syringe, with exclusion of moisture and vigorous stirring, 0.711 ml of acetyl chloride and then 0.8 ml of anhydrous pyridine in 15 ml of THF. The reaction mixture was stirred at 0–5° for 30 min. During this time a yellow gum which had separated was transformed into a white solid. The mixture was allowed to come to room temperature and was filtered under nitrogen pressure and exclusion of moisture to remove insolubles. The filtrate was concentrated to dryness *in vacuo* and the residue was treated with 20 ml of ethyl acetate followed by 10 ml of hexane. The supernatant was decanted from the solid which had separated. The supernatant was then treated with 20 ml of hexane to give a solid which crystallized on standing in a dry atmosphere. After crystallization had occurred, an additional 130 ml of hexane was added slowly with continued stirring. The crystalline solid was removed by filtration and was washed with a mixture of hexane–ethyl acetate (8:1). The resulting product (0.78 g, 31% yield) gave a negative Beilstein test and showed only weak absorption at 2.7 μ (OH) in the infrared in CH_3CN solution. The product was dissolved in 10 ml of ethyl acetate and treated with 10 ml of hexane. The supernatant was decanted from a small amount of gummy solid which adhered to the flask. The solution was then treated with an additional 50 ml of hexane and the resulting crystalline product was removed by filtration and washed with hexane–ethyl acetate (6:1) to afford 0.7 g; dec pt 122° (lit. 99–100°,[34] dec pt 122–123° [35]); $[\alpha]^{25}_{589}$ −37.2° (*c* 1.06, CH_3CN); ir (CH_3CN) 2.95 (NH), 5.37, 5.59, 5.62, 8.38 μ. *Anal.* Calcd for $C_{12}H_{11}NO_5$: C, 57.82; H, 4.45; N, 5.62. Found: C, 57.77; H, 4.40; N, 5.61.

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Studies on Polynucleotides. XCVIII.[1] A Convenient and General Method for the Preparation of Protected Dideoxyribonucleotides Containing 5′-Phosphate End Groups[2]

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Abstract: Protected dinucleotides carrying 5′-phosphomonoester end groups (*e.g.*, $pC^{An}pA^{Bz}$) are required as intermediates in the blockwise synthesis of deoxyribopolynucleotides of defined sequence. A rapid and general method for their preparation is now described, the primary feature of which is that it obviates the time-consuming separation by anion exchange or gel filtration columns and, instead, uses convenient solvent extraction procedures. The synthetic steps used were as follows. (1) The N-protected 5′-deoxyribomononucleotides were converted to the corresponding 5′-phosphoramidates by reaction with dicyclohexylcarbodiimide and the highly lipophilic aromatic amine, *p*-aminophenyltriphenylmethane. The phosphoramidates were obtained in 60–65% yield. (2) The phosphoramidates were condensed with suitably protected mononucleotides (*e.g.*, pA^{Bz}-OAc) using triisopropylbenzenesulfonyl chloride as the condensing agent. The resulting dinucleotide phosphoramidates were isolated by solvent extraction in yields of 50–65%. The phosphoramidate protecting group was selectively removed by treatment with isoamyl nitrite in a pyridine–acetic acid mixture. All of the required 16 dinucleotides were thus prepared in reasonably satisfactory yields (50–65%).

Synthesis of high molecular weight double-stranded DNA's with defined nucleotide sequences has been accomplished by a combination of chemical and enzymatic methods.[3–7] Thus, when chemically synthesized short-chain deoxyribopolynucleotides with repeating nucleotide sequences are used as templates for the DNA polymerase of *E. coli*, macromolecular DNA-like polymers containing the repeating sequences present in the short templates are obtained. The availability

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(3) H. G. Khorana, *Pure Appl. Chem.*, 17, 349 (1968).

(4) H. G. Khorana, *Proc. Int. Congr. Biochem., 7th 1967*, 17 (1968).

(5) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Amer. Chem. Soc.*, **87**, 2954 (1965).

(6) H. G. Khorana, H. Buchi, T. M. Jacob, H. Kössel, S. A. Narang, and E. Ohtsuka, *ibid.*, **89**, 2154 (1967).

(7) K. L. Agarwal, H. Buchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. RajBhandary, J. H. van de Sande, V. Sgaramella, H. Weber, and T. Yamada, *Nature (London)*, **227**, 27 (1970).