tion of glycerol and galactose in the hydrolysate revealed a molar ratio of 1.0:0.95. When the substance was oxidized in 0.02 *M* sodium metaperiodate solution in the usual way, formic acid (0.94 mole per mole of substance) was liberated, and 1.99 moles periodate per mole of substance was consumed. This, together with the fact that formaldehyde could not be detected in the reaction mixture, indicated that the galactose was linked through its reducing group to C2 in the glycerol; thus showing that the substance was galactosyl-2-glycerol, and corroborating the work of Putman and Hassid.[1] The glycoside was obtained from *Gracilaria confervoides* in 0.8% yield.

This note is published with the permission of the South African Council for Scientific and Industrial Research.

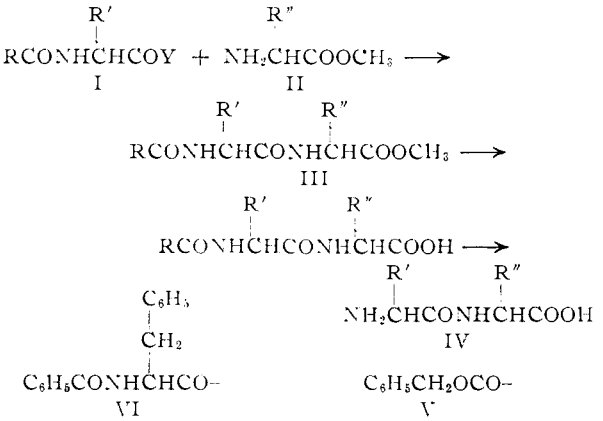
NATIONAL CHEMICAL RESEARCH LABORATORY
SOUTH AFRICAN COUNCIL FOR
SCIENTIFIC AND INDUSTRIAL RESEARCH
PRETORIA, SOUTH AFRICA

Enzymatic Removal of the Protecting Group in Peptide Synthesis[1]

BY ROBERT W. HOLLEY

RECEIVED DECEMBER 28, 1954

Peptide synthesis, in the laboratory, usually involves the reaction of an N-acylamino acid derivative I with an amino acid ester, II. The product III is saponified, and the acyl protecting group is removed to give the peptide IV. The classical example of an N-acyl protecting group is the carbobenzoxy group V.



In the synthesis of large peptides, particularly those containing amino acids with reactive side chains, a variety of protecting groups is needed in order to permit the selective removal of the protecting group from one reactive group in the molecule, allowing reaction with this group while other reactive groups remain protected. With this need in mind, we have been searching for new protecting groups which might be removed under highly selective conditions, and have investigated the possibility of using a protective group which can be removed by a hydrolytic enzyme. In the initial experiments, chymotrypsin was chosen as the enzyme and the benzoyl-L-phenylalanyl group VI was chosen as the protecting group. It is known that chymotrypsin catalyses the hydrolysis of benzoyl-L-phenylalanine amides.[2]

L-Leucyl-L-leucine (IV, R′ = R″ = $(CH_3)_2$-$CHCH_2$—) has been prepared in this way. Benzoyl-L-phenylalanine ethyl ester was converted to the hydrazide, and to the azide, and the azide was allowed to react with L-leucine methyl ester. The product, benzoyl-L-phenylalanyl-L-leucine methyl ester, was converted to the hydrazide, and the corresponding azide was reacted with L-leucine methyl ester. The benzoyl-L-phenylalanyl-L-leucyl-L-leucine methyl ester, which was obtained, was saponified, and the benzoyl-L-phenylalanyl group was removed by hydrolysis catalyzed by chymotrypsin. Yields throughout the synthesis were good. An 80% yield of L-leucyl-L-leucine hydrate was obtained in the final, enzyme-catalyzed step. As would be anticipated from the known specificity of chymotrypsin, the only products were benzoyl-L-phenylalanine and L-leucyl-L-leucine.

To the best of our knowledge, the enzymatic removal of a protecting group has not been used before in the synthesis of peptides. The present work establishes that the general approach can be used successfully. However, work with various hydrolytic enzymes and various protecting groups will be required to establish the scope of the procedure. One limitation is clear. If a proteolytic enzyme such as chymotrypsin is used, the peptide which is to be synthesized must not contain a linkage susceptible of hydrolysis by the enzyme. Also, in some instances the separation of the enzyme from the peptide which has been synthesized may cause difficulty.

Although it is not anticipated that enzymatic removal of a protecting group, as illustrated here with the benzoyl-L-phenylalanyl group, will compete with the use of the carbobenzoxy group in the synthesis of simple peptides, it is possible that this type of approach may be of value in special problems of peptide synthesis.

Experimental[3]

Benzoyl-L-phenylalanine Hydrazide.—A mixture of 2.2 g. (7.4 mmoles) of benzoyl-L-phenylalanine ethyl ester,[4] 8 ml. of absolute ethanol and 0.80 ml. of hydrazine hydrate was heated at 70–80° for 4 hr. The mixture was cooled and filtered, and the crystalline hydrazide was washed with cold absolute ethanol. It was recrystallized from 34 ml. of 95% ethanol; 1.6 g. (76%); m.p. 196–199°. It was recrystallized for analysis.

Anal. Calcd. for $C_{16}H_{17}N_3O_2$: N, 14.8. Found: N, 14.8.

Benzoyl-L-phenylalanyl-L-leucine Methyl Ester.—A mixture of 1.1 g. (6.0 mmoles) of L-leucine methyl ester hydrochloride and 25 ml. of ether was cooled in an ice-bath and 12.5 ml. of cold 50% (w./v.) potassium carbonate solution was added. The mixture was shaken and the ethereal solution was separated. The aqueous layer was washed with 5 ml. more ether and the ethereal solutions were combined and dried over anhydrous sodium sulfate at 0°, during preparation of the azide described below.

(1) Journal Paper No. 994, New York State Agricultural Experiment Station. This investigation was supported in part by research grants, G-3435 and G-3435(C), from the National Institutes of Health, Public Health Service.

(2) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(3) All melting points were determined on a microscope hot-stage and are corrected. Analyses were performed by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England.

(4) S. Kaufmann and H. Neurath, *Arch. Biochem.*, **21**, 437 (1949).

A solution of 1.42 g. (5.0 mmoles) of benzoyl-L-phenylalanine hydrazide in 25 ml. of 1 *N* hydrochloric acid and 7.5 ml. of glacial acetic acid was cooled in an ice–salt-bath and a cold solution of 350 mg. (5.0 mmoles) of sodium nitrite in 2 ml. of water was added and the mixture was shaken. The azide precipitated and was extracted with two 50-ml. portions of cold ether. The ethereal solution was washed at 0° with 25 ml. of water and twice with 50 ml. of 5% sodium bicarbonate solution (final wash was basic to litmus). The ethereal solution was dried over anhydrous sodium sulfate at 0° for 5 min.

The ethereal solution of L-leucine methyl ester was filtered through a cotton plug, and the solution of the azide was poured through the same plug. A small amount of solid, presumably azide, which had separated in the ethereal solution during the sodium bicarbonate washes was dissolved from the cotton by the addition of 1 ml. of dimethylformamide. The resulting solution was evaporated *in vacuo* to approximately 50 ml. and was left at 0° overnight and then at room temperature for 24 hours.

The solution was washed with 1 *N* hydrochloric acid, water and 5% sodium bicarbonate solution, and was dried over anhydrous sodium sulfate. The solution was evaporated to dryness *in vacuo;* weight 1.52 g. (77%), m.p. 130–165°. The product was recrystallized from 20 ml. of 95% ethanol and 20 ml. of water (cooled to room temperature only); weight 1.25 g. (63%), m.p. 163–166°. It was recrystallized for analysis.

Anal. Calcd. for $C_{23}H_{28}N_2O_4$: N, 7.1. Found: N, 7.2.

Benzoyl-L-phenylalanyl-L-leucine Hydrazide.—A mixture of 1.10 g. (2.8 mmoles) of benzoyl-L-phenylalanyl-L-leucine methyl ester, 2.6 ml. of absolute ethanol and 1.3 ml. of hydrazine hydrate was heated at 75° for 4 hr. The mixture was cooled, 6 ml. of water was added, and the solid was collected by filtration, washed with water, and dried *in vacuo* to constant weight; 1.07 g. (97%); m.p. 208–212°.

Anal. Calcd. for $C_{22}H_{28}N_4O_3$: N, 14.1. Found: N, 14.0.

Benzoyl-L-phenylalanyl-L-leucyl-L-leucine Methyl Ester.—A solution of L-leucine methyl ester was prepared from 3.0 mmoles of the hydrochloride, as in the above preparation of benzoyl-L-phenylalanyl-L-leucine methyl ester.

A solution of 990 mg. (2.5 mmoles) of benzoyl-L-phenylalanyl-L-leucine hydrazide in 20 ml. of 1 *N* hydrochloric acid and 20 ml. of glacial acetic acid was used in the preparation of the azide. The procedure was the same as in the preparation of benzoyl-L-phenylalanine azide, except that a total of 110 ml. of sodium bicarbonate solution was necessary for the last wash to be basic, and 2 ml. of dimethylformamide was added after the azide and ester solutions were combined. The product crystallized out at 0° and the reaction mixture was solid after 20 hr. at 0°. The mixture was left 24 hr. at room temperature. The mixture was cooled, mixed with 5 ml. of 1 *N* hydrochloric acid, and was filtered. The solid was washed on the funnel with water, 1 *N* hydrochloric acid, water, 5% sodium bicarbonate solution and water. It was dried in the air, and finally *in vacuo;* 1.03 g. m.p. 165–200°. The product was recrystallized from 10 ml. 95% ethanol and 5 ml. of water; 772 mg. (61%); m.p. 199–202°. It was recrystallized for analysis, m.p. 200–203°.

Anal. Calcd. for $C_{29}H_{39}N_3O_5$: N, 8.25. Found: N, 8.1.

For comparison, benzoyl-L-phenylalanyl-L-leucyl-L-leucine methyl ester was also prepared from L-leucyl-L-leucine methyl ester. A solution of 392 mg. of carbobenzoxy-L-leucyl-L-leucine methyl ester in 4.0 ml. of dimethylformamide was mixed with 200 mg. of palladium black, and hydrogen was bubbled through, with shaking, for one-half hour. The catalyst was filtered off, and to the cold solution was added a solution of benzoyl-L-phenylalanine azide prepared as described above. Once recrystallized product (248 mg.) had the same melting point and mixed melting point as the benzoyl-L-phenylalanyl-L-leucyl-L-leucine methyl ester described above.

Benzoyl-L-phenylalanyl-L-leucyl-L-leucine.—A mixture of 637 mg. (1.25 millimoles) of benzoyl-L-phenylalanyl-L-leucyl-L-leucine methyl ester, 5.0 ml. of methanol and 1.25 ml. of 1.12 *N* sodium hydroxide was heated in a bath at 75° five minutes to dissolve all the solid, and the solution was left at room temperature one-half hour. The excess alkali was neutralized by the addition of 0.15 ml. of 1.00 *N* hydrochloric acid and the methanol was evaporated *in vacuo*. The residue was dissolved in 8 ml. of water, and the solution was extracted with four 5-ml. portions of ether. The aqueous solution was acidified with 0.4 ml. of 5 *N* hydrochloric acid, and was extracted with four 5-ml. portions of ether. This ethereal solution was washed with 2 ml. of water, dried over magnesium sulfate and evaporated to dryness *in vacuo*. The product was a glass, 580 mg. (90% yield, based on a neutralization equivalent of 515 (theory 496)).

L-Leucyl-L-leucine.—To a solution of 535 mg. (1.04 mmoles) of benzoyl-L-phenylalanyl-L-leucyl-L-leucine in 8.2 ml. (1.09 mequiv.) of 0.133 *N* sodium hydroxide solution was added 28 mg. of chymotrypsin (Armour, salt-free crystalline) and 33.5 ml. of water. The *p*H of the solution was 7.3. The solution was left at room temperature for 8 hr. The solution was acidified with 1.06 ml. of 1.03 *N* hydrochloric acid and the benzoyl-L-phenylalanine was extracted with three 50-ml. portions of ether (278 mg., m.p. 134–141°, neut. equiv. 287 (authentic benzoyl-L-phenylalanine, m.p. 141–143°, neut. equiv. 269)). The aqueous solution, containing L-leucyl-L-leucine, chymotrypsin and sodium chloride, was evaporated to dryness *in vacuo* over phosphorus pentoxide. The residue, 350 mg., was extracted with 10 and 3 ml. of boiling 95% ethanol. L-Leucyl-L-leucine hydrate crystallized as the solution cooled; 229 mg. (84%). Analyses indicated that this still contained approximately 2% sodium chloride. The sodium chloride was removed by another recrystallization (80% recovery) from 95% ethanol; m.p. 258–263°, $[\alpha]^{23}D$ −13.4° (*c* 1, *N* sodium hydroxide).[5]

Anal. Calcd. for $C_{12}H_{24}N_2O_3H_2O$: N, 10.7; Cl, 0.0. Found: N, 10.7; Cl, 0.0.

Paper chromatograms (butanol–acetic acid–water (4:1:5)) of samples taken at various stages in the preparation, as well as chromatograms of the isolated crystalline L-leucyl-L-leucine, indicated that L-leucyl-L-leucine (R_f 0.85) was the only compound which reacted with ninhydrin that was formed in the reactions. (As little as 1% of L-leucine or L-phenylalanine would have been detected on the chromatograms.)

L-Leucyl-L-leucine hydrate prepared from carbobenzoxy-L-leucyl-L-leucine had properties identical with those of the above material.

(5) Calculated rotation for the anhydrous material: E. Fischer (*Ber.*, **39**, 2893 (1906)) reported m.p. 270° and $[\alpha]^{20}D$ −13.4° (*c* 8.1, *N* sodium hydroxide). F. H. Carpenter and D. T. Gish (THIS JOURNAL, **74**, 3818 (1952)) reported m.p. 252–254° (uncor.) and $[\alpha]^{22}D$ −13.7° (*c* 0.95, *N* sodium hydroxide).

NEW YORK STATE AGRICULTURAL EXPERIMENT STATION
CORNELL UNIVERSITY
GENEVA, NEW YORK

The Methoxymercuration of 1,3-Butadiene

BY K. H. MCNEELY AND GEORGE F WRIGHT

RECEIVED DECEMBER 17, 1954

In order to evaluate an hypothesis that oxymercuration of alkenes proceeds *via* an alkanemercurinium salt[1] we have studied the reaction of 1,3-butadiene with one equivalent of mercuric acetate in methanol. This addition ought to be analogous with that of bromine which is thought by some to proceed *via* an alkanebromonium ion intermediate.

A good yield of 4-chloromercuri-3-methoxy-1-butene is obtained when the rapidly reacting system is finally poured into aqueous sodium chloride. The structure of this product has been demonstrated by ozonization, which yields formaldehyde, and by conversion to the known 1,4-diacetoxymercuri-2,3-dimethoxybutane.[2] None of the 1,4-addition product can be found, although it might have

(1) H. J. Lucas, F. R. Hepner and S. Winstein, THIS JOURNAL, **61**, 3102 (1939).

(2) J. R. Johnson, W. H. Jobling and G. W. Bodamer, *ibid.*, **63**, 131 (1941).