Organic Syntheses

A Publication
of Reliable Methods
for the Preparation
of Organic Compounds

Working with Hazardous Chemicals

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These paragraphs were added in September 2014. The statements above do not supersede any specific hazard caution notes and safety instructions included in the procedure.

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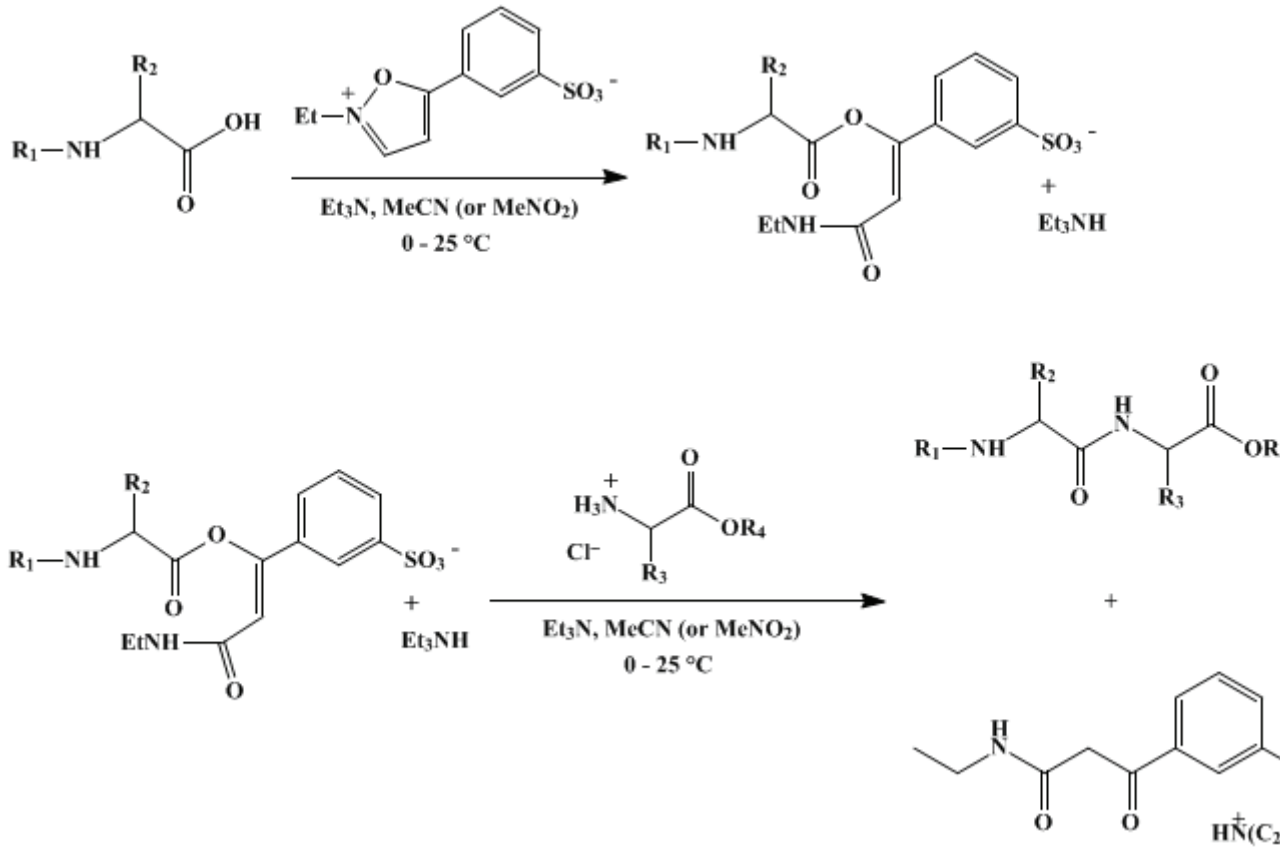
PEPTIDE SYNTHESES USING *N*-ETHYL-5-PHENYLISOXAZOLIUM-3'-SULFONATE: CARBOBENZOXY-L-ASPARAGINYL-L-LEUCINE METHYL ESTER AND *N*-CARBOBENZOXY-3-HYDROXY-L-PROLYLGLYCYLGLYCINE ETHYL ESTER

[L-Leucine, *N*-[*N*^{2}-[(phenylmethoxy)carbonyl]-L-asparginyl]-, methyl ester and Glycine, *N*-[3-hydroxy-1-[(phenylmethoxy)-carbonyl]-L-prolyl]-, ethyl ester]



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1. Procedure

A. *Carbobenzyloxy*-L-*asparaginyl*-L-*leucine methyl ester.* A mixture of 2.024 g. (0.00800 mole) of *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Note 1) and 20 ml. of nitromethane (Note 2) is prepared in a 50-ml., glass-stoppered Erlenmeyer flask and stirred vigorously, at room temperature, with a magnetic stirrer (Note 3). A solution of 2.128 g. (0.00800 mole) of carbobenzyloxy-L-asparagine (Note 4) and 810 mg. (0.00802 mole) of triethylamine (Note 5) and (Note 6) in 15 ml. of nitromethane (Note 2)) is added. Stirring is continued until dissolution of the isoxazolium salt is practically complete, giving a pale yellow solution (*ca.* 8 minutes is required; (Note 3), before 1.452 g. (0.00800 mole) of L-leucine methyl ester hydrochloride (Note 7) is added, followed by a solution of 810 mg. (0.00802 mole) of triethylamine (Note 5) in 5 ml. of nitromethane (Note 2). The resulting mixture is stirred overnight at room temperature, during which time some solid may separate from the solution. The mixture is then transferred to a 100-ml., round-bottomed flask and concentrated with a rotary evaporator. The residue is

triturated with warm (*ca.* 60°) aqueous 0.5% sodium hydrogen carbonate, and the resulting suspension is cooled and allowed to stand at 5° for at least 4 hours. The precipitate is collected on a filter, washed thoroughly with a total of 25 ml. of cold water, and dried, leaving 2.36–2.65 g. (75–84%) of the crude peptide, which melts within the range of 168–178°. Recrystallization from acetone–water provides 2.09–2.21 g. (66–70%) of pure carbobenzyloxy-L-asparaginyl-L-leucine methyl ester as fine, colorless crystals, m.p. 175–176.3°, $[\alpha]_D^{24}$ −27.7° (*c* 1.0, methanol) (Note 8).

B. N-*Carbobenzyloxy*-3-*hydroxy*-L-*prolylglycylglycine ethyl ester*. A mixture of 760 mg. (0.00300 mole) of *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Note 1) and 5 ml. of acetonitrile (Note 9) is prepared in a 25-ml., glass-stoppered Erlenmeyer flask. The mixture is cooled to 0° with an ice bath and stirred vigorously with a magnetic stirrer (Note 3). A solution of 796 mg. (0.00300 mole) of *N*-carbobenzyloxy-3-hydroxy-L-proline (Note 10) and 304 mg. (0.00301 mole) of triethylamine (Note 5) and (Note 6) in 5 ml. of acetonitrile (Note 9) is added. The cold (0–5°) mixture is stirred vigorously until almost all of the isoxazolium salt has dissolved (*ca.* 1–1.5 hours; (Note 3)), then treated with 590 mg. (0.00300 mole) of glycylglycine ethyl ester hydrochloride (Note 11), followed by addition of a solution of 304 mg. (0.00301 mole) of triethylamine (Note 5) in 6 ml. of acetonitrile (Note 9). After this mixture has been stirred for 1 hour at 0–5°, the resulting pale yellow solution is allowed to warm to room temperature and stirred overnight. The reaction solution is then transferred to a 100-ml., round-bottomed flask and concentrated with a rotary evaporator. The residue is partitioned between 10 ml. of aqueous 1% sodium hydrogen carbonate and 50 ml. of ethyl acetate. After the phases are separated, the aqueous phase is extracted with three 10-ml. portions of ethyl acetate, and the combined organic extracts are dried over anhydrous sodium sulfate. The organic solution is concentrated with a rotary evaporator, the residual white crystalline solid is triturated with 20 ml. of water, and the resulting slurry is cooled to 5° and allowed to stand overnight in a refrigerator. Filtration of the cold mixture provides a solid, which is washed with a small amount of cold water and dried. The combined aqueous filtrates are concentrated with a rotary evaporator, and the residual solid is again triturated with 10 ml. of water, allowed to stand overnight at 5°, and filtered. The solids collected total 958–980 mg. (78–80%) of *N*-carbobenzyloxy-3-hydroxy-L-prolylglycylglycine ethyl ester, m.p. 145–146°, $[\alpha]_D^{24}$ −18.5° (*c* 1.0, ethanol) (Note 12).

2. Notes

1. This isoxazolium salt reagent is commonly known and sold as Woodward's Reagent K (see, for example: The Merck Index, 9th ed., 1976, entry 9715). This salt (10 g.) (obtained from the Aldrich Chemical Company, Inc.) was dissolved in 45 ml. of 1 *M* hydrochloric acid and reprecipitated by the slow addition with swirling of 400 ml. of acetone. The salt was collected, washed with 300 ml. of acetone, and dried overnight at 25° under reduced pressure (<1 mm.) to give a fluffy product, m.p. 206–208° (dec.). An isomeric salt, *N*-ethyl-5-phenylisoxazolium-4'-sulfonate, which may be obtained by the usual synthetic procedure,[3,4] is also useful in peptide synthesis.
2. An anhydrous spectral grade of nitromethane, obtained from Fisher Scientific Company, was used without purification.
3. Since the initially formed enol ester rearranges slowly to an imide,[5] the yield depends on the rate at which the isoxazolium salt reacts, and that rate is increased by vigorous stirring. The reaction time for the activation step is approximately 8 minutes in nitromethane at 25° and approximately 1 hour in acetonitrile at 0°. In reactions performed in acetonitrile, the checkers did not obtain complete solution. The reaction flask should be kept in a water bath to minimize heat transfer from the magnetic stirrer to the reaction mixture.
4. Carbobenzyloxy-L-asparagine (obtained from the Aldrich Chemical Company, Inc.) was recrystallized from acetone–water to give the pure acid, m.p. 162–163°.
5. Commercial triethylamine (obtained from Eastman Organic Chemicals), was distilled (b.p. 89–90°) from phosphorus pentoxide and stored under a nitrogen atmosphere. Since the presence of even a small excess of triethylamine is deleterious in these reactions, the quantities of this amine used should be measured by weight rather than volume.
6. The triethylamine salts of peptide acids are often relatively insoluble in acetonitrile or nitromethane; therefore, the supersaturated solution formed on mixing the amine and the acid should be added to this reaction mixture immediately, before crystallization occurs. If crystallization does occur, the mixture should be heated to dissolve the salt, cooled rapidly, and added to the reaction mixture immediately. If it

is impossible to obtain a solution of the salt, the peptide acid and then the triethylamine solution may be added separately to the reaction mixture with only a small sacrifice in yield.

7. L-Leucine methyl ester hydrochloride (obtained from the Mann Research Laboratories, Inc.) was recrystallized from methanol–ether, m.p. 149–150°.

8. The reported[6] rotation for this product is $[\alpha]_D^{23}$ −26.3° (*c* 2.0, methanol). IR (KBr) cm.$^{-1}$: 3455, 3400, 3295 (amide NH stretching), 1737 (urethane and ester C=O), 1692 (amide C=O), 1647, 1535 (amide NH bending); ^{1}H NMR (dimethyl sulfoxide-d_6), δ (multiplicity, coupling constant *J* in Hz., number of protons, assignment): 0.7–1.1 (m, 6H, 2CH_3), 1.4–1.8 (m, 3H, aliphatic C*H*), 2.3–2.7 (m, 2H, CH_2CO), 3.62 (s, 3H, OCH_3), 4.1–4.7 (m, 2H, 2 NC*H*CO), 5.03 (s, 2H, benzylic CH_2), 6.92 (broad, 1H, N*H*), 7.25 (broad, 2H, NH_2), 7.35 (s, 5H, aromatic *H*), 8.17 (broad d, *J* = 7, 1H, N*H*); mass spectrum *m/e* (relative intensity): 393 (M^+, 1), 316 (5), 210 (12), 177 (15), 108 (37), 91 (100), 86 (50), 43 (28).

9. Reagent grade acetonitrile (obtained from Eastman Organic Chemicals) was dried over Linde type 4A molecular sieves, decanted, and used without further purification.

10. *N*-Carbobenzyloxy-3-hydroxy-L-proline (purchased from either Mann Research Laboratories or Sigma Chemical Company) was recrystallized prior to use. The submitters recommend recrystallization from water, but the checkers found it easier to recrystallize the material from a mixture of ethyl acetate and petroleum ether (b.p. 30–60°). In either case, the checkers added a seed crystal to induce crystallization. The recrystallized product melted at 106–107°.

11. Glycylglycine ethyl ester hydrochloride (obtained from Nutritional Biochemicals Corporation) was recrystallized twice from mixtures of ethanol and ether to separate the pure salt, m.p. 181–182°.

12. The reported[7] rotation for this product (m.p. 144–145°) is $[\alpha]_D^{21}$ −11.1° (*c* 1.0, ethanol). The submitters report that the melting point of the product is not changed by recrystallization. IR ($CHCl_3$) cm^{-1}: 3390, 3295 (OH and NH stretching), 1730 (ester and urethane C=O), 1620 (broad, amide C=O), 1520 (broad, amide NH bending); ^{1}H NMR ($CDCl_3$), δ (multiplicity, coupling constant *J* in Hz., number of protons, assignment): 1.2 (t, *J* = 7, 3H, OCH_2CH_3), 2.0–2.4 (m, 3H, aliphatic C*H*), 3.5–4.7 (m, 10H, O*H* and aliphatic C*H*), 5.08 (s, 2H, benzylic CH_2), 7.30 (s, 5H, aromatic *H*), 7.6 (broad, 2H, 2N*H*); mass spectrum, *m/e* (relative intensity): 207 (6), 149 (17), 108 (93), 107 (64), 91 (18), 79 (100), 77 (55), 65 (13).

3. Discussion

These procedures illustrate the use of *N*-ethyl-5-phenylisoxazolium-3'-sulfonate as a reagent for peptide synthesis.[3,4,5] Procedure A is recommended for peptides not soluble in either organic solvents or water. Procedure B illustrates the formation of a peptide that is soluble both in organic solvents and in water. For peptides soluble in organic solvents and insoluble in water, the submitters recommend the use of Procedure B, except that the peptide product may be recovered directly from its solution in ethyl acetate after this organic solution has been washed successively with aqueous 5% sodium hydrogen carbonate, water, 1 *M* hydrochloric acid, and water. Table I summarizes the preparation of various peptides by these procedures. Additional complex examples from other laboratories are listed elsewhere.[4]

Since there are a number of excellent and extensive reviews of peptide chemistry,[8,9,10,11] [12] no attempt will be made here to describe the known methods of peptide synthesis. Absolute comparisons of the procedure presented herein with other methods are impossible due to a number of factors: (a) the practice of many authors of reporting only yields of crude materials of unknown purity, (b) the necessities that prompt many experimenters to use a large excess of either the carboxyl component or the amine component in peptide synthesis, and (c) the difficulty of comparing this one-step synthesis of the amide bond with the ordinary two- and three-step syntheses (carboxyl activation, isolation of the free amine component from its hydrohalide, and aminolysis of the activated carboxyl group).

The method illustrated here does have, however, several excellent features.[13] The yields are very good even in the synthesis of asparaginyl and glutaminyl peptides, which are ordinarily very difficult to prepare in reasonable yield. Furthermore, all by-products are water-soluble and, therefore, easily removed from the product peptide derivative. One recrystallization, even under conditions of almost complete precipitation, usually suffices to yield pure material. A stringent test of this statement is the synthesis of carbobenzyl-oxyhydroxy-L-prolylglycylglycine ethyl ester, a peptide which is itself very soluble in water. The water solubility of the starting isoxazolium salt and of the by-products from

coupling has also been useful in studies of the reaction of proteins with the isoxazolium salt in aqueous solution[14,15] and to effect intermolecular cross linking of polypeptides.[16] Finally, use of the isoxazolium salt procedure for activation of the carboxyl groups of serine, tyrosine, and threonine offers the advantage that protection of the hydroxyl groups is often unnecessary.[12] Among the disadvantages of this method of peptide synthesis are the high cost of the isoxazolium salt and the limitations in the choice of solvent.

TABLE I[3,4]

Peptide	Reaction Procedure	Yield
Z-(N^{ε}-Z)-Lys·Gly-OEt[Glycine, *N*-[N^{2},N^{6}-bis-[(phenylmethoxy) carbonyl]-L-lysyl]-, ethyl ester]	B	95%
Z-Phe·Gly-OEt[Glycine, *N*-[*N*-[(phenylmethoxy)-carbonyl]-L-phenylalanyl]-, ethyl ester]	B	93%
Z-Phe·Leu-OMe[L-Leucine, *N*-[*N*-[(phenylmethoxy)-carbonyl]-L-phenylalanyl]-, methyl ester]	B	90%
Z-Met·Gly·Gly-OEt[Glycine, *N*-[*N*-[*N*-(phenylmethoxy)carbonyl]-L-methionyl]glycyl-, ethyl ester]	B	86%
Phth-Gly·Gly-OEt[Glycine, *N*-[(1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)acetyl]-, ethyl ester]	B	88%
Z-Gly·Gly·Tyr-OMe[L-Tyrosine, *N*-[*N*-[*N*-[(phenylmethoxy)carbonyl] glycyl]glycyl]-, methyl ester]	B	84%
Z-Gly·DL-Phe·Gly-OEt[Glycine, *N*-[*N*-*N*-[(phenylmethoxy)carbonyl] glycyl-DL-phenelalanyl]-, ethyl ester]	A	89%
Z-Gly·Gly·Gly-OEt[Glycine, *N*-[*N*-[*N*-[(phenylmethoxy)carbonyl] glycyl]glycyl]-, ethyl ester]	A	91%
Z-Gly-NHBz[Carbamic acid, [2-oxo-2-[(phenylmethyl)amino]ethyl]-, phenylmethyl ester]	A	94%
Z-Asp·Gly-OEt [Glycine, *N*-[N^{2}-[(phenylmethoxy)carbonyl]-L-asparaginyl]-, ethyl ester]	A	80%
Z-Gln·Val-OMe[L-Valine, *N*-[N^{2}-[(phenylmethoxy)carbonyl]-Lglutaminyl]-, methyl ester]	A	77%
Z-Gln·Tyr-OMe[L-Tyrosine, *N*-[N^{2}-[(phenylmethoxy)carbonyl]-L-glutaminyl]-, methyl ester]	A	75%

In tests devised to determine the amount of racemization to be expected in peptide syntheses in which the carboxyl component is a di- or higher peptide, this method ranks below the racemization-resistant azide procedure but above almost all other standard methods. Using a very sensitive and accurate isotope dilution assay, 1% racemization was observed[17] in the formation of the Anderson test peptide (Z-Gly·Phe·Gly-OEt)[18] and 7% racemization was observed[17] in the formation of the Young test peptide (Bz-Leu·Gly·OEt)[19] under optimized conditions. (The Young test was designed to exaggerate racemization problems, thus permitting more accurate studies of the effects of reaction condition variations.)

References and Notes

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Appendix
Chemical Abstracts Nomenclature (Collective Index Number); (Registry Number)

L-Leucine, N-[N^2-[(phenylmethoxy)carbonyl]-L-asparginyl]-, methyl ester

Glycine, N-[N^2,N^6-bis-[(phenylmethoxy)carbonyl]-L-lysyl]-, ethyl ester

Glycine, N-[N-N-[(phenylmethoxy)carbonyl]glycyl-DL-phenelalanyl]-, ethyl ester

ethanol (64-17-5)

hydrochloric acid (7647-01-0)

ethyl acetate (141-78-6)

methanol (67-56-1)

ether (60-29-7)

acetonitrile (75-05-8)

sodium hydrogen carbonate (144-55-8)

sodium sulfate (7757-82-6)

nitrogen (7727-37-9)

acetone (67-64-1)

tyrosine (60-18-4)

Nitromethane (75-52-5)

serine (56-45-1)

threonine (72-19-5)

triethylamine (121-44-8)

L-leucine methyl ester hydrochloride

glycylglycine ethyl ester hydrochloride (2087-41-4)

phosphorus pentoxide (1314-56-3)

L-Tyrosine, N-[N-[N-[(phenylmethoxy)carbonyl]glycyl]glycyl]-, methyl ester

CARBOBENZOXY-L-ASPARAGINYL-L-LEUCINE METHYL ESTER,
Carbobenzyloxy-L-asparaginyl-L-leucine methyl ester

N-Carbobenzyloxy-3-hydroxy-L-prolylglycylglycine ethyl ester,
N-CARBOBENZOXY-3-HYDROXY-L-PROLYLGLYCYLGLYCINE ETHYL ESTER (57621-06-4)

N-ETHYL-5-PHENYLISOXAZOLIUM-3'-SULFONATE

Glycine, N-[3-hydroxy-1-[(phenylmethoxy)-carbonyl]-L-prolyl]-, ethyl ester

carbobenzyloxy-L-asparagine

N-carbobenzyloxy-3-hydroxy-L-proline

N-ethyl-5-phenylisoxazolium-4'-sulfonate

carbobenzyl-oxyhydroxy-L-prolylglycylglycine ethyl ester

Carbamic acid, [2-oxo-2-[(phenylmethyl)amino]ethyl]-, phenylmethyl ester

Glycine, N-[(1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)acetyl]-, ethyl ester

Glycine, N-[N^2-[(phenylmethoxy)carbonyl]-L-asparaginyl]-, ethyl ester

Glycine, N-[N-[N-[(phenylmethoxy)carbonyl]glycyl]glycyl]-, ethyl ester

Glycine, N-[N-[(phenylmethoxy)-carbonyl]-L-phenylalanyl]-, ethyl ester

L-Valine, N-[N^2-[(phenylmethoxy)carbonyl]-Lglutaminyl]-, methyl ester

Glycine, N-[N-[N-(phenylmethoxy)carbonyl]-L-methionyl]glycyl-, ethyl ester

L-Leucine, N-[N-[(phenylmethoxy)-carbonyl]-L-phenylalanyl]-, methyl ester

L-Tyrosine, N-[N^2-[(phenylmethoxy)carbonyl]-L-glutaminyl]-, methyl ester