A CONVENIENT METHOD FOR THE ISOLATION OF FREE AMINO ACIDS FROM MARINE INVERTEBRATES

JOHN MATSOUKAS,[1] Y.E. MOHARIR, and JOHN A. FINDLAY*

Department of Chemistry, University of New Brunswick, Fredericton, New Brunswick, Canada E3B 6E2

ABSTRACT.—A simple and efficient method for the isolation of free amino acids in derivatized form from marine invertebrates is described. It has been used to isolate free amino acids as their *N-t*-Boc amino acid benzyl esters from the echinoderms *Asterias forbesi, Astropecten aurantiacus, Astropecten irregularis, Cucumaria frondosa, Echinaster sepositus, Luidia ciliaris, Marthasterias glacialis,* and *Stichopus regalis*. Major free amino acids present were identified, and relatively high levels of sarcosine were found in *A. aurantiacus* and *A. irregularis*.

In recent years there has been much discussion about the role of free amino acids in nerve tissue. Apart from possible metabolic functions involving protein synthesis and energy transfer, amino acids have also been implicated as neurotransmitters (1-3).

The customary procedures (4) for the isolation of free amino acids (neutral, basic, and acidic) from marine organisms and other sources usually involve aqueous alcohol extraction, solvent partitioning, passage through a series of ion exchange resins followed by further purification via chromatography, recrystallization, or derivatization. In our experience, these processes are sometimes made more tedious by the presence of large amounts of inorganic salts, saponins, or sterol sulfates such as are present in many echinoderms.

As part of a study in marine natural products, we have examined the free amino acids of a number of marine organisms. We report here the development of a convenient method for isolating these substances and for identifying the major free amino acids obtained in this way from eight echinoderms.

In our procedure, the crude, water-soluble extract of macerated echinoderm is treated with triethylamine and *t*-butyl-S-4,5-dimethyl pyrimid-2-yl thiocarbonate (5) to form the ethyl-acetate-soluble *N-t*-Boc amino acids, which are benzylated in high yield with benzyl bromide and triethylamine to provide the *N*-Boc amino acid benzyl esters. Such derivatized mixtures may then be analyzed by gas chromatography (gc) or high performance liquid chromatography (hplc), subjected to silica gel chromatography (column or layer), or hydrolyzed efficiently to free amino acids and subjected to automatic amino acid analysis. The particular merits of this method are the facile separation of free amino acids from other polar substances and their ready detection during subsequent chromatographic techniques employing uv detectors. In addition, these derivatives are readily recognized by ^{1}H-nmr because they are $CDCl_3$ soluble and provide a sharp singlet [9H, $(CH_3)_3C$] at δ 1.4 as well as signals at 5.2 and 7.3 ppm for the benzylic and aromatic protons, respectively.

This procedure has been employed to isolate the free amino acids from eight echinoderms, and table 1 indicates the major amino acids found in each. To test the efficiency of this procedure and to provide pure authentic standard derivatized amino acid samples, we have prepared the *N-t*-Boc amino acid benzyl esters of the eight amino acids in table 2. Also, we have ascertained, by adding known quantities of several amino acids to crude isolates from marine invertebrates, that the recovery of individual *N-t*-Boc amino acids is generally in excess of 90% yield, consistent with literature data (5) for this derivatization reaction performed on pure amino acids.

[1]Present address: Department of Chemistry, University of Patras, Patras, Greece.

TABLE 1. Major free amino acids in echinoderms (% wet weight x 10^{-2})

Organism	Alanine	Glycine	Leucine	
Asterias forbesi	0.2[a]	1.6	—	trace (phenylalanine trace (methionine)
Astropecten aurantiacus	—	—	—	5.3[b] (sarcosine)
A. irregularis	—	—	—	4.8[a] (sarcosine)
Cucumaria frondosa	0.4[b]	0.1	0.5	trace (methionine)
Echinaster sepositus	0.2[a]	1.8	—	
Luidia ciliaris	0.3[a]	2.4	1.6	
Marthasterias glacialis	0.3[a]	2.1	0.3	
Stichopus regalis	0.2[b]	2.0	0.3	1.3 (glutamic acid)

[a]Values obtained by integration of characteristic nmr signals of derivatized amino acids in mixtures.
[b]Composition determined by gc analysis.

TABLE 2. *N-t*-Boc amino acid benzyl esters

N-t-Boc amino acid	yield of benzyl ester (%)	RRt[a]	Rf[b]
Alanine	91	0.94	0.45
Aspartic acid	77		0.5
Glutamic acid	82		0.55
Glycine	95	1.00	0.35
Leucine	73	1.34	0.7
Methionine	70	2.05	0.5
Phenylalanine	66	2.40	0.55
Sarcosine	85	0.89	0.4

[a]Relative retention time for gc on 8′ OV-101 column (3% on high performance chromosorb W, 80/100 mesh) at 160-220° (6/min) using He (30 ml/min).
[b]On 0.25 mm silica gel plates in hexane-ethyl acetate (7:3).

In cases where economy of derivatizing reagents is important the crude extract from a marine organism may be introduced onto a column of Dowex 50WX8 (H^+) and eluted with water to remove salts, saponins, free sugars, and other neutral contaminants. Elution with ammonium hydroxide (2 N) affords the amino acid mixture ready for derivatization with calculated quantities of reagents.

We believe that this method of amino acid isolation offers some advantages over alternate methods in terms of simplicity, efficiency, and ease of detection and analysis. In the course of our exploration of this procedure, we have also isolated oligopeptides, novel amino acids, and amines, which are currently under investigation and will be reported elsewhere.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—A Varian XL200 instrument was used to record ^{1}H-nmr spectra in $CDCl_3$, using tetramethylsilane as internal standard (coupling constants *J* are in Hz). Infrared spectra were recorded with Perkin-Elmer 727B and 598 instruments, while mass spectra were obtained with a Hitachi Perkin-Elmer RMU-6D spectrometer. Gas chromatographic analyses were performed on a Perkin-Elmer 990 instrument equipped with flame ionization detector.

MATERIALS.—Specimens of *A. forbesi* and *C. frondosa* were collected from Passamaquoddy Bay, New Brunswick, Canada, in June 1982, while all other organisms were collected from the Gulf of Patras, Greece, in August 1981 and stored in deep-freeze until used.

EXTRACTION AND DERIVATIZATION OF FREE AMINO ACIDS.—The procedure for isolating free amino acids in derivatized form from *A. aurantiacus* described below was applied to all other organisms in this study.

Chopped, wet, whole-body *A. aurantiacus* (690 g) was treated with methanol (500 ml) at room temperature for 24 h and the extract evaporated under reduced pressure to yield a solid residue (12.5 g). A portion (2 g) of this residue was dissolved in water (30 ml) and washed with hexane (3×50 ml) to remove traces of lipid. To the aqueous phase (containing inorganic salts, saponins, and sterol sulfates, in addition to amino acids), triethylamine (2 ml) was added followed by a solution of *t*-butyl-S-4,6-dimethyl pyrimid-2-yl thiocarbonate (2 g) in dioxane (30 ml). This mixture was stirred for 12 h, and then water (30 ml) was added and the mixture extracted with ethyl acetate (2×25 ml) from which extract *N-t*-Boc amines, if present, are recoverable. The aqueous layer was acidified with 5 N HCl at 0° and extracted with ethyl acetate (3×50 ml). The organic layer was evaporated at room temperature, affording the *N-t*-Boc amino acid mixture (0.39 g), which was then dissolved in ethyl acetate (20 ml). Triethylamine (1 ml) and benzyl bromide (1 ml) were then added and the mixture was stirred at room temperature for 12 h and finally refluxed for 0.5 h. After cooling, a solution of sodium bicarbonate (50 ml, 5%) in water was added. This was followed by extraction with ethyl acetate (2×50 ml). The ethyl acetate extract was washed with water (2×50 ml), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue (0.46 g) was subjected to column chromatography on silica gel using hexane-ethyl acetate (4:1) as eluant, which afforded a major product (0.16 g) identified as *N-t*-Boc sarcosine benzyl ester; ir, ν ($CHCl_3$), 1740 (ester), 1685 (urethane) cm^{-1}; ms, 279 (3, M^+), 230 (7), 224 (50), 180 (11), 144 (30), 91 (63), 57 (100).

HYDROLYSIS OF *N-t*-BOC AMINO ACID BENZYL ESTERS.—Recovery of free amino acids from their *N-t*-Boc benzyl esters was carried out as described for recovery of sarcosine below.

Hydrochloric acid (1 ml, 5 N) was added to a solution of *N-t*-Boc sarcosine benzyl ester (0.05 g) in dioxane (1 ml), and the mixture was heated at reflux for 2 h, cooled to room temperature, and evaporated *in vacuo*. The residue was redissolved in a minimum of water, and the solution was examined by silica gel tlc using: (a) methanol-water (4:1) and (b) *n*-butanol-acetic acid-water (3:1:1) as eluants. The free amino acid was detected by ninhydrin and showed: (a) Rf=0.7, (b) Rf=0.2 in these solvent systems and showed a single spot when admixed with an authentic sample of sarcosine (*N*-methylglycine).

PREPARATION OF PURE *N-t*-BOC AMINO ACID BENZYL ESTERS.—*N-t*-Boc amino acid benzyl esters were prepared from authentic amino acids as described for *N-t*-Boc leucine-*O*Bz below, by treatment with benzyl bromide-triethylamine of the pure *N-t*-Boc amino acids available from commercial sources (Sigma Chemical Co., St. Louis, MO) or prepared by known methods (5) from the authentic amino acid. An alternative procedure (6), described earlier, employs benzyl alcohol in an acidic medium to make first the *O*-benzyl ester followed by protection of the amino function.

GENERAL METHOD FOR THE BENZYLATION OF *N-t*-BOC AMINO ACIDS.—*N-t*-Boc-leucine (0.231 g, 1 mmol) was added to a solution of triethylamine (0.101 g, 1 mmol) in ethyl acetate (10 ml). Benzyl bromide (0.181 g, 1.1 mmol) was then added, and the mixture stirred at room temperature for 6 h. Reaction mixture was then heated under reflux for 30 min. After cooling, it was treated with a solution of sodium bicarbonate (5%. 5 ml) followed by extraction with ethyl acetate (2×10 ml). The ethyl acetate extract was washed with water (3×10 ml) and was dried over anhydrous sodium sulfate; the solvent removed *in vacuo*. Crude *N-t*-Boc-leucine-*O*-Bz derivative thus obtained was purified by preparative tlc (silica gel, ethyl acetate-hexane, 1:4) to give pure *N-t*-Boc-leucine-*O*-Bz (0.225 g, yield=73%).

^{1}H-NMR SPECTRAL DATA FOR *N-t*-BOC AMINO ACID BENZYL ESTERS.—*N-t*-Boc alanine benzyl ester (oil): δ_{TMS} 1.40 (d, 3H, J=8, CH_3CH), 1.43 [s, 9H, $(CH_3)_3C$], 4.37 (m, 1H, -CH-), 5.06 (bs, 1H, -NH), 5.15, 5.22 (2d, 1H each, J_{AB}=12, -$CH_2C_6H_5$), 7.37 (s, 5H, C_6H_5).

N-t-Boc aspartic acid dibenzyl ester (7) (9): δ_{TMS} 1.44 [s, 9H, $(CH_3)_3C$], 2.88, 3.08 (2dd, 1H each, -CH_2-C_6H_5, J=16, J=6), 4.58-4.72 (m, 1H, -CH-), 5.09 (s, 2H, -CH_2-C_6H_5), 5.15 (s, 2H, -CH_2-C_6H_5), 5.50-5.58 (b, 1H, -NH), 7.30-7.42 (m, 2 x 5H, 2 x C_6H_5).

N-t-Boc glutamic acid dibenzyl ester (mp, 69-71°): δ_{TMS} 1.44 [s, 9H, $(CH_3)_3C$], 1.90-2.32 (m, 2H, -CH_2-CH-), 2.39-2.52 (m, 2H, -CH_2-COO-), 4.32-4.48 (m, 1H, -CH-), 5.12 (s, 2H, -CH_2-C_6H_5), 5.18 (s, 2H, -CH_2-C_6H_5), 5.10-5.22 (1H, -NH), 7.38 (s, 10H, 2 x C_6H_5).

N-t-Boc glycine benzyl ester (mp, 72-73°): δ_{TMS} 1.46 [s, 9H, $(CH_3)_3C$], 3.96 (d, 2H, J=6, -CH_2NH), 5.20 (s, 2H, -$CH_2C_6H_5$), 7.39 (s, 5H, -C_6H_5).

N-t-Boc leucine benzyl ester (oil): δ_{TMS} 0.91, 0.92 [2d, 3H each, J=6, $(CH_3)_2$CH-], 1.44 (s, 9H, $(CH_3)_3$C-), 1.45-1.80 (m, 3H, -$CH_2CH(CH_3)_2$), 4.30-4.46 (m, 1H, -CHNH-), 4.86-5.02 (m, 1H, -NH), 5.14, 5.22 (2d, 1H each, J_{AB}=12, -$CH_2C_6H_5$). 7.38 (s, 5H, -C_6H_5).

N-t-Boc methionine benzyl ester (oil): δ_{TMS} 1.45 [s, 9H, $(CH_3)_3$C-], 2.05 (s, 3H, CH_3S-), 1.80-2.24 (m, 2H, -CH_2CH-), 2.36-2.58 (m, 2H, -CH_2S-), 4.40-4.52 (m, 1H, -CH-), 5.15, 5.23 (2d, 1H each, J_{AB}=12, -$CH_2C_6H_5$), 5.2 (b, 1H, -NH), 7.38 (s, 5H, -C_6H_5).

N-t-Boc phenylalanine benzyl ester (mp, 59-62°): δ_{TMS} 1.42 [s, 9H, $(CH_3)_3$C-], 3.09 (d, 2H, J=6, -CH_2CH-), 4.56-4.70 (m, 1H, -CH-), 4.92-5.04 (b, 1H, -NH), 5.11, 5.19 (2d, 1H each, J_{AB}=12, -$CH_2C_6H_5$), 7.02-7.24 (m, 10H, $2C_6H_5$).

N-t-Boc sarcosine benzyl ester (oil):[2] δ_{TMS} 1.38, 1.48 [2s, 2 x 4.5H each), $(CH_3)_3C$-], 2.92, 2.95 (2s, 2 x 1.5H each, CH_3N-), 3.94, 4.04 (2s, 1H each, $-CH_2N$-), 5.20 (s, 2H, $-CH_2-C_6H_5$), 7.40 (s, 5H, $-C_6H_5$).

N-t-Boc serine benzyl ester (oil) (7) (8): δ_{TMS} 1.46 [2, 9H, $(CH_3)_3C$-]. 2.14-2.26 (b, 1H, -OH), 3.90-4.04 (m, 2H, $-CH_2OH$), 4.38-4.50 (b, 1H, -CH-), 5.24 (s, 2H, $-CH_2C_6H_5$), 5.38-5.54 (b, 1H, -NH), 7.38 (s, 5H, $-C_6H_5$).

ACKNOWLEDGMENTS

This study was supported, in part, by a strategic grant from the Natural Sciences and Engineering Research Council of Canada. One of us (JM) is grateful to NATO and the Greek government for providing financial assistance.

LITERATURE CITED

1. N.N. Osborne, *Comp. Biochem. Physiol.*, **43B**, 579 (1972).
2. F.V. De Feudis, in:"International Review of Neurobiology, Vol. 21." Eds. J.R. Synthies and R.J. Bradley, Academic Press Inc., New York, 1979, pp 129-216.
3. M.R. Hanley, *Experientia*, **31**, 881 (1975).
4. E. Fattorusso and M. Piattelli, in: "Marine Natural Products Vol. III." Ed. Paul J. Scheuer, Academic Press Inc., New York, 1980, pp 95-97.
5. T. Nagasawa, K. Kuroiwa, K. Narita, and Y. Isowa, *Bull. Chem. Soc. Jap.*, **46**, 1269 (1973).
6. L. Zervas, M. Winitz, and J. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).
7. E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).
8. E. Wunsch and A. Zwick, *Chem. Ber.*, **97**, 2497 (1964); also, R. Schwyzer, B. Iselin, H. Kappeler, W. Rittel, and P. Sieber, US Patent 3,247,182, April 19 (1966).
9. E. Sandrin and R.A. Boissonas, *Helv. Chim. Acta*, **46**, 1637 (1963).

Received 29 November 1982

[2]The duplicity of some ^{1}H-nmr signals is probably due to geometrical isomerism at the amidic bond. Similar duplicity of signals is noted in the ^{13}C-nmr spectrum of sarcosine, but is absent from all nmr spectra of the other derivatized amino acids, which differ from sarcosine by not having an N-CH_3 feature.