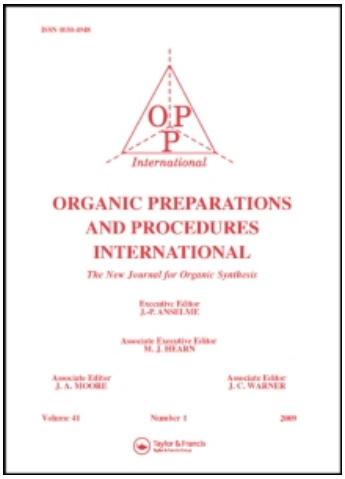

Organic Preparations and Procedures International
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t902189982

A SIMPLIFIED PROCEDURE FOR PREPARING THE CHOLINERGIC NEUROTOXIN 1-ETHYL-1-(2-EYDROXYETHYL)AZIRIDINIUM ION (AF64A)

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To cite this Article Moos, Walter H. , Davis, Robert E. and Schwarz, Roy D.(1989) 'A SIMPLIFIED PROCEDURE FOR PREPARING THE CHOLINERGIC NEUROTOXIN 1-ETHYL-1-(2-EYDROXYETHYL)AZIRIDINIUM ION (AF64A)', Organic Preparations and Procedures International, 21: 3, 315 — 319
To link to this Article: DOI: 10.1080/00304948909356385
URL: http://dx.doi.org/10.1080/00304948909356385

ORGANIC PREPARATIONS AND PROCEDURES INT. 21(3), 315-319 (1989)

A SIMPLIFIED PROCEDURE FOR PREPARING THE CHOLINERGIC NEUROTOXIN 1-ETHYL-1-(2-HYDROXYETHYL)AZIRIDINIUM ION (AF64A)

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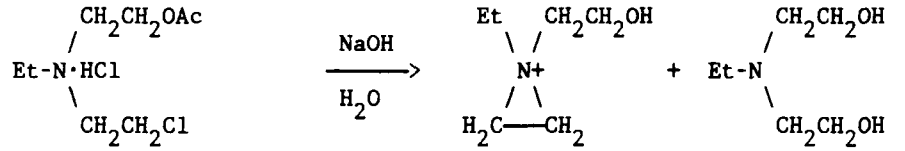
Since the original proposal[1] of the AF64A-treated mouse as a biological model[2] for central cholinergic hypofunction, and therefore perhaps for Alzheimer's disease,[3] the neurochemical deficits produced by AF64A (1-ethyl-1-(2-hydroxyethyl)aziridinium ion; ECMA; ECM Az; ADCh-1; 2) have been replicated in many laboratories. However, appropriate attention to the preparation and standardization of AF64A was somewhat lacking until several workers began to address the problem systematically.[4] During the course of our work in this area we developed a simplified process for preparing AF64A, which is reported herein.



AF64A Precursor (1) AF64A (2) Side Product (3)

The chemistry[5] and pharmacology[6] of nitrogen mustards have been well known for many years. However, the resurgence of interest in nitrogen mustards and aziridinium salts as pharmacological tools in cholinergic pharmacology has prompted a reinvestigation of their synthesis and properties, in this series and in others.[7] In particular, conditions associated with the optimal generation and

stabilization of AF64A have been studied recently by other workers. For example, general pH control[8] or careful titration[9] can be used effectively to generate AF64A, and decomposition is a pH, concentration, and temperature-dependent process. It was reasoned that stoichiometric (i.e., 200 mol%) base would be sufficient for the preparation of AF64A from its precursor hydrochloride salt (**1**), that is, the first 100 mol% base would free the hydrochloride salt, with cyclization to the aziridinium ion occurring spontaneously thereafter. The second 100 mol% base would hydrolyze the acetate, thus providing AF64A without the need for pH monitoring, a result of intrinsic stoichiometric control. Indeed, the hypothesis was borne out in practice. Characterization of AF64A prepared using stoichiometric base (aqueous NaOH) by ^{1}H NMR,[1,4] TLC,[8] thiosulfate titration,[4,5] and bioassay[10] confirmed the identity and purity of the cholinotoxin. AF64A prepared using this method is 90-95% pure, and, as a 1 mM solution, can be used for up to 60 min when kept at 0-5°C. If used within 30-45 min, the most reproducible results are obtained.

The half-life of AF64A solutions is dependent upon method of preparation, concentration, and temperature. Using the stoichiometric method, the AF64A half-life in the initial 100 mM solution at 20-26°C is >11 hrs by ^{1}H NMR. In our hands, 2,2'-(ethylimino)bis[ethanol] (**3**), the major degradation product, does not interfere with the toxicity of AF64A when co-administered in equimolar quantities. However, other as yet undetermined degradation products apparently do interfere with the toxicity of AF64A, thus necessitating its immediate use despite an extended half-life under these conditions. As an example of the latter point, 1.0-1.5 mM solutions of AF64A frozen for 30 days retain thiosulfate titer, but lose toxicity.

All reported methods for preparing AF64A have operational similarities, for example, in the use of NaOH as the base. Based on our experience, the most reproducible, previously reported process for preparing AF64A involves a micro-scale procedure that employs a micro-pH electrode and a syringe titrator.[9] The present method is simpler to execute, and provides material of comparable purity.

In conclusion, a simplified procedure for preparing the cholinergic neurotoxin AF64A has been developed, employing stoichiometric base. Because the new method requires neither special equipment nor careful monitoring, AF64A should now be routinely available to any laboratory.

EXPERIMENTAL SECTION

^{1}H and ^{13}C NMR spectra were recorded in D_2O containing TSP (3-(trimethylsilyl)propanoic-2,2,3,3-d_4 acid, sodium salt; Aldrich) as reference, using Varian XL-200 or IBM-100 Fourier transform spectrometers. TLC was performed with precoated glass plates (EM reagents silica gel 60 F-254). All volumes were measured using digital pipettes. AF64A precursor hydrochloride (1) was obtained from Research Biochemicals. The purity of the precursor (1), which is readily assessed using high field NMR (^{1}H and/or ^{13}C), is essential to the successful production of AF64A. Aqueous sodium hydroxide was obtained from Fisher Scientific.

AF64A (2). CAUTION: **AF64A** and **its** precursor **are** potent **toxins. Use care in handling these agents.** Twenty-five mg of precursor hydrochloride (1) was dissolved in 1 ml of triple-distilled water at 20-26°C, and the resulting solution was cooled in an ice/water bath. To this ice/water bath-cooled solution was added, with stirring or shaking, 12.5 µl of cool 50% w/w (19.1 M) aqueous sodium hydroxide solution. After 60 sec of stirring or shaking, 10 µl was withdrawn and added with stirring or shaking to 990 µl of triple-distilled water at 20-26°C. The resulting diluted solution was allowed to stand at 20-26°C for 30 min. After these 30 min had expired, the solution was placed on ice and used immediately (i.e., within 30-60 min). This

provides a solution of ≈1 mM AF64A. Each µl provides ≈1 nmol of AF64A.

Characterization of AF64A prepared using stoichiometric base (aqueous NaOH) by ^{1}H NMR, TLC, and thiosulfate titration confirmed the identity and purity of the cholinotoxin, which was further characterized via bioassay.[10] ^{1}H NMR data for educts and products have been reported, and thus are not duplicated in detail here.[1,4] Briefly, reaction progress can be monitored by disappearance of the chloromethylene triplet (ca. δ 4.0 ppm) in the precursor (**1**), and appearance of the key aziridinium methylene multiplet (ca. δ 3.2-3.4 ppm). TLC on silica gel is possible using 3:2:1 EtOAc:HOAc:H_2O.[8] After the TLC plate has been developed, visualization entails spraying dry plates with a dilute acetone solution of 4-(4'-nitrobenzyl)pyridine, heating the plates briefly at 100°C, and then spraying cooled plates with a dilute ethanolic solution of NaOH. Alkylating agents turn blue under these conditions; approx. R_f values are: **2**, 0.1; **1**, 0.6. Aziridinium ion formation can also be monitored by thiosulfate titration, a method that has been described by several groups.[4,5,8] Briefly, addition of acetic acid, thiosulfate, and starch indicator to the reaction solution allows colorimetric titration with iodine.

Acknowledgments.- We thank the following scientists for many helpful discussions: A. J. Bridges and F. M. Hershenson at Parke-Davis; S. M. Miller at the University of Michigan.

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(Received August 2, 1988; in revised form February 23, 1989)