Diethyl Azodicarboxylate Oxidation of Some Carcinogenic Arylhydroxylamines to Nitroso Derivatives

It has been demonstrated that 2-nitrosonaphthalene[1-3], 1-nitrosonaphthalene[4] and 4-nitrosobiphenyl[5] are urinary metabolites of the corresponding arylamines in the dog. 2-Naphthylamine and 4-aminobiphenyl are both powerful bladder carcinogens in man and the dog whereas 1-naphthylamine is presumably non-carcinogenic in these species.

In order to study the carcinogenicity of these nitroso compounds in several species, a rapid and efficient method of their synthesis was sought. Existing methods are quite tedious and result in low yields.

TAYLOR and YONEDA[6] reported the oxidation of N-phenylhydroxylamine to nitrosobenzene in 89% yield with diethyl azodicarboxylate. Extending this observation, we wish to report the rapid and efficacious oxidation of N,1- and N,2-naphthylhydroxylamine and N,4-biphenylhydroxylamine to their corresponding nitroso derivatives in high yield with this reagent.

To a stirred ether solution of N,1- or N,2-naphthylhydroxylamine[7,8] cooled to 0 °C is added dropwise an ether solution containing one equivalent of freshly distilled diethyl azodicarboxylate (Aldrich). The reactions were followed by thin layer chromatography on silica gel G with petroleum ether (40–60°), acetone (4:1) and the nitroso compounds detected with 5% aqueous trisodium acetacyanoamino ferrate. After completion (under 1 h), the precipitate of diethyl hydrazodicarboxylate is removed by filtration, the ether removed in vacuo, and a benzene solution of the residue applied to a silica gel column and developed with n-hexane-benzene (7:1)[3]. The bright emerald green band of nitroso compound was collected, the solvent removed in vacuo to yield light yellow-green crystals in 90–95% depending on the purity of the naphthylhydroxylamines. The 1- and 2-nitrosonaphthalenes[1,3,7] were identical to those prepared by the $KMnO_4$ oxidation of the ammonium salts of the corresponding N-nitrosonaphthylhydroxylamines (mixed mp, IR- and UV-spectra and Rf values).

Using chloroform as a solvent, 4-nitrosobiphenyl mp 73–74 (lit.[9] mp 73–74) was prepared from N,4-biphenylhydroxylamine[10] in 90–92% yields.

Zusammenfassung. Eine schnelle und wirksame Methode für die Oxydation von N,1- und N,2-Naphthylhydroxylamin und N,4-Biphenylhydroxylamin wird beschrieben, bei der diese Hydroxylamine durch Diethylazodicarboxylat zu den entsprechenden Nitrosoderivaten, in hoher Ausbeute umgewandelt werden.

E. BRILL

Department of Pharmacology,
University of Miami School of Medicine,
Coral Gables (Florida, USA), 3 March 1969.

[1] E. BOYLAND and D. MANSON, Biochem. J. *101*, 84 (1966).
[2] E. BRILL and J. L. RADOMSKI, in *Bladder Cancer* (Ed. K. F. LAMPE; Aesculapius Publishing Co., Birmingham, USA 1967), p. 90.
[3] H. UEHLEKE and E. BRILL, Biochem. Pharm. *17*, 1459 (1968).
[4] E. BRILL and J. L. RADOMSKI, unpublished results.
[5] E. FEFER, E. BRILL and J. L. RADOMSKI, Pharmacologist *9*, 241 (1967).
[6] E. C. TAYLOR and F. YONEDA, Chem. Commun. *199* (1967).
[7] R. WILLSTATTER and H. KUBLI, Ber. dt. chem. Ges. *41*, 1936 (1908).
[8] O. BAUDISH and R. FURST, Ber. *50*, 324 (1917).
[9] H. UEHLEKE and K. NESTEL, Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. *257*, 151 (1967).
[10] E. FEFER, *The Urinary Metabolites of the Carcinogen 4-Aminobiphenyl*, unpublished Ph. D. Thesis, Coral Gables, Florida. The University of Miami (1968).

[4-Proline, 8-Isoleucine]-Oxytocin and [4-Leucine, 8-Isoleucine]-Oxytocin, Possible Intermediates in the Evolutionary Series of Neurohypophysial Hormones: Synthesis and Some Pharmacological Properties

The biogenesis of the neurohypophysial hormones appears to involve at one stage the standard ribosomal mechanism of protein synthesis[1]. It is therefore not surprising that most of the structural differences between the homologous hormones of this group involve amino-acid replacements which can be accounted for by single base changes in the appropriate codons[2,3]. The single exception to this rule is the occurrence, in sequence position 4, of either glutamine (oxytocin, the vasopressins, vasotocin, mesotocin) or serine (isotocin, glumitocin). None of the known codons for glutamine on the one hand and for serine on the other can be interrelated by single base changes. This suggests the occurrence, as an evolutionary intermediate, of a peptide with an amino acid in position 4 which can be related to both glutamine and serine by single base changes. Proline meets this condition (Table I) but substitution of this imino acid for an amino acid (serine or glutamine) in the already conformationally constrained cyclic portion of the molecule would be expected to cause profound changes in the topochemistry of the molecule and hence probably loss of biological activity; such a mutant molecule would be unlikely to survive long enough to undergo further evolution.

Alternative links between the 4-serine and 4-glutamine series through a *single* intermediate peptide could be formulated by postulating *three* successive single base changes, one of these changes relating 2 codons for the same intermediate amino acid. Leucine and arginine meet these requirements (Table I) and of these 2 the neutral leucine seems the most likely in terms of the 'conservative' character of the change.

To facilitate the search for possible evolutionary intermediates in lower vertebrates we have synthetised the 2 peptides regarded as the most likely candidates, [4-proline, 8-isoleucine]-oxytocin (Ia) and [4-leucine,

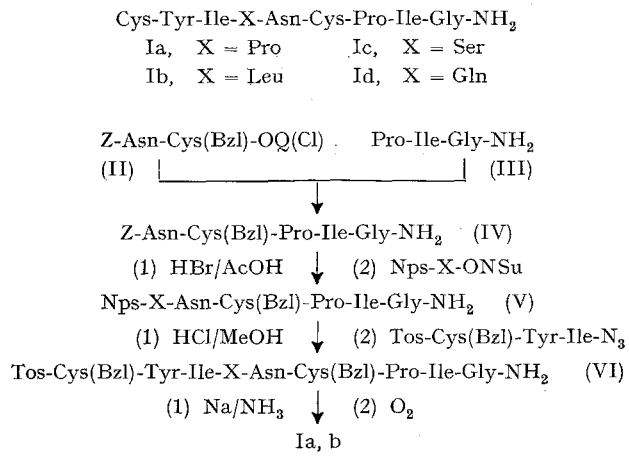
[1] H. SACHS and Y. TAKABATAKE, Endocrinology *75*, 943 (1964).
[2] J. F. G. VLIEGENTHART and D. H. G. VERSTEEG, J. Endocr. *38*, 3 (1967).
[3] I. I. GESCHWIND, Am. Zool. 7, 89 (1967).

8-isoleucine]-oxytocin[4] (Ib) and studied their physical and pharmacological properties. The 8-isoleucine series was chosen because in this series alone both the 4-serine and the 4-glutamine derivatives (Ic and Id, respectively) are known. After completion of our syntheses (cf. [5]) the preparation of [4-leucine]-oxytocin was reported[6] in a different context; [4-proline]-oxytocin has also been prepared recently[7].

The key intermediate[8] IV [mp 232–234°; lit. 233-235° (ref. [9]) or 212° (ref. [10])] was prepared by condensation of prolyl-isoleucyl-glycine amide III (mp 168–171°, $[\alpha]_D$ −67.8° (AcOH); lit.[11] mp 118°, $[\alpha]_D$ −63 ± 1° (AcOH)), obtained by a novel route[12], with benzyloxy carbonyl-asparaginyl-S-benzylcysteine 5-chloro-8-hydroxyquinoline ester II (mp 199–200°, $[\alpha]_D$ −46.6°), prepared in turn from benzyloxycarbonylasparagine and S-benzyl-cysteine 5-chloro-8-hydroxyquinoline ester[13] by a mixed anhydride procedure[14]. Removal of the benzyloxycarbonyl group with hydrogen bromide in acetic acid followed by acylation with the N-hydroxysuccinimide esters of *o*-nitrophenylsulphenylproline[15] and *o*-nitrosulphenylleucine (oil) afforded the hexapeptide derivatives Va (mp 221–223°, $[\alpha]_D$ −78°) and Vb (mp 221–223°, $[\alpha]_D$ −70°). Removal of the *o*-nitrophenylsulphenyl group with 1 equivalent of hydrogen chloride in methanol[16] followed by acylation with tosyl-S-benzylcysteinyl-tyrosyl-isoleucyl azide[17] afforded the protected nonapeptides VIa (dihydrate; mp 132–136°, $[\alpha]_D$ −42°) and VIb (monohydrate; mp 234–236°, $[\alpha]_D$ −31.7°). The free peptides Ia and Ib were obtained by reduction with sodium in liquid ammonia followed by oxidation with air[18] and isolated by countercurrent distribution and gel filtration.

Table I. Some possible evolutionary transitions between glutamine and serine

By 2 mutations[2,3]			By 3 mutations, one 'silent'			
CAA(G)	CCA(G)	UCA(G)	CAA(G)	CUA(G)	UUA(G)	UCA(G)
Gln	Pro	Ser	Gln	Leu		Ser
CAA(G)	UAA(G)	UCA(G)	CAA(G)	CGA(G)	CGC(U)	AGC(U)
Gln	'stop'	Ser	Gln	Arg		Ser



[4] Analogues of oxytocin are denoted in accordance with the IUPAC-IUB tentative rules (Biochemistry *6*, 362 (1967)). Standard abbreviations are used for amino-acid residues and protecting groups (cf. Biochemistry *5*, 2485 (1966)); in addition, OQ(C) stands for 5-chloro-8-quinolyloxy and ONSu for succinimidoxy. All amino acids (except glycine) are of the L configuration.

[5] J. RUDINGER, O. V. KESAREV and K. PODUŠKA, Abstracts of Communications, 5th FEBS Meeting, Prague, July 1968, No. 983.

[6] W. Y. CHAN, V. J. HRUBY, G. FLOURET and V. DU VIGNEAUD, Science *161*, 280 (1968).

[7] Personal communication from Drs. M. MANNING and W. H. SAWYER; M. MANNING, communication at the 5th FEBS Meeting, Prague 1968.

[8] For all numbered compounds, satisfactory elemental analyses were obtained. Optical rotations were measured with a photoelectric polarimeter using 0.2–0.5% solutions in dimethylformamide unless otherwise stated.

[9] A. JÖHL, A. HARTMANN and H. RINK, Biochim. biophys. Acta *69*, 193 (1963).

[10] S. GUTTMANN, B. BERDE and E. STÜRMER, Experientia *18*, 445 (1962); S. GUTTMANN, Helv. chim. Acta *45*, 2622 (1962).

[11] P.-A. JAQUENOUD and R. A. BOISSONNAS, Helv. chim. Acta *44*, 113 (1961).

[12] O. V. KESAREV, K. PODUŠKA and J. RUDINGER, Colln Czech. chem. Commun., in press.

[13] H.-D. JAKUBKE and A. VOIGT, Chem. Ber. *99*, 2944 (1966).

[14] M. ZAORAL, Colln. Czech. chem. Commun. *27*, 1273 (1962).

[15] J. MEIENHOFER, Nature *205*, 73 (1965).

[16] K. PODUŠKA, Colln. Czech. chem. Commun. *33*, 3779 (1968).

[17] J. HONZL and J. RUDINGER, Colln. Czech. chem. Commun. *20*, 1190 (1955); J. RUDINGER, J. HONZL and M. ZAORAL, Colln. Czech. chem. Commun. *21*, 202 (1956).

[18] V. DU VIGNEAUD, C. RESSLER, J. M. SWAN, C. W. ROBERTS and P. G. KATSOYANNIS, J. Am. chem. Soc. *76*, 3115 (1954).

Table II. Physical and biological properties of isotocin, mesotocin and 2 analogues

Peptide	Physical properties		Biological activities, units/mg[a]					
	K^b Rf[c]	$[\alpha]_D$[d]	Rat uterus in vitro without Mg^{2+}	with Mg^{2+}	Milk ejection (rat)	Chicken depressor	Antidiuretic (rat)	Toad bladder
[Pro^4, Ile^8]-oxytocin, Ia	0.37 0.72	− 8.8°	0.011 (0.008–0.014)	0.029 (0.025–0.033)	0.038 (0.032–0.044)		$<2.5\times10^{-5}$	<0.1
[Leu^4, Ile^8]-oxytocin, Ib	1.25 0.88	− 35.8°	4.4 (3.9–4.9)	31 (29–33)	4.9 (4.1–5.7)	32 (29–35)	$<5\times10^{-5}$[e]	<1
Isotocin	0.47	− 11.0°	120	352	530	382	0.29	30
Ic	0.65		(107–136)	(332–372)	(340–720)	(344–423)		
Mesotocin	0.33	− 31.8°	389	436	264	724	0.53	1190
Id	0.66		(355–426)	(413–458)	(175–347)	(664–953)		

[a] Values in brackets are 95% fiducial limits; 1 mg is 1 μmole for all peptides within the limits of experimental error. [b] Distribution coefficient in the solvent system 2-butanol – 0.05% aqueous acetic acid. [c] Descending chromatography on Whatman No. 1 paper in 1-butanol – acetic acid – water (4:1:5) at 22 °C. [d] In 1 *M* acetic acid (c = 0.10–0.15), recalculated for anhydrous peptide. [e] On occasions produced a diuretic response.

Alternative syntheses of isotocin (Ic) and mesotocin (Id) were carried out by the same route[12].

The free peptides showed satisfactory elemental and amino-acid analyses. The optical rotations, distribution coefficients, and Rf values are given in Table II. The gel filtration experiment showed that all the products were monomeric.

Oxytocic activity was measured with isolated uteri from stilboestrol-treated rats[19] by the method of HOLTON[20] using suspension fluids[21] containing no magnesium or 0.5 m*M* $MgCl_2$. Milk ejection activity was determined in the anaesthetized rat[22]. Chicken vasodepressor potencies were determined by a modification[23] of the method of COON[24]. Antidiuretic activity was measured in ethanol-anaesthetized rats by a modification[25] of the method of JEFFERS, LIVEZEY and AUSTIN[26]. The effect on water permeability was measured with isolated toad (*Bufo marinus*) bladders according to BENTLEY[27]. The results are given in Table II. Where comparable assay conditions were used the biological activities of our isotocin and mesotocin are comparable with those reported earlier[9-11, 28-30], particularly if it is kept in mind that the peptide content of the earlier samples is uncertain.

Of the newly prepared peptides, [4-proline, 8-isoleucine]-oxytocin (Ia) showed very low activities in all the standard assay systems, as had been anticipated. A similar low degree of activity is also shown by [4-proline]-oxytocin[7]. Although the (as yet unknown) physiological target organs of the oxytocin-like principles in lower vertebrates may well have specificity requirements different from any of those represented in the standard range of assays, all of the peptides isolated to date have at least moderate potencies in these tests. It therefore seems unlikely that a peptide 10^4 times less active than the natural hormones in all the assays would be a member of the same biological series. On the other hand, the [4-leucine, 8-isoleucine]-oxytocin (Ib), though less active than mesotocin or isotocin in all the standard assays, has appreciable potency in several tests so that its occurrence as a natural hormone cannot be discounted on the same grounds.

Characteristic properties of [4-leucine, 8-isoleucine]-oxytocin which should help in any search for its natural occurrence are the rather high partition coefficient and Rf value; the high ratio of avian depressor activity to activity on the rat uterus in vitro in the absence of magnesium; the high degree of magnesium potentiation (though in the case of glumitocin[31] this parameter has recently proved less constant than had been assumed); and the diuretic rather than antidiuretic effect together with natriuretic action[32] in the hydrated rat, properties which it shares with [4-leucine]-oxytocin[6].

If an evolutionary intermediate of the type discussed still survives, it is likely that it will be found among the elasmobranch fishes, since the primitive holocephalians seem to elaborate oxytocin itself[33] (with glutamine in position 4) while the more recent selachians secrete glumitocin (with serine in position 4)[34].

Zusammenfassung. [4-Prolin, 8-Isoleucin]-Oxytocin und [4-Leucin, 8-Isoleucin]-Oxytocin wurden als mögliche Glieder in der entwicklungsgeschichtlichen Reihe der Neurohypophysenhormone synthetisiert und pharmakologisch geprüft.

J. RUDINGER[35], O. V. KESAREV[36], K. PODUŠKA, B. T. PICKERING, R. E. J. DYBALL, D. R. FERGUSON and W. R. WARD

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Praha 6 (Czechoslovakia) and Department of Pharmacology, University of Bristol, Bristol (England), 17 March 1969.

[19] B. K. FOLLETT and P. J. BENTLEY, J. Endocr. *29*, 277 (1964).
[20] P. HOLTON, Br. J. Pharmac. *3*, 328 (1948).
[21] R. A. MUNSICK, Endocrinology *66*, 451 (1960).
[22] G. W. BISSET, B. J. CLARK, J. HALDAR, M. C. HARRIS, G. P. LEWIS and M. ROCHA E SILVA, Br. J. Pharmac. *31*, 537 (1967).
[23] B. K. FOLLETT and H. HELLER, J. Physiol. (Lond.) *172*, 74 (1964).
[24] J. M. COON, Arch. int. Pharmacodyn. Thér. *62*, 79 (1939).
[25] R. E. J. DYBALL, G. J. LANE and R. G. MORRIS, J. Physiol. (Lond.) *186*, 43P (1966).
[26] W. A. JEFFERS, M. M. LIVEZEY and J. H. AUSTIN, Proc. Soc. exp. Biol. Med. *50*, 184 (1942).
[27] P. J. BENTLEY, J. Endocr. *17*, 201 (1958).
[28] B. BERDE and H. KONZETT, Medna exp. *2*, 317 (1960).
[29] R. ACHER, J. CHAUVET, M. T. CHAUVET and D. CREPY, Biochim. biophys. Acta *58*, 624 (1962).
[30] H. RASMUSSEN, I. L. SCHWARTZ, R. YOUNG and J. MARC-AURELE, J. gen. Physiol. *46*, 1171 (1963).
[31] W. H. SAWYER, M. MANNING, E. HEINICKE and A. M. PERKS, Gen. comp. Endocr., in press.
[32] Unpublished results by Dr. J. H. CORT, Prague.
[33] W. H. SAWYER, R. J. FREER and T.-C. TSENG, Gen. comp. Endocr. *9*, 31 (1967).
[34] The authors are obliged to Academician F. ŠORM and Professor H. HELLER for their interest and encouragement, and to Professor W. H. SAWYER for information about his results in advance of publication.
[35] Present address: Laboratorium für Molekularbiologie, ETH, CH-8006 Zürich (Switzerland).
[36] Permanent address: Department of Natural Product Chemistry, Leningrad University, Leningrad (USSR).

Hexalure, an Insect Sex Attractant Discovered by Empirical Screening

Chemical attractants have become an indispensable tool in the detection[1] and control[2] of certain destructive insect pests. The sex attractants emitted by the virgin females of several species of Lepidoptera have been shown to be C_{12-16} alkenol acetates[3,4]. Isolation and identification of minute amounts of natural lure is an arduous, often frustrating task, and we therefore undertook to supplement our isolation program with a search for new insect attractants by a strictly empirical approach.

A large number of C_{12}, C_{14} and C_{16} alken-1-ol acetates were synthetized and evaluated as attractants for several insect species. One of these, *cis*-7-hexadecen-1-ol acetate, has proved to be an outstanding attractant for male

[1] M. JACOBSON, *Insect Sex Attractants* (Interscience Publishers, Inc., New York 1965), p. 104.
[2] M. JACOBSON, *Insect Sex Attractants* (Interscience Publishers, Inc., New York 1965), p. 112. – L. K. GASTON, H. H. SHOREY and C. A. SAARIO, Nature *213*, 155 (1967).
[3] R. S. BERGER, Ann. ent. Soc. Am. *59*, 767 (1966). – A. A. SEKUL and A. N. SPARKS, J. econ. Ent. *60*, 1270 (1967). – W. L. ROELOFS and H. ARN, Nature *219*, 513 (1968).