Vol. 95, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1980 Pages 491-498

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF SUBSTANCE P IODINATED DERIVATIVES

Robert Michelot, Henri Gozlan, Jean-Claude Beaujouan, Marie-Jo Besson, Yvette Torrens and Jacques Glowinski

Institut de Chimie des Substances Naturelles,CNRS 91190 Gif sur Yvette and INSERM U.114, Collège de France 11,place Marcelin Berthelot, 75231 Paris cedex 5, France

Received April 14,1980

SUMMARY

The synthesis of four substance P (SP) derivatives obtained by coupling the monoiodo and diiodo Bolton and Hunter reagents with synthetic SP is described. These compounds were proved to be good ligands for SP antibodies. As seen for SP, they exhibit a high biological activity in the guinea pig ileum bioassay.

In a previous study (1) we reported the use of a radioactive tracer related to substance (SP) in a SP radioimmunoassay. We describe here the synthesis of this compound and of three other derivatives obtained by coupling to synthetic SP the mono- and diiodo-Bolton and Hunter reagents (3-(3-iodo-, or 3,5-diiodo-, 4-hydroxyphenyl) propionic acid N-hydroxysuccinimidyl ester)(2). The corresponding non radioactive compounds were synthesized for chemical characterization and for evaluation of their biological activities in the guinea pig ileum assay. In addition, the binding properties of these ligands towards SP-antibodies raised in rabbits were investigated.

MATERIALS AND METHODS

Materials

Synthetic SP was purchased from Peninsula Laboratories (Mallet, France). Bolton and Hunter reagents were supplied from Amersham (monoiodo, 1500 Ci/mmole) or NEN (diiodo, 4000 Ci/mmole). Other reagents were of analytical grade. HPLC analysis was performed on a Waters equipment (pump 6000, injector U 6K and UV detector 440, columns: micro Bondapak C 18 and CN). Albino male guinea pigs weighing about 300 g and starved for 24 hours were used for bioassays.

Immunochemical reactivities of the derivatives were measured with SP antibodies prepared in our laboratory using a modification of the method originally described by Powell et al.(3). ^{125}I-Tyr-Gly-SP was a generous gift from Dr P. Pradelles (4).

0006-291X/80/140491-08$01.00/0

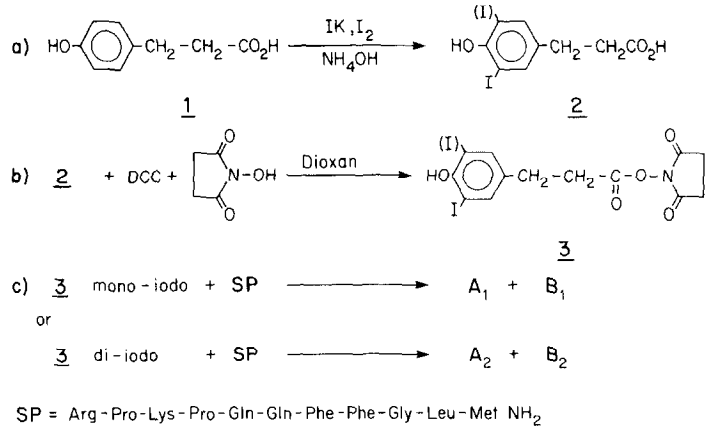

Fig 1 : Pathway for the synthesis of the non radioactive ligands

Methods

The pathways of the synthesis of SP derivatives is summarized in Fig 1.

Synthesis of the radioactive ligands

SP (0.5 mg) dissolved in 150 µl distilled water and 50 µl borate buffer (0.5 M, pH 8.5) was reacted with 1 mCi of commercial mono- or diiodo-Bolton and Hunter reagent shortly after their evaporation to dryness at 0°C for 30 min in a conic tube containing a magnetic stirrer. The reaction was stopped by adding 100 µl ammonium acetate (N, pH 4). Radioactive ligands were purified by HPLC by two successive runs on a micro Bondapak-CN column (solvent : methanol/water/ammonium acetate, N, pH 4.0, 1850/1350/30, V/V/V, rate 2ml/min). The elution volumes were 7.9 ml for the monoiodo derivative A_1 and 10.4 ml for the diiododerivative A_2. In addition to these major radioactive peaks, we could also observe in each case a weak peak appearing at 13 ml (B_1) or at 22 ml (B_2) (amounts less than 5%).

As sufficient material was not available for detailed characterization of the radioactive compounds the corresponding non radioactive ligands were synthesized.

Synthesis of the non-radioactive ligands

3-(iodo-3 or 3-5 diiodo-4-hydroxyphenyl) propionic acid 2

An aqueous solution (50 ml) of IK (20 g) and I_2 (6.35 g) was prepared. Part of this solution (23 ml) was slowly added (30 min) to 4-hydroxyphenyl propionic acid (4 g, 2.4 10^{-2}mole) dissolved in a 34% ammonia solution (480 ml). The resulting colorless mixture was stirred at room temperature for an additional hour, then concentrated to a final volume of about 100 ml. After acidification to pH 4.0 with concentrated 12 N HCl, the solution was extracted with ethyl ether. The organic layer was washed, dried over Na_2SO_4 and evaporated to dryness yielding a pale yellow solid which was recristallized from toluene. Yield: 68%, F : 109-111°C (Lit. 112-113°C (5)). (3-5 diiodo-

-4-hydroxyphenyl) propionic acid was obtained as above except that 46 ml of the initial solution of IK and I_2 were used. Yield : 56%, F = 165-167°C (Lit. 166-168°C (6)).

3-(iodo-3 or diiodo 3-5, 4-hydroxyphenyl) propionic acid N-hydroxysuccinimidyl ester 3

Dicyclohexylcarbodiimide (DCC) ($1.1\ 10^{-2}$mole) in cold dry dioxane (10 ml) was poured slowly into a mixture of the derivative 2 (10^{-2}mole) and N-hydroxysuccinimide (10^{-2}mole) in 50 ml dry dioxane kept at 0°C for one hour, and the reaction mixture was stirred overnight at room temperature. The excess of DCC was destroyed by adding acetic acid (60 ul). N,N' dicyclohexyl urea was discarded and the dioxane solution was evaporated under vacuum. The crop was recrystallized from propanol.

Monoiodo 3 : Yield:57%;F : 214-216°C. Thin layer chromatography (TLC) on silicagel 60 Merck F-254 in AcOH/toluene ; 10/9, V/V Rf : 0.44 UV (Me OH): $\varepsilon = 23.10^3$ at 211 nm and $\varepsilon = 3.10^3$ at 290 nm. PMR (DMSO-d6) : two multiplets for the aromatic protons at 7.55 (1 H) and 6.83 (2 H) ppm. Analysis : $C_{13}H_{12}I\ NO_5$

Diiodo 3 : Yield : 55%; F : 183-185°C (decomp.). TLC as above Rf : 0.48, UV (MeOH): $\varepsilon = 22.10^3$ at 221 nm and $\varepsilon = 3.10^3$ at 290 nm. PMR : (DMSO-d6) singulet for the two aromatic protons at 7.60 ppm. Analysis $C_{13}H_{11}I\ NO_5$.

It is noteworthy that the iodination of 3-(4 hydroxyphenyl) propionic acid by IK, I_2, NH_4OH made according to Runeberg (5) led to higher yields of 3 than the original method of Bolton and Hunter (2) in which the iodination by (NaI, chloramine T) occurs at the last step onto the activated ester.

Synthesis of the ligands A and B

SP (0.5 mg, $3.7\ 10^{-7}$mole) in 180 µl borate buffer (0.05 M pH 8.5) was reacted with 3 (0.12 mg, $2.4\ 10^{-7}$mole) in 50 µl dimethyl formamide (DMF) at 0°C for 90 min. The reaction was stopped by adding 10 N acetic acid to pH 4.0 and the reaction mixture was processed for HPLC as described above. The monitoring was performed at 254 nm (chromophoretic group of the acylating radical : $\varepsilon = 3000$ at 285 nm).

RESULTS

Analytical identification of the derivatives

As illustrated in Fig 2 (for A_1) mixtures of solutions containing A or B and the corresponding radioactive ligands showed in a second HPLC analysis a close correspondance in the maxima of radioactivity and UV absorbance.

The hydrolysis of the peptidic ligands led to the same repartition in aminoacids as SP itself since the basic amino acids were modified only by acylation.

SP could theoretically be acylated either on lysin or on arginin N-terminal or on both. The determination of the

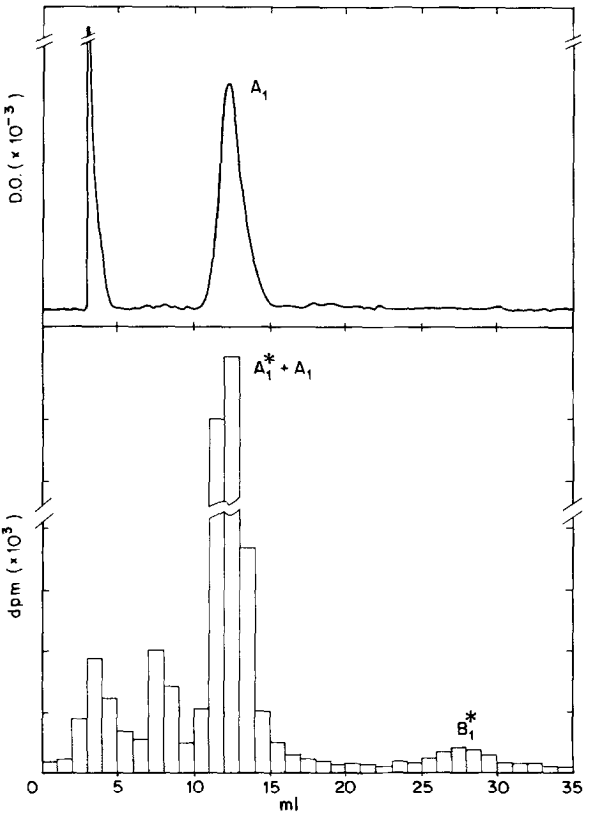

Fig 2 : Identification of the radioactive ligand A^*_1 to A_1 by HPLC on microbondapak C-18 (elution rate and solvent as in method) a) chromatography of A_1, UV detection at 254 nm b) chromatography of the diluted reaction mixture (SP + radioactive monoiodo Bolton and Hunter reagent - 0.005 µl) plus A_1 as a carrier (10^{-8} mole).

coupling site was achieved as follow : 1) Dansylation following Chen (7). TLC on Schleicher and Schüll F 1700 micro-polyamide plates, eluents: a) aqueous formic acid 1% ; b) ethyl acetate acetic acid methanol 20/1/1, V/V/V ; c) ammonium chloride (80mg) ammonium hydroxide (1.8% aqueous solution) (22 ml), ethanol (10 ml), solution completed to 100 ml. Authentic SP processed as a standard showed among other fluorescent spots, those corresponding to DNS-Arg and DNS-Lys while A_1 and A_2 led only to DNS-Arg. Neither B_1 nor B_2 gave any dansylated aminoacid. 2) Edman degradation (7). The resulting PTH were separated by HPLC (8). We could observe the PTH-Arg with SP, A_1 and A_2 but not with B_1 and B_2. 3) Sakaguchi test (9). The reaction was performed in order to verify the integrity of the guanidino group of the arginin residue after acylation by Bolton and

Hunter reagent. The compounds A_1, B_1, arginin and SP itself were rechromatographied on cellulose plates (5728 F Merck) in n-butanol, acetic acid, water, 75/20/25, V/V/V (Rf A_1: 0.65, B_1 : 0.78, arginin: 0.10, SP : 0.15). After spraying hydroxy-8-quinoleine, then hypobromite, orange red spots appeared on heating indicating that guanidino groups were not modified. According to the structure determination, compounds A_1 and A_2 are substituted on the ε lysin amino group. B_1 and B_2 are acylated on both arginin and ε lysin amino groups. Surprisingly we did not observe any acylated derivative at the N-terminal arginin only despite the difference in the PK_B of the two groups (8.99 for Arg and 10.79 for Lys (12)) in the conditions of the reaction (pH 8.5). This could be attributed to the solvation by DMF which enhances the basicity of the medium. The lack of acylated products on the guanidino group could be explained by their instability (13). By examining the evolution of the reaction at different time courses, we were able to observe a very rapid first substitution at the lysin group followed rather slowly by the second acylation at the N-terminal amino group. This observation explains also the yields obtained in the synthesis: 70-80% for A_1 or A_2 and 6-10% for B_1 and B_2 as measured by UV adsorption at 290 nm.

Biological tests

Contraction of the isolated guinea pig ileum

Animals were killed by cervical dislocation and 3-4 cm strips of intact gut were taken from the ileum 5-10 cm from the caecum. The tissue was placed in a 30 ml bath containing Tyrode's solution bubbled with 95% O_2-5% CO_2 at 37 $\pm$ 1°C and containing per ml 0.1 µg atropine, 1.0 µg mepyramine and 0.1 µg methysergide. Isotonic contractions under 0.5 g initial tension were recorded using a 386-A Harvard transducer coupled to a Hitachi potentiometric recorder. Synthetic SP was used as a standard in a cumulative dose assay (10,11)(Fig 3). The E_{50} for SP was 1.4 10^{-9}M and 1.5 10^{-9}M for A_1 and A_2 while B_1 and B_2 led to E_{50} 2.9 10^{-9}M and 3.6 10^{-9}M respectively.

Radioimmunoassay (RIA)

RIA were performed as originally described by Powell et al.(3) with minor modifications. The antiserum was raised in rabbits against synthetic SP coupled to serumalbumin with

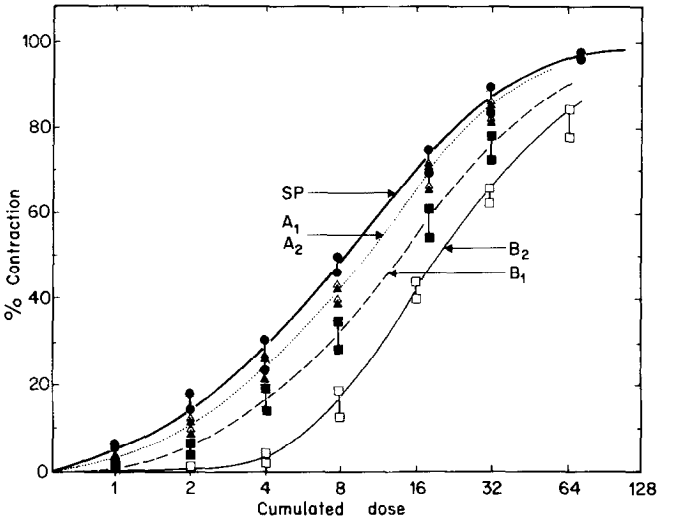

Fig 3 : Comparison of the effects of SP and of the iodinated derivatives on the contraction of the guinea pig ileum. SP and the synthetized iodinated derivatives A_1, A_2, B_1 and B_2 were added in cumulated doses. The initial dose being 6.3 10^{-12} mole. The data are the mean of results obtained in four distinct experiments.

1-ethyl-3(3-dimethyl-amino-propyl) carbodiimide. The tracer used was ^{125}I-Tyr-Gly-SP. After incubation (48 hours) of samples (0.1 ml) with diluted antiserum (0.4 ml, 1 : 150.000 final in phosphate buffer : 0.02 M, pH 7.4, 0.5% BSA) and the tracer (0.1 ml, 10000 dpm), the reacting mixture was precipitated by adding n-propanol (1 ml) at 0°C. The pellet was then processed for γ counting. In these conditions, corresponding to the equilibrium of the reaction mixture, maximal binding was 30%. The minimal detectable amount of SP was about 30 femtomoles (Fig 4). In the same assay, the half displacement of the ligand by A_1 was about 50 femtomoles reflecting a much higher potency of A_1 to bind the antibodies than SP. A_2, B_1 and B_2 displayed an affinity close to that of SP itself.

These results may be explained partly by the similarity of A_1 with the antigen used to generate the SP antiserum where the SP is probably bound to BSA by the lysin group. In addition the relative lower reactivity of B_1 and B_2 could be due to the increased hydrophobicity and to the steric hindrance due to the 4-hydroxyphenyl-3 iodo or 4-hydroxyphenyl 3,5 diiodo substituents.

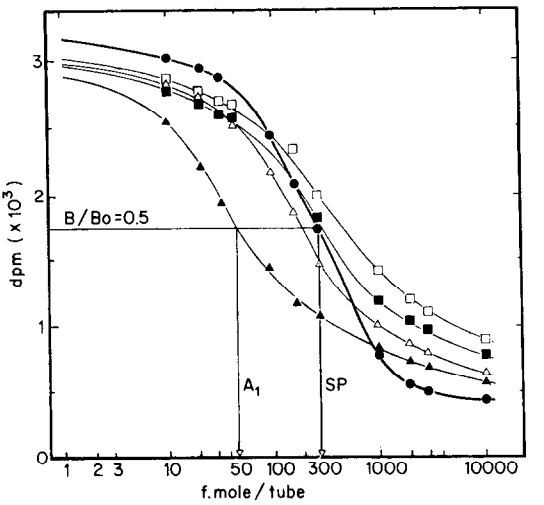

Fig 4 : Radioimmunoassay curves for SP and synthetized iodinated derivatives (SP —●—, A_1 —▲—, A_2 —△—, B_1 —■—, B_2 —□—).

DISCUSSION

Recently we have shown that labelling by tritium exchange or by deshalogenation of the (p-Cl)[7,8]SP derivative yielded products with specific radioactivities ranging from 15 Ci/mmole to 50 Ci/mmole respectively (16). These specific radioactivities were too low for the characterization of the SP receptors or for the radioimmunoassay of SP. Therefore, the aim of the present study was to synthesize a SP derivative with a high specific radioactivity retaining its biological properties. Since SP does not contain residues such as tyrosine or histidine, the direct iodination (^{125}I) was impossible. Furthermore, the Tyr-8-SP analogue leads to a iodinated product with rather low biological activity. This reduced activity can be explained by the concomitant oxidation of the methionine residue during the iodination, a phenomenon which also occurs during the iodination of Met-enkephalin (17) and proteins (18). These various limitations led us to couple a labelled iodinated substituent to the N-terminus of SP which is not involved in biological activity (14,15). We succeeded by using the Bolton and Hunter reagents. The best result was obtained with the monoiodo, monosubstituted derivative A_1 a compound which is stable over a period of two months when stored at -20°C in the chromatographic eluent. This ligand of high specific activity (1500 Ci/mmole) which has already been used in a SP radioimmunoassays (19) could be useful for the characterization of SP receptors.

REFERENCES

1. Michelot, R., Leviel, V., Torrens, Y., Glowinski, J. and Cheramy, A. (1979) Neuroscience Letters 15, 141-146.
2. Bolton, A.E. and Hunter, W.H.(1973) Biochem. J. 133, 529-539.
3. Powell, D. Leeman, S.E., Tregear, G.W., Niall, H.D. and Potts, Jr (1973) Nature New Biol. 241, 252-254.
4. Pradelles, Ph., Humbert, J., Cros, C. and Dray, F., submitted for publication
5. Runeberg, J., (1958) Acta Chem. Scand. 12, 188-192.
6. Barnes, J.H., Borrows, E.T., Elks, J., Hems, B.A. and Long, A.G. (1950) J. Chem. Soc. 2824-2833.
7. Chen, R., (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 873-876.
8. Abrahamsson, M.A., Groningsson,K. and Castesson, S. (1978) J. Chromatogr. 154, 313-317.
9. Heesing, A. and Hoppe, K., (1967) Chem. Ber. 100, 3649-3654.
10. Rossell, S., Björkroth, U., Chang, D., Yamaguchi, I., Wan, Y.P., Rackur, G., Fischer, G. and Folkers, K. (1977) in Substance P, eds U.S. von Euler and B. Pernow, Raven Press, N.Y. pp 83.
11. Van Rossum (1963) Arch. Int. Pharmacol. 143, 299-330.
12. Dawson R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M.(1972) in Data for Biochemical Research, Oxford, Oxford University Press G.B.
13. Bodansky, H., Klausner, Y.S., and Odetti, M.A. (1976) in Peptide Synthesis 2nd ed. Olah ed. John Wiley and Sons, New York. pp 67.
14. Yanaihara, N., Yanaihara, C., Horohashi, M., Sato, H., Lizuka,Y. Hashimoto, T. and Sakagami, M. (1977) in Substance P, ed. U.S. von Euler and B. Pernow, Raven Press, N.Y. pp 27-33.
15. Otsuka, M. and Konishi, S. (1977) in Substance P, ed. U.S. von Euler and B. Pernow, Raven Press, N.Y. pp 207-213.
16. Morgat, J.L., Fromageot, P., Michelot, R. and Glowinski, J., to be published in Febs Letter
17. Clement-Jones, V., Lowry, P.I., Rees, L.H. and Besser, G.M. (1980) Nature 283, 295-297.
18. Krohn, K.A., Knight, L.C., Harwig, J.F. and Welch, M.J. (1977) Biochemica et Biophysica Acta 490, 497-505.
19. Michelot, R., Leviel, V., Torrens, Y., Glowinski, J. and Cheramy, A. (1979) Neuroscience Letters 15,141-146.