Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 635–640

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SOLID PHASE ORGANIC SYNTHESIS OF POLYAMINE DERIVATIVES AND INITIAL BIOLOGICAL EVALUATION OF THEIR ANTITUMORAL ACTIVITY

Sophie Tomasi [a], Myriam Le Roch [a], Jacques Renault [a], Jean-Charles Corbel [a], Philippe Uriac [a,*] Bertrand Carboni [b], Damien Moncoq [c], Bénedicte Martin [c] and Jean-Guy Delcros [c]

[a] *Pharmacochimie de Molécules de Synthèse et de Produits Naturels, Faculté de Pharmacie, 2 Avenue du Professeur L. Bernard, 35043 Rennes Cedex, France.* [b] *Synthèse et Electrosynthèse Organiques, UMR CNRS, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes Cedex, France.*[c] *Groupe de Recherche en Thérapeutique Anticancéreuse, CNRS ESA 6027, Institut de Recherche Contre le Cancer, Faculté de Médecine, 2 Avenue du Professeur L. Bernard, 35043 Rennes Cedex, France.*

Received 11 December 1997; accepted 5 February 1998

Abstract - A series of N^1-monosubstituted putrescine and spermine derivatives was synthesised using a solid phase methodology. We evaluated their cytotoxicity, calmodulin antagonism and polyamine uptake inhibition, pharmacological properties shared by some antitumoral agents.

The natural polyamines, putrescine, spermidine, spermine have attracted considerable interest in recent years due to their essential role in cell growth and differentiation. Synthetic polyamine analogues and derivatives are promising tools for cancer chemotherapy.[1] Considerable effort has therefore been put forth by a number of researchers to investigate the structure-activity relationships of these molecules, resulting in the synthesis of a wide range of compounds selectively modified at their carbon chains or their amino functions.[2] Recently, we showed that pharmacological properties of N^1-dansylspermine and related sulfonamides of the natural polyamines, were not predictable from their constituents. This includes NMDA receptor antagonism, and properties such as cytotoxicity, calmodulin antagonism and polyamine uptake competitive inhibition, which make those conjugates potential antitumor agents.[3]

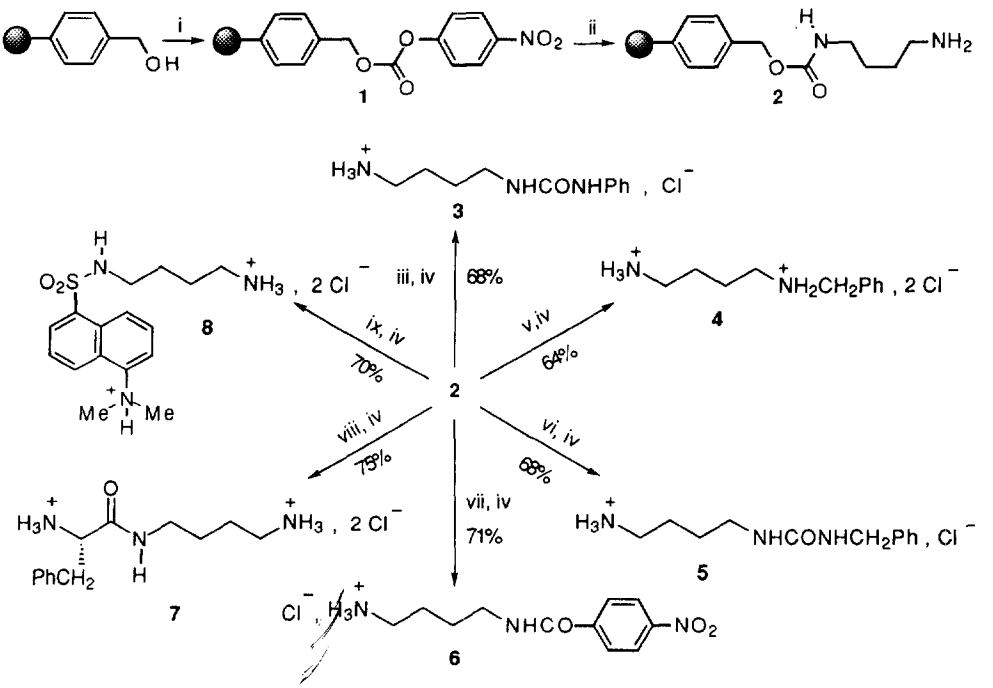
However, many synthetic problems are associated with their chemistry, most notably those of amine differentiation and purification of these highly polar compounds. In recent years, solid phase organic synthesis has emerged as a powerful methodology due to several advantages such as simplification of reaction procedures, easy separation of supported species and products, and application to automation systems.[4] In the course of our on-going program related to the synthesis and the biological evaluation of polyamine derivatives,[5] we envisaged to use this strategy to realise more efficient and selective reactions and to explore the elaboration of combinatorial libraries. Recent works concerning solid phase synthesis of polyamine conjugates [6] prompted us to report here our results dealing with the preparation of new N-monosubstituted putrescine and spermine and their initial biological evaluation.

Fax : (33) 02 99 28 68 88. E-mail : philippe.uriac@univ-rennes1.fr

0960-894X/98/$19.00
PII: S0960-894X(98)00086-9

Chemistry :

Initially, we were interested in preparing putrescine derivatives that led us to choose a route similar to the one reported by Leznoff et al.[7] As illustrated in Scheme 1, Wang resin was first reacted with 4-nitrophenyl chloroformate to give a polymer-bound carbonate **1**. Upon treatment with a tenfold excess of putrescine, **1** yielded the supported carbamate **2**.[7,8] This selectively protected diamine **2** is a convenient precursor of diversely mono *N*-substituted 1,4-diaminobutane and successful transformations have been carried out using various reagents such as isocyanate, aldehyde in the presence of sodium cyanoborohydride,[9] chloroformate followed by amine, acid chloride, aminoacid and sulfonyl chloride. Cleavage was achieved with trifluoroacetic acid in methylene chloride. Purification was achieved by conversion to the free amine and further treatment with an ethanolic solution of hydrogen chloride to afford the corresponding hydrochlorides **3** to **8** in good yields.[10]

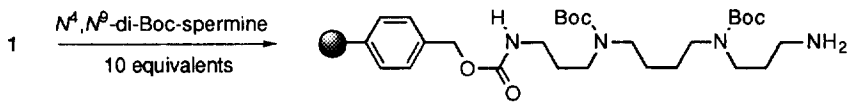


Reagents and conditions : i, 4-nitrophenyl chloroformate, *N*-methylmorpholine, CH_2Cl_2, 18h, rt. ii, putrescine (10 equivalents), CH_2Cl_2, 18h, rt. iii) PhNCO, CH_2Cl_2, rt,18h. iv, a) CF_3CO_2H/CH_2Cl_2 1/1, rt, 2h, b) aqueous 10% NaOH, c) ethanolic 2N HCl. v, a) PhCHO, $HC(OMe)_3$, rt,18h, twice, b) $NaBH_3CN$, $HC(OMe)_3$, rt,48h. vi, a) 4-nitrophenyl chloroformate, *N*-methylmorpholine, rt,18h, twice, b) $PhCH_2NH_2$, CH_2Cl_2, rt,18h. vii, 4-nitrobenzoyl chloride, *N*-methylmorpholine, rt,18h. viii, BOC-L-phenylalanyl, HOBt, DCC, THF, rt,18h. ix, Dansylchloride, *N*-methylmorpholine, CH_2Cl_2, rt,18h.

Scheme 1

Recognising the advantages of this solid-phase synthesis approach, we then became interested in the preparation of spermine derivatives. To prevent the possible addition to the secondary amino groups, we chose to use N^4,N^9-di-Boc-spermine as starting material. It was readily prepared by Michael addition of putrescine to acrylonitrile, followed by protection of the amino groups as *N*-Boc and hydrogenation over Raney Nickel.[11] The coupling to the resin was achieved by addition of a tenfold excess of this diprotected spermine to the 4-nitrophenyl carbonate **1**. As previously described, we then exploited the reactivity of the free amino group

to prepare *N*-alkyl, urea, amide, and sulfonamide derivatives. Deprotection and simultaneous cleavage from the resin afforded the corresponding mono N^1-substituted tetramine trifluoroacetates **10-15**.[10] In all cases, ^{1}H NMR revealed the presence of about 20% (^{1}H NMR) of spermine salt, which cannot be suppressed whatever the modifications of experimental conditions. This contamination could result from an initial double-binding (cross-linkage) to the polymer although we used a tenfold excess of reagent. But, it seems more conceivable that it was the consequence of the incomplete reaction of the attached spermine **9**. Indeed, the use of a 2-chlorotrityl chloride resin which was known to minimise cross-linkage, did not improve the purity of the resulting product. To eliminate spermine and to prepare the hydrochlorides, we have carried out preparative high voltage paper electrophoresis for **10-14** [12] and used the precedent procedure for **15**.



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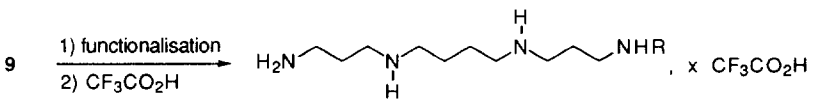

10-15

Compound	R	Reagents	yield (%)[a]
10	PhNHCO	PhNCO	70
11	$PhCH_2$	1) PhCHO 2) $NaBH_3CN$	50
12	$PhCH_2NHCO$	1) 4-NO_2-C_6H_4-OCOCl 2) $PhCH_2NH_2$	71
13	4-NO_2-C_6H_4-CO	4-NO_2-C_6H_4-COCl	70
14	Phe	Boc-PheOH, DCC, HOBt	58
15	Dansyl	DansylCl	56[b]

[a] Corrected yields taking into account the presence of spermine. [b] **15** was purified as its hydrochloride.

Reactions on polymers were monitored by FTIR and the complete linkage of putrescine and di-Boc-spermine was controlled by gel phase ^{13}C NMR. Structure of polyamine derivatives **3-15** was determined by ^{1}H NMR and HRMS and their purity (>98%) using HPLC.[13]

Biological activity

1 - Oxidative deamination by bovine serum amine oxidase (SAO) [14]

SAO is at high activity in ruminant serum, a usual component of tissue culture media. SAO deaminates the polyamines oxidatively and generates cytotoxic products (aldehyde, hydrogen peroxide, ammonia).[15] **4**, the only derivative retaining the two positive charges of the putrescine, was a very poor substrate of SAO as putrescine itself. In contrast, the other putrescine derivatives were oxidized by SAO. In a previous study, N^1-dansylspermine **15** was shown to be a substrate of SAO with a high affinity, but having a

slow reaction rate.[3] As expected, all spermine derivatives tested here were oxidised by SAO, but they were poorer substrates than spermine itself.

2 - Cytotoxicity [16]

To avoid the oxidation of the compounds by SAO, cells were cultured in artificial medium devoid of enzyme activities. None of the putrescine derivatives were active against L1210 leukemia cells, but all the spermine derivatives tested were found to inhibit their proliferation. Compounds **10** and **11** were the most potent (IC_{50}<2.5μM) and **15** was the least potent (IC_{50}=30μM).

3 - Inhibition of calmodulin-dependent phosphodiesterease (PDE) [17]

Spermine is known to bind to calmodulin with an affinity in the mM range and calmodulin antagonism property of N^1-dansyl spermine **15** has been reported.[3] Since we previously observed that putrescine derivatives exhibited lower activity than their spermine homologs, none of the putrescine derivatives prepared here were tested. Most spermine derivatives inhibited calmodulin-activated PDE. Their activities varied as follows : **15** (the most active : IC_{50}=65μM) > **13** > **11** > **10** > **14** (which is inactive).

4 - Inhibition of [^{14}C] putrescine uptake [18]

Among the putrescine derivatives, **4** was the only active compound. The inactivity of the other derivatives could be related to the lack of a second charged aminogroup in the putrescine moiety. All spermine derivatives were as potent as spermine itself to inhibit putrescine uptake. In contrast with putrescine derivatives, the conservation of all four positive charges of spermine was not a requirement to retain the activity. The cyclic nature of the substituent is likely to be essential for the activity since it has been shown that *N*-alkyl substituents on the terminal amino groups of spermine decrease the ability of the derivatives to compete for uptake [19].

Table I - Biological properties of *N*-substituted putrescine and spermine

	Oxidative desamination by SAO	Cytotoxicity on L1210 cells	Inhibition of calmodulin-activated PDE	Inhibition of [^{14}C]putrescine uptake
Coumpound No.	Fluorescence intensity (arbitrary unit)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Putrescine	2±1	-	-	-
3	41±5	>200	-	> 170
4	3±1	-	-	14.4 ± 6.1
5	44±2	>200	-	> 170
6	13±1	>200	-	> 170
7	24±2	>200	-	> 170
8	28±3	>200	-	> 170
Spermine	124±4	-	-	0.39 ± 0.05
10	86±9	2.2 ± 0.1	584 ± 58	0.29 ± 0.07
11	81±9	1.7 ± 0.1	313 ± 31	0.26 ± 0.05
13	73±3	5.6 ± 0.4	149 ± 14	0.39 ± 0.06
14	73±1	5.8 ± 0.6	>1500	0.37 ± 0.11
15	-	30 ± 2	65 ± 5	0.46 ± 0.04

In conclusion, if the synthesis described by Bycroft *et al* . [6] seems very attractive mainly because it can be achieved without any pre-protection of the secondary amino group of the spermine, the use of a Wang resin may be advantageous for combinatorial chemistry as this resin is less sensitive to acidic conditions than

the quite expensive chlorotrityl resin. We have also demonstrated that the substitution of a terminal aminogroup of a polyamine greatly affects its biological properties. Spermine derivatives may be calmodulin antagonists, polyamine uptake inhibitors or cytotoxic agents of considerable potency. Depending on the nature of the substituents (lipophilicity, charge, hindrance, size), one of these properties could be highlighted and the solid phase organic synthesis developed here is an efficient method to introduce diversity in order to target a specific activity.

Acknowledgements : We thank the Association de Recherche sur le Cancer (ARC) and the Ligue Nationale Contre le Cancer (Ille et Vilaine and Morbihan) for financial support and T. Bernard, J.F. Cupif, R. Havouis, N. Labbé for technical assistance.

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polymer **2** or **9** with 4-nitrophenylchloroformate (2 equivalents) in anhydrous CH_2Cl_2 in the presence of N-methylmorpholine (2 equivalents). The mixture was stirred for 18 h at rt, washed with CH_2Cl_2 (6x20 mL). The same treatment was repeated and the resin was dried under reduced pressure. Benzylamine (4 equivalents) was then added to a suspension of the resin. After strirring for 18 h at rt, the resulting mixture was treated as previously described for the addition of phenyl isocyanate. For the reaction with BOC-L-phenylalanine, this *N*-protected amino acid (3 equivalents) was first dissolved in anhydrous THF (5 mL) with 1-hydroxybenzotriazole (3 equivalents). A solution of *N,N'*-dicyclohexylcarbodiimide (3.equivalents) in anhydrous THF (3 mL) was added at 0°C and the solution was stirred 1 h at 0°C and 1 h at rt. The solid was removed by filtration and the clear solution was added to the suspension of the polymer **2** or **9** (1 g) in anhydrous THF (10 mL). The mixture was stirred for 18 h at rt. After washing as before, a second identical process was carried out. **Cleavage of the putrescine and spermine derivatives from the resin :** 1 g of the polymer was suspended in a mixture of CF_3COOH (5 mL) and CH_2Cl_2 (5 mL). The suspension was stirred for 2 h at rt. The polymer was removed by filtration, washed with CH_2Cl_2 (5x 20 mL),THF (2x20 mL) and water (2x20 mL). The filtrate (containing the trifluoroacetic solution combined with the washing solvents) was treated as follows : - *for putrescine derivatives 3-8 and for 15*: The filtrate was concentrated to dryness under reduced pressure, dissolved in the minimal amount of water (2 mL), alcalinised with aqueous sodium hydroxyde (NaOH 10%) and then extracted with CH_2Cl_2 (3x20 mL). The organic layer was dried over K_2CO_3 and the solvent removed under reduced pressure. To the crude residue dissolved in ethanol (2 mL) was added at 0°C a 1.5 N ethanolic solution of HCl (1.2 equivalents). After stirring 30 mn, ethanol was evaporated and the residue was triturated in diethyl ether to yield **3-8** and **15** as white solids which were collected and dried under reduced pressure.- *for spermine derivatives 10-14* : the filtrate was concentrated to dryness under reduced pressure. The residue was triturated in diethyl ether to yield **10-14** as white solids which were collected and dried under reduced pressure. Compounds **10-15** were purified by preparative high-voltage paper electrophoresis on a PHEROGRAPH model 64 using Whatman 3MM paper with a 3% HCOOH solution. The purity of putrescine and spermine derivatives was determined by HPLC after dansylation for **3, 4, 5, 6, 7, 10, 11, 12, 13, 14** and, after post-column derivatization with *o*-phthalaldehyde, for **8** and **15**.

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18 L1210 cells (2.10^6 cells) were incubated for 20 min at 37°C in Hanks' balanced salt solution in presence of [^{14}C]putrescine (0.5 µCi; 10µM) and the desired concentrations of the test compound (final volume : 600 µL). Blank samples were run in parallel at 4°C. Uptake of putrescine was stopped by layering the cell suspension on top of a mixture of corn oil + dibutylphthalate (3+1) and centrifugation at 15000 g for 1min. The cell pellet was dissolved in 1N NaOH. Aliquots of the pellet were also used for radioactivity measurement and protein determination using the Lowry method (Pöch, N. *Naunyn-Schmiedebergs Arch. Pharmacol.* **1971**, *268*, 272-299.

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