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Original article

Synthesis of 2-substituted β-cyclodextrin derivatives with a hydrolytic activity against the organophosphorylester paraoxon

Nicolas Masurier [a], François Estour [a], Marie-Thérèse Froment [b], Bertrand Lefèvre [c], Jean-Claude Debouzy [d], Bernard Brasme [c], Patrick Masson [b], Olivier Lafont [a,*]

[a] *Laboratoire de Pharmacochimie, Département de Chimie Organique Pharmaceutique, Faculté de Médecine et de Pharmacie de Rouen, 22 Boulevard Gambetta, 76183 Rouen cedex 1, France*
[b] *Unité d'Enzymologie, Centre de Recherches du Service de Santé des Armées, 24, avenue des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche cedex, France*
[c] *Service de Biospectrométrie, Centre de Recherches du Service de Santé des Armées, 24, avenue des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche cedex, France*
[d] *Unité de Biophysique Cellulaire et Moléculaire, Centre de Recherches du Service de Santé des Armées, 24, avenue des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche cedex, France*

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Abstract

β-Cyclodextrin was substituted by an iodosobenzoic acid derivative to create a catalytic hydrolytic activity against neurotoxic organophosphorus agents. The catalytic moiety was introduced on a secondary hydroxy group at the position 2 of a glucose unit. Several β-cyclodextrin derivatives were obtained. In these derivatives, the methylene linker occupied all potential positions on the aromatic ring. Kinetic assays were carried out with paraoxon as organophosphate model. Three regioisomers hydrolyzed paraoxon, although the paraoxon-leaving group, *para*-nitrophenol, was not released from the β-cyclodextrin torus.

Keywords: Cyclodextrin; Iodosobenzoic acid; Organophosphorus agents; Catalyst; Paraoxon

1. Introduction

The acute toxicity of neurotoxic organophosphorus (OP) agents is mainly due to their ability to inhibit cholinesterases [1]. These enzymes play a key role in the cholinergic system in terminating the action of the neurotransmitter acetylcholine. Therefore, intoxication by OPs leads to a severe cholinergic syndrome which can cause seizures and the death by respiratory failure [2]. Although prophylaxis and treatment of intoxication by OPs have been improved in the past 20 years, they are imperfect and can induce side effects [3]. To overcome these disadvantages, the concept of scavenger capable of degrading OPs has emerged as a new approach to reduce toxicity of chemical neurotoxic agents. The supporting idea was to neutralize OP molecules before they reached their neurological targets. Protection against OP poisoning can be achieved through the use of scavengers in active topical skin protectants, in decontaminant devices and in prophylactic injectable solutions [4,5]. Bioscavengers are enzymes that have the ability to react with OPs [6–8]. However, enzymes cause numerous problems: large-scale production, pharmaceutical formulations, cost, storage stability, immunogenicity and residence time in the bloodstream. Thus, the use of chemically accessible artificial enzymes as OPs scavengers for decontamination offer an attractive approach.

Cyclodextrins (CD) derivatives are potentially interesting for skin protection. Their chemical structure with a hydrophobic cavity is known to mimic the binding and catalytic activity of various enzymes [9–11]. Various esters showed the ability to bind into the CD cavity and then to react with a CD hydroxy group [12–18]. In the specific case of phosphate esters, an oxyanion obtained from a CD hydroxy group, reacted with the phosphate function, leading to phosphorylated CDs [19]. CD itself increased the cleavage of bis(*para*-nitrophenyl)phosphate by almost two orders of magnitude

* Corresponding author. Tel.: +33 2 35 14 86 51; fax: +33 2 35 14 84 00.
E-mail address: Olivier.Lafont@univ-rouen.fr (O. Lafont).

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[20]. Some improvements could also be obtained by capping [21,22]. But, the best method to obtain a suitable enzymomimetic model consisted in introducing a reactive functional group on the CD torus [23–25]. Even if different CDs were efficient, the choice of β-CD was justified by the fact that soman and sarin showed a better affinity for β-CD than for α- or γ-CD [26]. It had also been proved that some fluorophosphates were able to acetylate rapidly β-CD [27–29].

The present study was especially based on interactions of β-CD with soman and paraoxon. Generally, intoxications caused by soman are highly dangerous because they are resistant to usual treatment of anticholinesterase poisoning. In order to test the activity of protectants against soman, the most commonly used model is a less toxic organophosphorylester: paraoxon (Fig. 5), the hydrolysis of which can easily be monitored by UV spectroscopy.

The essential properties of the inclusion into a cyclodextrin catalyst of an organophosphonate were explained in terms of the geometries of the CD–OP inclusion complexes [30]. Data in relation with these parameters had been already published concerning soman and paraoxon. The hydrophobic nature of soman and its size compatible with β-CD made it a substrate for the oligosaccharide molecule [31]. Soman reaction with a secondary hydroxy group, leading to its inclusion in the hydrophobic cavity of the β-CD, had been described [32]. Soman and β-CD form a 1:1 complex [31]. Moreover, it had been observed that soman is hydrolyzed about 2500 times faster by the monoanion formed from a secondary CD hydroxy group than by the hydroxide ion [31]. Concerning paraoxon, its promotive catalysis by β-CD is probably caused by sufficient proximity of the paraoxon phosphorus atom to the secondary hydroxy groups surrounding the top of β-CD torus [30]. It had been proved that the *para*-nitro phenoxy ring of paraoxon was axially included in β-CD with the nitro-end first, and with its phosphate group exposed in solvent [33]. Moreover, the catalytic effect of cyclodextrin is essentially dependent on the hydrogen-bonding interaction between the secondary hydroxy groups of paraoxon [30].

Ortho-iodosobenzoic acid (IBA) is known to react as an anionic nucleophile on phosphorus atom of various OPs [34–38], including nerve active agents [39]. Moreover this compound is efficient in neutral aqueous media. The choice of IBA as catalytic system was then justified. In the β-cyclodextrin (β-CD) structure, three hydroxy groups can be potentially used to introduce IBA system, i.e. positions 2, 3 and 6 (Fig. 1). β-CD presenting a functional group branched on a secondary alcohol (C-2 or C-3) or on a primary alcohol (C-6) had shown different catalytic effects [40]. It was then necessary to choose the best position to branch IBA. Introduction of IBA as a catalytic moiety on a secondary hydroxy group had already been made it a catalyst for soman inactivation. But, these results were only partially published without description of synthesis protocols, separation methods, identification and structure of compounds [41].

In order to functionalize β-CD by IBA, we decided to introduce this reactive moiety in position 2, the hydroxy group in

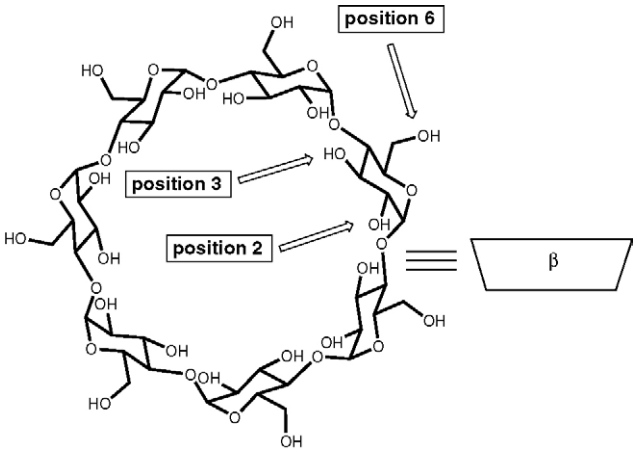
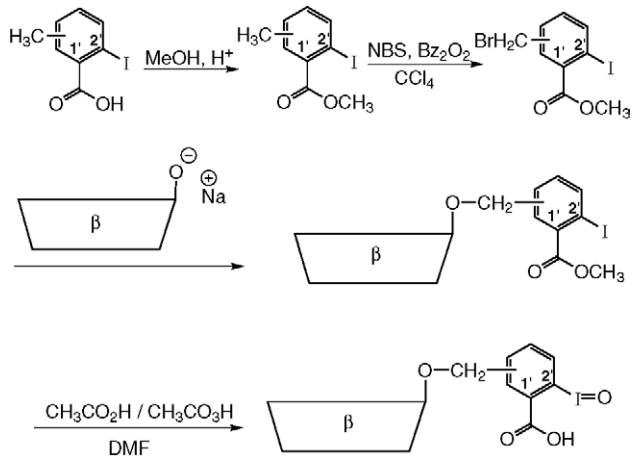


Fig. 1. The three categories of hydroxy groups in β-CD glucosyl unit.

that position being more reactive (vide infra) than the hydroxy group in positon 3. In that purpose, methyl bromomethyl-2-iodobenzoate derivatives were synthesized. Starting materials were 2-iodomethyl-benzoic acid isomers with the methyl group in various positions. The carboxylic function was protected as a methyl ester, then the methyl group was converted in a bromomethyl group by treatment by *N*-bromosuccinimide (NBS). Sodium hydride was first used to generate mono sodium salt of β-CD which reacted with the bromomethyl compound to give the alkylated β-CD. This latest was then oxidized by peracetic acid, to yield the iodoso derivative. The synthetic pathway is described in Scheme 1.

2. Synthesis

Each of the various steps of this synthesis made difficulties appear. The first step consisted in the synthesis of variously substituted precursors of IBA. In order to study the influence of the position of the linker between IBA and β-CD on the OP hydrolytic activity, the bromoalkyl group was successively introduced in the four free positions (3–6) of the methyl 2-iodomethylbenzoate ring (Fig. 2).



Scheme 1. Synthetic pathway for alkylation of β-CD.

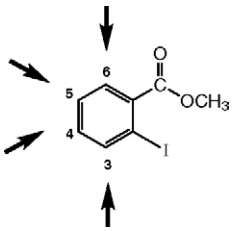


Fig. 2. The free positions of the methyl 2-iodobenzoate ring.

2.1. Synthesis of bromomethyl aromatic precursors of IBA

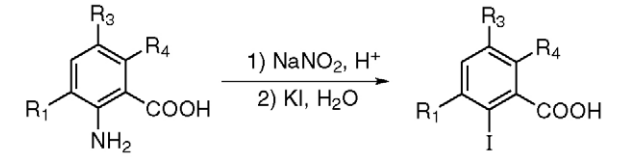
For isomeric positions 3, 5 and 6, iodination of the aromatic ring was carried out via a diazoic intermediate by treatment of the corresponding 2-aminomethylbenzoic acid **1**–**3** by sodium nitrite in acidic medium and then by potassium iodide to give compounds **4**–**6** (Scheme 2) [42].

For the 4-bromomethyl derivative, another pathway had to be used. The starting material was then 4-methylbenzoic acid. Iodination was performed by thallation followed by subsequent halogenation with potassium iodide and led to compound **7** (Scheme 3) [43].

All 2-iodo-methylbenzoic derivatives **4**–**7** were then esterified by methanol in acidic medium. Only in the case of compound **6**, the reaction needed an acyl chloride intermediate before reaction with methanol. For all compounds **8**–**11**, radical bromination of the methyl group led, in a last step, to the corresponding bromomethyl aromatic precursors of IBA **12**–**15** (Scheme 4).

2.2. Substitution of β-CD

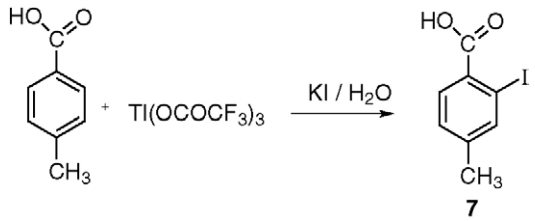
The three sorts of hydroxy groups in β-CD present different behaviors in alkaline medium. The hydroxy group in position 6 which corresponds to primary alcohol is the less acidic (pK_a ~ 15–16) [44]. Amongst the two secondary alcohols, the most acidic is situated in position 2 (pK_a = 12.1) [44]. In the case of hydroxy group situated in position 3, the main factor



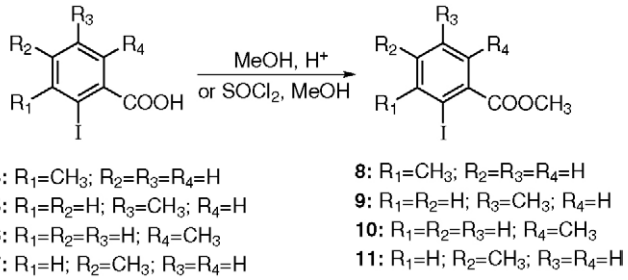
1: R_1=CH_3; R_3=H; R_4=H
2: R_1=H; R_3=CH_3; R_4=H
3: R_1=H; R_3=H; R_4=CH_3

4: R_1=CH_3; R_3=H; R_4=H
5: R_1=H; R_3=CH_3; R_4=H
6: R_1=H; R_3=H; R_4=CH_3

Scheme 2. Iodination of the aromatic ring via diazotation.

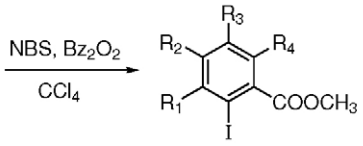


Scheme 3. Iodination of the aromatic ring via thallation.



4: R_1=CH_3; R_2=R_3=R_4=H
5: R_1=R_2=H; R_3=CH_3; R_4=H
6: R_1=R_2=R_3=H; R_4=CH_3
7: R_1=H; R_2=CH_3; R_3=R_4=H

8: R_1=CH_3; R_2=R_3=R_4=H
9: R_1=R_2=H; R_3=CH_3; R_4=H
10: R_1=R_2=R_3=H; R_4=CH_3
11: R_1=H; R_2=CH_3; R_3=R_4=H



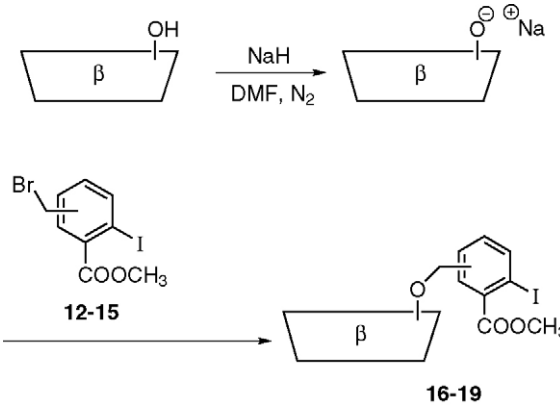
12: R_1=CH_2Br; R_2=R_3=R_4=H
13: R_1=R_2=H; R_3=CH_2Br; R_4=H
14: R_1=R_2=R_3=H; R_4=CH_2Br
15: R_1=H; R_2=CH_2Br; R_3=R_4=H

Scheme 4. Synthesis of bromomethyl precursors of catalytic moiety.

is the structure, which makes its access difficult. The choice to substitute position 2 in alkaline medium was confirmed by the fact that it was the best possibility to obtain a monosubstitution. This choice was strengthened by the strategy developed by Rong and D'Souza: deprotonation of a single hydroxy group to afford an oxyanion followed by nucleophilic substitution of a halogeno derivative. The degree of functionalization was controlled by the amount of alkali [44].

In order to synthesize 2-substituted derivatives, β-CD was then monosubstituted by the bromomethyl precursors (**12**–**15**) (Scheme 5).

This reaction led predominantly, as expected, to the substitution of a hydroxy group in position 2 (Table 1). The best selectivity was detected with methyl 6-bromomethyl-2-iodobenzoate **14**. In that case the reaction led to the isolation of one single compound monoalkylated in position 2 of a glucose unit. In the case of methyl 3-bromomethyl-2-iodobenzoate **12**, two regioisomers were obtained. The 2-substituted compound was the major product, and the by-product was monosubstituted in position 3. The 4-bromomethyl analog **15** led also to two regioisomers, but in that



Scheme 5. Alkylation of β-CD.

Table 1
Yields in 2-substituted compounds

Bromomethyl precursor	Total yield (%)	Analytic evaluation of 2-substitution rate (%)	Isolated 2-substituted compound yield
Compound **12**	24	77	18% (compound **16**)
Compound **13**	37	57	Not isolated (compound **17**)
Compound **14**	16	100	16% (compound **18**)
Compound **15**	43	77	31% (compound **19**)

Conditions for substitution of β-cyclodextrin: NaH in DMF.

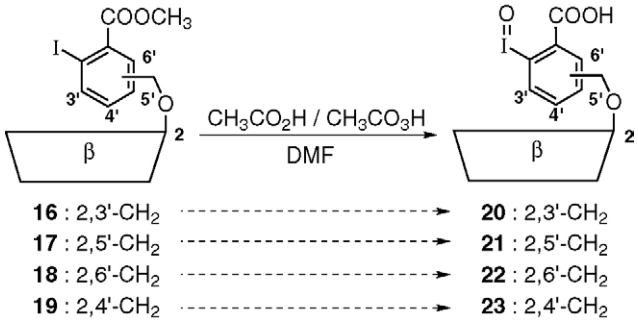
case, the by-product was monosubstituted in position 6. The lowest selectivity was observed in the case of the 5-bromomethyl compound **13** and then three regioisomers, respectively, monosubstituted in positions 2, 3 and 6 were formed.

These differences in the reactivity could be explained by two phenomenons. First of all sodium hydride is a strong base and the differences in acidity of hydroxy groups were not in the best conditions to be revealed. Then, the structures of the variously substituted benzyl bromides were not equivalent. For example, in the case of compound **18**, the linker in *ortho* position of the carboxymethyl group could orient its reactive site towards a hydroxy group in position 2. On the other hand, in the case of compound **17**, the linker in *meta* position of the carboxymethyl group could allow the nucleophilic attack by an anion of the β-CD situated either in position 2, 3 or 6. Anyway substitution in position 2, 3 or 6 of β-CD with this base–solvent system, i.e. sodium hydride in dimethylformamide, had already been described [45,46].

In order to get reproducible kinetic assays and to compare OP-hydrolyzing efficiency of the different derivatives, it was essential to test hydrolytic activity on a single derivative and not on a mixture of regioisomers. Compound **18** being devoided of any side product, it was then easily isolated by column chromatography. For compounds **16** and **19**, they were separated from the respective side products by column chromatography. No condition for chromatographic separation could be found in the case of compound **17**. It was then necessary to optimize the experimental conditions of β-CD substitution in order to obtain this product as a single isomer. To prevent a deprotonation of the three kinds of alcohols, the reaction was realized with sodium hydroxide which is a weaker base than sodium hydride. No substitution was observed in dimethylformamide, but the same experiment with dimethylsulfoxide as solvent allowed to obtain compound **17** as a single 2-substituted β-CD isomer. With sodium hydroxide the yields were lower than with sodium hydride, but regioselectivity was better.

β-CD derivatives monoalkylated in position 2 were then converted into corresponding iodoso derivatives by peracetic acid oxidation in acetic acid medium (Scheme 6). The lack of reactivity of alcohols in the β-CD with peracetic acid was proved by treatment of β-CD itself under the same conditions. It was known that *ortho*-iodoso carboxylic acid derivatives underwent an equilibrium leading to a cyclic structure which was the active form (Fig. 3) [39].

The four iodoso regiosisomers **20**–**23** which were tested are presented in Fig. 4 under this cyclic form.



Scheme 6. Conversion into iodoso derivatives.

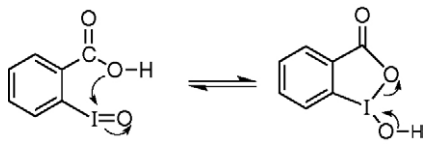


Fig. 3. Active form of 2-iodosobenzoic acid.

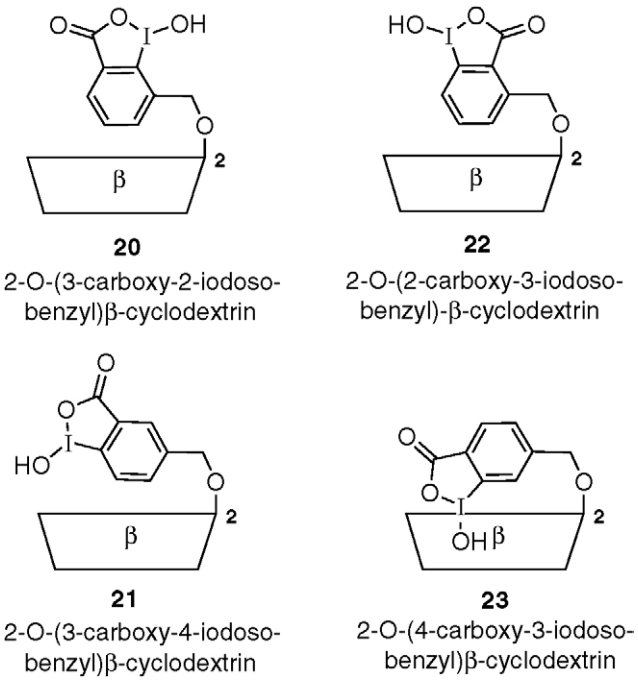
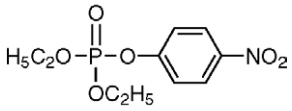


Fig. 4. Tested compounds against paraoxon.

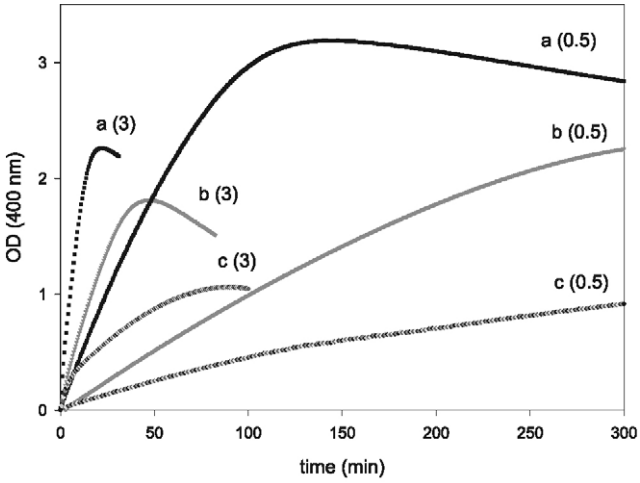
3. Protective activity

Hydrolysis assays were carried out with paraoxon as organophosphate model (Figs. 5 and 6). Compound **21** was active against paraoxon. Compounds **22** and **23** showed a weak hydrolytic activity. Compound **20** was inactive. This pro-



paraoxon

Fig. 5. Chemical structure of paraoxon.

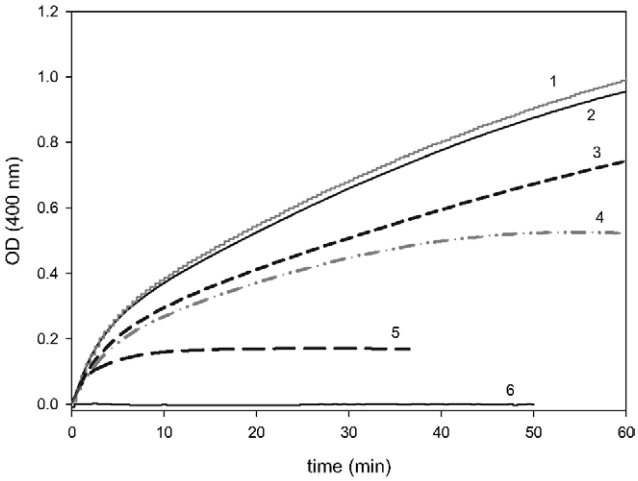


Hydrolysis of paraoxon (3mM and 0.5mM: figures in parentheses on curves) by iodoso-substituted β-CD derivatives (1mM). a: compound 21; b: compound 23; c: compound 22.

Fig. 6. Hydrolysis of paraoxon by iodoso-substituted β-CD derivatives.

vided evidence that hydrolytic activity of β-CD derivatives was regio-controlled. The relative position of the ether linker between the IBA and β-CD (*ortho*, *meta* or *para* to the iodoso group) had an influence. No activity was observed when the linker was in *ortho* to the iodoso group, the steric hindrance being maximum. An intermediate activity was observed when the linker was in *meta*-position but the best hydrolytic activity was obtained when iodoso group was in *para*-position. It is likely that the ether linker in *para*-position of the iodoso group optimized interactions between the catalytic moiety and the OP compound scavenged into the hydrophobic cavity of β-CD.

The paraoxon molecule being included in CD cavity in the case of the four isomers, it was then proved that hydrolytic activity against paraoxon was depended only on the orientation of the iodoso group in the surroundings of β-CD, and on the flexibility of the linker. Nevertheless, hydrolysis of paraoxon showed saturation kinetics with the three active compounds **21**–**23**. The higher the paraoxon concentration, the earlier the saturation plateau appeared (Fig. 6). Hypothesis was made that the β-CD catalyst could be poisoned by the paraoxon leaving group, *para*-nitrophenol which was not released from the torus of the CD molecule. The size of *para*-nitrophenol molecule is suitable for being included in β-CD, and it is known that phenol derivatives can form more or less stable complexes with β-CD [47,48]. Kinetic measurements were then performed with compound **22**, using 3 mM paraoxon in the presence of various potential competitors (final concentration: 0.5 mM) of paraoxon (Fig. 7). Phenol itself did not alter CD-catalyzed hydrolysis of paraoxon. Phe-



Effect of different competitors (0.5mM) on hydrolysis of paraoxon (3mM) by β-CD derivative (1 mM) **22**. **1**: no competitor; **2**: phenol; **3**: acetyl salicylic acid; **4**: *ortho*-nitrophenol; **5**: *meta*-nitrophenol; **6**: *para*-nitrophenol (paraoxon leaving-group).

Fig. 7. Effect of competitors on hydrolysis of paraoxon by β-CD derivative **22**.

nols substituted by electron-withdrawing groups, acetylsalicylic acid, *ortho*- and *meta*-nitrophenol caused increasing inhibition of paraoxon hydrolysis. It is important to notice that *para*-nitrophenol led to complete inhibition of hydrolysis. These observations provided evidence that *para*-nitrophenol formation during paraoxon hydrolysis limitated the efficiency of β-CD catalyst. The complex formation constants were then determined for both protonated and deprotonated forms of phenol, *ortho*-, *meta*- and *para*-nitrophenols with β-CD. In the single case of the *para* isomer of nitrophenol, the acid-base (phenol-phenolate) equilibrium was shifted toward the ionic form by addition of β-CD in the media [49]. The particular high stability (410 ± 40 dm^3 mol^{-1}) of the complex between *para*-nitrophenolate anion and β-CD could be explained by polarizability and the resonance delocalization in this anion which increased the electron density of the nucleus. This concept allowed to explain the difficulty of the β-CD catalyst to release the paraoxon leaving group.

4. Conclusion

Different β-CD derivatives, monosubstituted by IBA on a secondary hydroxy group, in position 2 of a glucose unit were prepared. The reactivity of hydroxy groups in position 2, 3, and 6 being different, it was possible to substitute mainly position 2 with sodium hydride as a base and bromobenzyl derivatives as nucleophile acceptors. But, when by-products were difficult to separate from the 2-substituted derivative, sodium hydroxide allowed a better regioselectivity. Their capacity to hydrolyze OP was tested with paraoxon. Several IBA-monosubstituted β-CDs displayed a hydrolytic activity against paraoxon. It was proved that catalytic activity was strongly dependent on the position of the linker between IBA and β-CD on the aromatic moiety. Compound **21** substituted on posi-

tion 5′ of the nucleus proved to be the most potent compound. The efficacy of the OP-hydrolyzing was limited due to poisoning of the catalyst by *para*-nitrophenol, the paraoxon-leaving group. This competitive inhibition is related to paraoxon use as model OP and should not be observed with OPs devoided of similar leaving group.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Kofler hot-plate melting point apparatus and were not corrected. IR spectra were obtained on a Shimadzu IR-408 spectrometer. Absorption bands are expressed in cm^{-1} and only noteworthy absorptions are listed. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DPX 300 spectrometer working at 300 MHz (^{1}H-NMR) and 75 MHz (^{13}C-NMR). Chemical shifts are reported in ppm downfield δ from tetramethylsilane. The electrospray ionization mass spectrometry (ESI-MS) experiments were performed on a MICROMASS Quattro II (altrincham, G.B.). Solutions were introduced through a Z-spray source. This one was continuously fed (5 μl min^{-1}) with a solution (1 mg ml^{-1}) of the studied compound which was diluted in a mixture of acetonitrile/water/formic acid (60:40:0.05; v/v/v). This solution was delivered by a syringe pump HARVARD APPARATUS type 22A. The temperature of the source was set at 80 °C. The electrospray probe (capillary) voltage was optimized in the range 2.5–4 kV for positive or negative ion electrospray. The sample cone was set within the range 40–160 V. Microanalyses are indicated only by symbols of the elements analyzed. The results obtained had a maximum deviation of 0.4% from the theoretical value. For iodoso derivatives **20**–**23** no correct analytical data were obtained, probably as a result of their highly hygroscopic nature and their complexation with solvents: their purity was established by TLC in two different systems and NMR spectroscopy. 2-Iodomethylbenzoic acids **4**–**7** [42,43,50], methyl 2-iodomethylbenzoate **8** [50] and **9** [38] and methyl 5-bromomethyl-2-iodobenzoate **13** [38] were synthesized as described in the literature.

5.1.1. Methyl 2-iodo-6-methylbenzoate (*10*)

A solution of 2.8 g (10.7 mmol) of 2-iodo-6-methylbenzoic acid (**6**) in 3 ml (41 mmol) of thionyl chloride was refluxed for 2 h. Twenty-five milliliters (617 mmol) of methanol were added and the mixture was refluxed for 3 additional h. After cooling, the reaction mixture was neutralized with aqueous $NaHCO_3$ solution, first extracted by ethyl acetate (50 ml), then extracted again by ethyl acetate (20 ml). The organic layers were collected, washed with a 20% aqueous solution of sodium thiosulfate, then with a 10% aqueous solution of sodium hydroxide (50 ml), and finally with distilled water (50 ml). The organic solution was separated and dried over sodium sulfate. The solvent was evaporated under reduced pressure and the residual oil was chromatographied on silica gel; solvent: cyclohexane/ethyl acetate (4:1; v/v). Compound **10** was obtained as a yellow oil (yield 70%); IR (NaCl): ν (cm^{-1}) 1740; ^{1}H-NMR ($CDCl_3$): δ 2.34 (s, 3H, CH_3), 3.96 (s, 3H, OCH_3), 6.99 (dd, 1H, J = 7.9 Hz, J = 7.4 Hz, H-4), 7.17 (d, 1H, J = 7.4 Hz, H-5), 7.64 (d, 1H, J = 7.9 Hz, H-3); ^{13}C-NMR ($CDCl_3$): δ 20.0 (CH_3), 52.5 (OCH_3), 91.8 (C-2), 129.6 (C-5), 130.6 (C-4), 136.3 (C-3), 136.5 (C-1), 140.1 (C-6), 169.4 (CO). Anal. ($C_9H_9O_2I$): C, H.

5.1.2. Methyl 2-iodo-4-methylbenzoate (*11*)

To a solution of 3 g (11.4 mmol) of 2-iodo-4-methylbenzoic acid (**7**) in 15 ml (370 mmol) of methanol, were added 1.5 ml of concentrated sulfuric acid. The mixture was refluxed for 5 h. After cooling, the reaction mixture was neutralized with aqueous $NaHCO_3$ solution, first extracted by ethyl acetate (50 ml), then extracted again by ethyl acetate (20 ml). The organic layers were collected, washed with a 20% aqueous solution of sodium thiosulfate, then with distilled water (50 ml). The organic solution was separated and dried over sodium sulfate. The solvent was evaporated under reduced pressure and the residual oil was chromatographied on silica gel; solvent: chloroform. Compound **11** was obtained as a yellow oil (yield 85.5%); IR (NaCl): ν (cm^{-1}) 1750; ^{1}H-NMR ($CDCl_3$): δ 2.25 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 7.11 (d, 1H, J = 7.9 Hz, H-5), 7.66 (d, 1H, J = 7.9 Hz, H-6), 7.76 (s, 1 H, H-3); ^{13}C-NMR ($CDCl_3$): δ 20.9 (CH_3), 52.6 (OCH_3), 94.7 (C-2), 129.0 (C-5), 131.3 (C-6), 131.9 (C-1), 142.3 (C-3), 143.9 (C-4), 167.0 (CO). Anal. ($C_9H_9O_2I$): C, H.

5.1.3. Methyl bromomethyl-2-iodobenzoate (*12, 14* and *15*)

Seven hundred thirty milligrams (41 mmol) of NBS and two drops of bromine were added to a solution of 1.2 g (4.35 mmol) of the corresponding methyl 2-iodomethylbenzoate (**8**–**11**) in 45 ml of carbon tetrachloride. Then 310 mg (1.3 mmol) of benzoyl peroxide were added to the medium. The mixture was refluxed for 24 h. After cooling and filtration, the solution was washed with 20% aqueous sodium thiosulfate (2 × 50 ml), then with brine (50 ml), and finally with distilled water (50 ml). The organic layer was dried over sodium sulfate. The solvent was evaporated under reduced pressure. The crude product was chromatographied on silica gel; solvent: cyclohexane/tetrahydrofuran (14:1; v/v).

Methyl 3-bromomethyl-2-iodobenzoate (**12**) was obtained as a yellow oil (yield 34.5%); IR (NaCl): ν (cm^{-1}) 1740; ^{1}H-NMR ($CDCl_3$): δ 3.94 (s, 3H, CH_3), 4.69 (s, 2H, CH_2), 7.34 (t, J = 7.5 Hz, H-5), 7.48 (dd, J = 7.5, 1.5 Hz, H-4), 7.57 (dd, J = 7.5, 1.5 Hz, H-6); ^{13}C-NMR ($CDCl_3$): δ 39.7 (CH_2), 52.8 (OCH_3), 99.3 (C-2), 129.7 (C-5), 130.6 (C-6), 132.5 (C-4), 139.3 (C-1), 142.1 (C-3), 168.2 (CO). Anal. ($C_9H_8O_2BrI$): C, H.

Methyl 6-bromomethyl-2-iodobenzoate (**14**) was obtained as a yellow solid (yield 45%); m.p. 67 °C; IR (nujol): ν (cm^{-1}) 1755; ^{1}H-NMR ($CDCl_3$): δ 3.99 (s, 3H, CH_3), 4.47 (s, 2H, CH_2), 7.09 (dd, 1H, J = 8.0, 7.5 Hz, H-4), 7.40 (d, 1H, J = 7.5 Hz, H-3), 7.78 (d, 1H, J = 8.0 Hz, H-5); ^{13}C-NMR ($CDCl_3$): δ 30.0 (CH_2); 52.9 (OCH_3); 93.0 (C-2); 130.0 (C-5);

131.3 (C-4); 136.8 (C-1); 139.5 (C-3); 140.1 (C-6); 175.4 (CO). Anal. ($C_9H_8O_2BrI$): C, H.

Methyl 4-bromomethyl-2-iodobenzoate (**15**) was obtained as a yellow solid (yield 36%); m.p. 98 °C; IR (nujol): ν (cm^{-1}) 1740; ^{1}H-NMR ($CDCl_3$): δ 3.93 (s, 3H, CH_3), 4.39 (s, 2H, CH_2), 7.42 (dd, 1H, J = 8.0, 1.0 Hz, H-5), 7.78 (d, 1H, J = 8.0 Hz, H-6), 8.01 (d, 1H, J = 1.0 Hz, H-3); ^{13}C-NMR ($CDCl_3$): δ 30.7 (CH_2), 52.7 (OCH_3), 94.4 (C-2), 128.6 (C-5), 131.3 (C-6), 134.7 (C-1), 141.7 (C-3), 142.6 (C-4), 166.4 (CO). Anal. ($C_9H_8O_2BrI$): C, H.

5.1.4. 2-Substitution of β-CD

To a solution of 1 g (0.88 mmol) of β-cyclodextrin (previously dried for 48 h under vacuum at 120 °C) in 40 ml of dry DMF, were added 35 mg (0.88 mmol) of sodium hydride (60% in mineral oil, in toluene and DMF). The solution was stirred under nitrogen overnight. A solution of 320 mg (0.90 mmol) of the corresponding methyl bromomethyl-2-iodobenzoate (**12**–**15**) in 5 ml of dry DMF was then added drop wise in the medium. The mixture was stirred for 9 additional h. Acetone (500 ml) was added and the crude product precipitated. After filtration, the solid residue was chromatographied on silica gel; solvent: ethyl acetate/isopropanol/water (12:7:4; v/v/v). Identification of by-products was realized by analysis of ^{13}C-NMR spectra of their mixture and their ratio was evaluated by comparison of the integrations of their respective aromatic signals on ^{1}H-NMR spectra. The following compounds were isolated.

2-O-(3-carboxymethyl-2-iodobenzyl)-β-cyclodextrin (**16**) (from **12**) was obtained as a yellow solid (yield 18%); IR (nujol): ν (cm^{-1}) 3400, 3100, 1740; ^{1}H-NMR (DMSO-d_6): δ 3.31–3.62 (m, 83H, 46H from H_2O), 3.85 (s, 3H, OCH_3), 4.50 (m, 7H, OH-6), 4.82 (bs, 7H, H-1), 6.02 (m, 13H, OH-2 and 3), 7.23–8.00 (m, 3H, ArH); ^{13}C-NMR (DMSO-d_6): δ 52.3 (OCH_3), 54.7 (OCH_2), 59.7 (C-6), 70.1 (C-3′), 71.8 (C-5), 72.1 (C-2), 72.9 (C-3), 79.0 (C-2′), 81.5 (C-4), 81.7 (C-4′), 93.1 (C-I), 99.8 (C-1′), 101.7 (C-1), 129.4, 132.1, 135.3, 138.3, 140.4 (5× C-Ar), 166.6 (CO); MS ES+: 1431 $(M + Na)^+$; MS ES–: 1407 $(M - H)^-$, 1443 $(M + Cl)^-$, 1445 $(M + Cl)^-$. Anal. ($C_{51}H_{77}O_{37}I$): C, H.

2-O-(2-carboxymethyl-3-iodobenzyl)-β-cyclodextrin (**18**) (from **14**) was obtained as a yellow solid (yield 16%); IR (nujol): ν (cm^{-1}) 3400, 3100, 1735; ^{1}H-NMR (DMSO-d_6): δ 3.31–3.45 (m, 67H, 30H from H_2O), 3.87 (s, 3H, OCH_3), 4.40 (m, 7H, OH-6), 4.64 (bs, 7H, H-1), 6.17 (m, 13H, OH-2 and 3), 7.22–7.85 (m, 3H, ArH); ^{13}C-NMR (DMSO-d_6): δ 48.5 (OCH_3), 52.8 (OCH_2), 60.0 (C-6), 69.0 (C-3′), 71.8 (C-5), 72.5 (C-2), 73.2 (C-3), 79.0 (C-2′), 81.6 (C-4), 82.0 (C-4′), 93.3 (C-I), 100.0 (C-1′), 102.0 (C-1), 128.8, 131.4, 136.7, 138.3, 138.8 (5× C-Ar), 167.3 (CO); MS ES+: 1431 $(M + Na)^+$; MS ES–: 1407 $(M - H)^-$, 1443 $(M + Cl)^-$, 1445 $(M + Cl)^-$. Anal. ($C_{51}H_{77}O_{37}I$): C, H.

2-O-(4-carboxymethyl-3-iodo)benzyl-β-cyclodextrin (**19**) (from **15**) was obtained as a yellow solid (yield 31%); IR (nujol): ν (cm^{-1}) 3450, 3150, 1730; ^{1}H-NMR (DMSO-d_6): δ 3.43–3.53 (m, 103H, 66H from H_2O), 3.80 (s, 3H, OCH_3), 4.54 (m, 7H, OH-6), 4.79 (bs, 7H, H-1), 5.95 (m, 13H, OH-2 and 3), 7.51 (d, 1H, J = 7.5 Hz, H-6Ar), 7,66 (d, 1H, J = 7.5 Hz, H-5Ar), 7.99 (s, 1H, H-2Ar); ^{13}C-NMR ($CDCl_3$): δ 52.6 (OCH_3), 55.1 (OCH_2), 59.9 (C-6), 71.7 (C-3′), 72.1 (C-5), 72.4 (C-2), 73.1 (C-3), 79.5 (C-2′), 81.6 (C-4), 81.9 (C-4′), 94.6 (C-I), 101.7 (C-1′), 101.9 (C-1), 127.4, 130.3, 134.8, 139.5, 143.3 (5× C-Ar), 167 (CO); MS ES+: 1431 $(M + Na)^+$; MS ES–: 1407 $(M - H)^-$, 1443 $(M + Cl)^-$, 1445 $(M + Cl)^-$. Anal. ($C_{51}H_{77}O_{37}I$): C, H.

Compound **17** (from **13**) could not be isolated from the mixture and another method was used.

*5.1.5. 2-O-(3-Carboxymethyl-4-iodo)benzyl-β-cyclodextrin (**17**)*

To a solution of 1 g (0.88 mmol) of β-cyclodextrin (previously dried for 48 h under vacuum at 120 °C) in 40 ml of dry DMSO, were added 88 mg (2.20 mmol) of sodium hydroxide. The solution was stirred under nitrogen for 48 h. A solution of 320 mg (0.90 mmol) of methyl 2-iodo-5-bromomethylbenzoate (**13**) in 5 ml of dry DMSO was then added drop wise to the media. The mixture was stirred for 24 additional h. Acetone (500 ml) was added and the crude product precipitated. After filtration, the solid residue was chromatographied on silica gel; solvent: ethyl acetate/isopropanol/water (12:7:4; v/v/v). Compound **17** was obtained as a yellow solid (yield 16%); IR (nujol): ν (cm^{-1}) 3400, 3100, 1760; ^{1}H-NMR (DMSO-d_6): δ 3.40–3.67 (m, 93H, 56H from H_2O), 3.80 (s, 3H, OCH_3), 4.51 (m, 7H, OH-6), 4.78 (bs, 7H, H-1), 6.17 (m, 13H, OH-2 and 3), 7.38 (dd, 1H, J = 8.0, 2.0 Hz, H-6Ar), 7.76 (d, 1H, J = 2.0 Hz, H-2Ar), 7.99 (d, 1H, J = 8.0 Hz, H-5Ar); ^{13}C-NMR (DMSO-d_6): δ 52.4 (OCH_3), 55.8 (OCH_2), 59.7 (C-6), 71.5 (C-3′), 71.8 (C-5), 72.2 (C-2), 72.9 (C-3), 79.7 (C-2′), 81.3 (C-4), 81.9 (C-4′), 93.2 (C-I), 99.8 (C-1′), 101.8 (C-1), 129.4, 132.2, 135.3, 138.3, 140.5 (5× C-Ar), 166.7 (CO); MS ES+: 1431 $(M + Na)^+$; MS ES–: 1407 $(M - H)^-$, 1443 $(M + Cl)^-$, 1445 $(M + Cl)^-$. Anal. ($C_{51}H_{77}O_{37}I$): C, H.

*5.1.6. 2-O-(Carboxy-iodosobenzyl)-β-cyclodextrin (**20**–**23**)*

Sixteen milliliters (204 mmol) of peracetic acid were added over a period of 1 h to a solution of 300 mg (0.20 mmol) of the corresponding 2-*O*-(carboxymethyl-iodobenzyl)-β-cyclodextrin (**16**–**18** or **19**) in 3 ml of DMF and 20 ml of glacial acetic acid. The solution was stirred at room temperature for 24 h. Acetone (500 ml) was added and the crude product precipitated. The solution was filtered, and the precipitate was washed with acetone (20 ml), then with diethyl ether (20 ml).

2-O-(3-carboxy-2-iodosobenzyl)-β-cyclodextrin (**20**) was obtained as a white solid (yield 78%); IR (nujol): ν (cm^{-1}) 3500, 3250, 1770; ^{1}H-NMR (DMSO-d_6): δ 3.35–3.85 (m, 85 H, 48H from H_2O), 4.49 (m, 7H, OH-6), 4.82 (bs, 7H, H-1), 5.73 (m, 13H, OH-2 and 3), 7.46–7.81 (m, 3H, H-Ar); ^{13}C-NMR (DMSO-d_6): δ 52.7 (OCH_2), 60.0 (C-6), 69.0 (C-3′), 72.1 (C-5), 72.5 (C-3), 73.4 (C-2), 73.1 (C-2′), 81.6 (C-4), 102.0 (C-1′), 102.6 (C-1), 119.3 (C-I), 128.3, 130.8, 131.8, 134.2, 138.9 (5× C-Ar), 168.5 (CO).

2-O-(3-carboxy-4-iodosobenzyl)-β-cyclodextrin (**21**) was obtained as a white solid (yield 61%); IR (nujol): ν (cm^{-1}) 3550, 3300, 1770; ^{1}H-NMR (DMSO-d_6): δ 3.34–3.85 (m, 85H, 48H from H_2O), 4.49 (m, 7H, OH-6), 4.82 (bs, 7H, H-1), 5.71 (m, 13H, OH-2 and 3), 7.83–8.04 (m, 3H, H-Ar); ^{13}C-NMR (DMSO-d_6): δ 58.2 (OCH_2), 60.0 (C-6), 71.6 (C-5), 73.4 (C-3), 73.5 (C-2), 80.8 (C-2′), 82.4 (C-4), 82.8 (C-4′), 99.2 (C-1′), 101.1 (C-1), 121.0 (C-I), 127.1, 131.2, 131.4, 140.0, 145.4 (5× C-Ar), 159.8 (CO).

2-O-(2-carboxy-3-iodosobenzyl)-β-cyclodextrin (**22**) was obtained as a white solid (yield 65%); IR (nujol): ν (cm^{-1}) 3500, 3200, 1770; ^{1}H-NMR (DMSO-d_6): δ 3.38–3.61 (m, 81H, 44H from H_2O), 4.51 (m, 7H, OH-6), 4.85 (bs, 7H, H-1), 5.77 (m, 13H, OH-2 and 3), 7.20–7.97 (m, 3H, H-Ar); ^{13}C-NMR (DMSO-d_6): δ 53.1 (OCH_2), 60.2 (C-6), 71.1 (C-3′), 72.4 (C-5), 72.8 (C-3), 73.4 (C-2), 81.8 (C-4), 100.3 (C-1′), 102.2 (C-1), 122.5 (C-I), 130.0, 131.7, 137.0, 138.6, 139.7 (5× C-Ar), 167.0 (CO).

2-O-(4-carboxy-3-iodosobenzyl)-β-cyclodextrin (**23**) was obtained as a white solid (yield 81%) IR (nujol): ν (cm^{-1}) 3400, 3100, 1730; ^{1}H-NMR (DMSO-d_6): δ 3.39–3.70 (m, 77H, 40H from H_2O), 4.57 (m, 7H, OH-6), 4.90 (bs, 7H, H-1), 5.75 (m, 13H, OH-2 and 3), 7.61–8.1 (m, 3H, H-Ar); ^{13}C-NMR (DMSO-d_6): δ 48.2 (OCH_2), 60.2 (C-6), 72.3 (C-5), 72.6 (C-3), 73.3 (C-2), 79.6 (C-2′), 80.3 (C-4), 82.8 (C-4′), 100.2 (C-1′), 102.2 (C-1), 121.0 (C-I), 127.1, 131.2, 131.4, 140.0, 145.4 (5× C-Ar), 168.0 (CO).

5.2. OP hydrolytic activity of β-CD derivatives

Paraoxon was the organophosphate model: its stock solution was 100 mM in anhydrous methanol. Stock solutions of β-CD derivatives (5 mM) were in a mixture of 10% v/v DMSO in 20 mM sodium phosphate buffer pH 7.65. Kinetic assays were carried out with paraoxon (3, 1, 0.5 and 0.1 mM) at 25 °C, in 20 mM phosphate buffer pH 7.65 in the presence of 13 mM cetyltrimethylammonium chloride (CTAC) [34]. The final concentration of methanol in assays was 3% v/v and that of DMSO was 2% v/v. The final concentration of β-CD derivatives in assays was 1 mM. Hydrolysis of paraoxon was monitored up to 120 min by following the release of the leaving group *para*-nitrophenol (λ = 400 nm; double beam spectrophotometer). The control cuvette contained all assay components except the β-CD derivative.

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