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Generation of Polyclonal Catalytic Antibodies Against Cocaine Using Transition State Analogs of Cocaine Conjugated to Diphtheria Toxoid

Garo P. BASMADJIAN,*[,a] Satendra SINGH,[a] Budiono SASTRODJOJO,[a] Blaine T. SMITH,[a] Kwasi S. AVOR,[a] Fengchun CHANG,[a] Stanley L. MILLS,[a] and Thomas W. SEALE[b]

Department of Medicinal Chemistry and Pharmaceutics, College of Pharmacy[a] and Department of Pediatrics, College of Medicine,[b] University of Oklahoma Health Sciences Center, Oklahoma City, OK 73117, U.S.A.

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Six novel transition state analogs (TSAs) of cocaine (10—14 and 17) and one non-cocaine, *p*-aminophenylphosphonyl ester of cyclohexanol (19), were synthesized and characterized by ^{1}H- and ^{13}C-NMR and FAB-MS. (1*R*)-ecgonine methyl ester or cyclohexanol were subjected to phenylphosphonylation in the presence of dicyclohexyl carbodiimde (DCC) and 4-*N*,*N*-dimethyl aminopyridine (4-DMAP). TSA-IV (10), however, was synthesized from norcocaine which was protected with dibromoethane to yield 4 before acid hydrolysis, esterification and phenylphosphonylation were carried out. TSA-III (11) TSA-I (12) and (19), using various length spacer arms, were coupled with the immunogenic protein, diphtheria toxoid (DT). The TSAs coupled with DT were used to immunize mice and after appropriate boosts their sera were tested for the presence and titer of anti-TSA polyclonal antibodies using ELISA. Preliminary results show that the mice immunized with these TSAs produced high titers of polyclonal catalytic antibodies, except for (19), with the ability to hydrolyze the substrate ^{125}I-4′-iodococaine in an *in vitro* assay, even in the presence of noncatalytic anti-TSA antibodies.

Key words cocaine transition state analog; anti-cocaine catalytic antibody; polyclonal antibody; immunization; esterase activity

In the late nineteenth century, Sigmund Freud was one of the first investigators who studied the effects of cocaine and proposed its use in the treatment of alcohol and opiate abuse. Interestingly, for reasons beyond comprehension, it was then thought to be safe and nonaddictive. Even though this error was soon realized cocaine has continued to be abused. Currently, the United States is in the largest cocaine epidemic in its history, and cocaine has been proclaimed the drug of greatest national health threat.[1-3]

Cocaine has many physiological effects. It is a local anesthetic and vasoconstrictant. The central nervous system effects include the feelings of well being and euphoria which occur without hampering motor activity.[4] The reinforcing properties, *i.e.*, increase in the probability of repeated abuse of cocaine are believed to be associated with dopaminergic neurotransmission.[5,6] According to the dopamine hypothesis, cocaine binds to the dopamine transporter site in a way which inhibits dopamine transport. The building-up of dopamine in the synaptic cleft significantly potentiates dopaminergic transmission which presumably is responsible for the reinforcing properties of cocaine.[7] Therefore, a compound capable of competing with cocaine, as a partial agonist or antagonist, for its binding site(s) at the dopamine transporter site has potential clinical applications. At the present time no compound capable of blocking cocaine binding without blocking dopamine uptake, *i.e.*, an antagonist, is known to exist.

An alternative approach which originated in 1986[8,9] and has received wide attention today[10,11] is to develop catalytic antibodies with the ability to hydrolyze esters.[12,13] Cocaine is a diester. Hydrolysis of either ester, produces hydrophilic metabolites that are inactive. Cocaine and crack when sniffed, smoked, swallowed or applied to mucus membrane is absorbed from all sites of exposure. Cocaine is hydrolyzed by serum esterases in 20—60 min and partially metabolized in the liver. Hydrolysis of the methyl ester produces benzoylecgonine a common inactive metabolite of cocaine detected in the blood and the urine and hydrolysis of the benzoyl ester produces ecgonine methyl ester and benzoic acid. All three metabolites do not possess cocaine's stimulant or reinforcing properties[14] and also do not cross the blood brain barrier. Antibodies with esterase activity approaching that of neutral enzymes have been reported.[10,15] Since the hydrolysis of the benzoyl ester occurs *via* a tetrahedral intermediate (Chart 1), it can be mimicked by the phosphonate moiety because it can stably impart oxyanionic and tetrahedral features to the transition state.[8,16] Recently, some transition state analogs of cocaine have been reported and shown to generate catalytic antibodies.[17,18] Landry *et al.*[17] showed that immuniza-

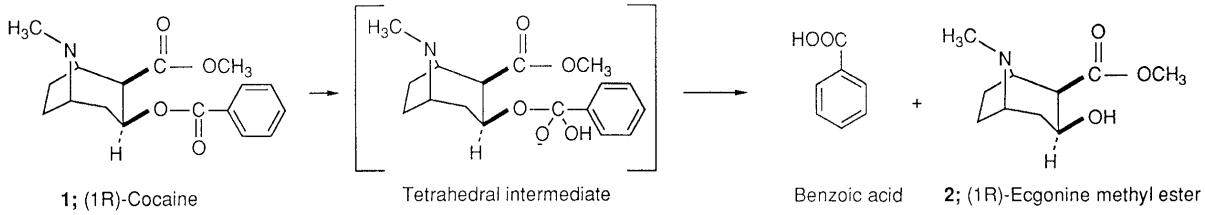


Chart 1. Hydrolysis of the Benzoyl Ester of Cocaine

* To whom correspondence should be addressed.

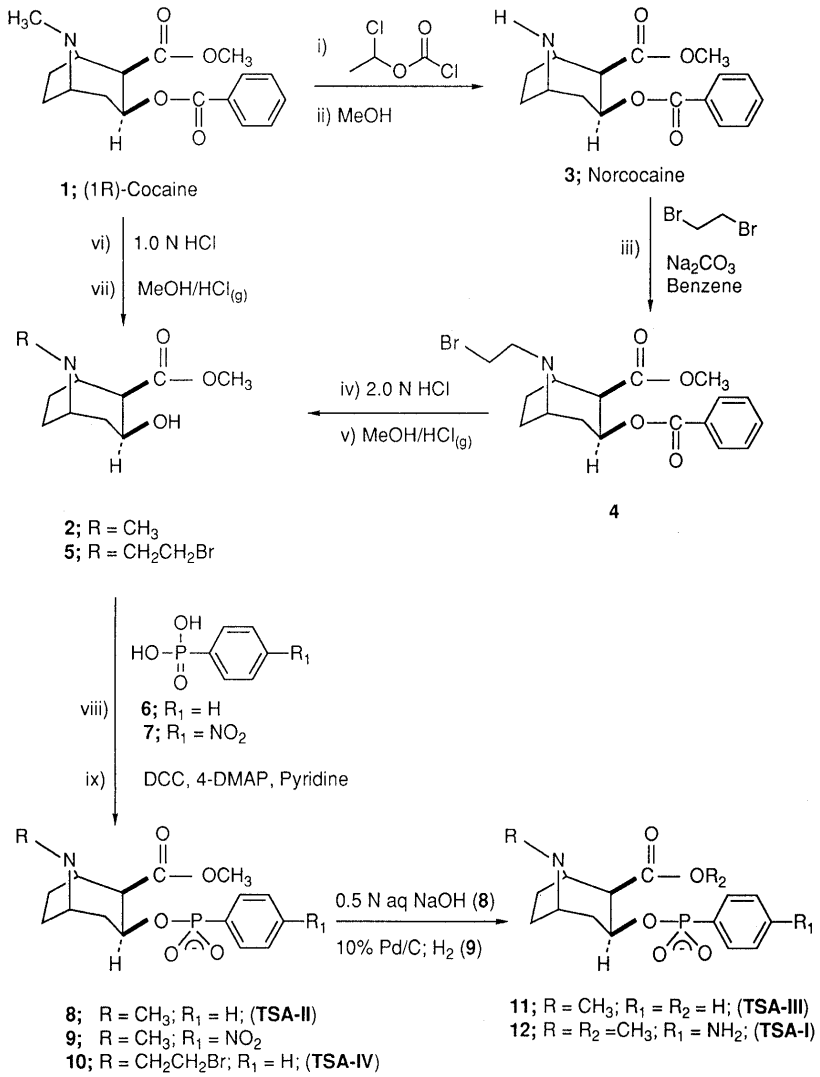


Chart 2

tion of mice with a phosphonate monoester transition state analog (TSA) of cocaine, TSA-III (Chart 2) conjugated to a protein, provided monoclonal antibodies capable of catalyzing the hydrolysis of the cocaine benzoyl ester group. He advocated that passive immunization with an anti-cocaine catalytic antibody could help in curbing cocaine abuse. Chandrakumar *et al.*[18] generated monoclonal antibodies that recognize the hapten TSA-II (Chart 2) when mice were immunized with the same hapten conjugated to a protein through the -N of the tropane ring with a 4C spacer arm, but no catalytic properties were described.

In this publication we report the synthesis of new transition state analogs of cocaine including a neutral transition state analog with the phosphonyl group protected as an ethyl ester and a non-cocaine benzoyl ester hydrolysis phosphonate TSA to test if anti-sera generated against this TSA would have anti-cocaine hydrolytic activity. Preliminary biological evaluation of five TSA protein-conjugates for the generation of polyclonal catalytic and non-catalytic antibodies are presented. Chart 3 shows the structures of the transition state analogs of cocaine conjugated to the immunogenic protein, diphtheria toxoid. Diphtheria toxoid (DT) was chosen because

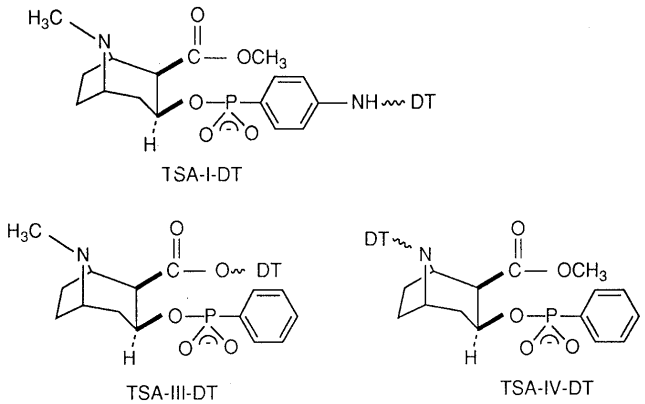


Chart 3. Structures of the Immunogenic Haptens Singly or Collectively Comprising of the Vaccine

most humans above the age of fifteen months should have memory B and T lymphocytes against this toxoid due to childhood diphtheria/pertussis/tetanus (DPT) immunizations. Our approach will involve the use of the conjugates, singly or in a mixture, in a vaccine formulation, for pseudo-active immunization to produce a host of circulating antibodies, some non-catalytic and some catalytic to better rid the system of any circulating cocaine molecules. Previous studies have succeeded in generating

polyclonal catalytic antibodies from either transition state analog haptens conjugated to immunogens,[19-21] or from anti-idiotypes or internal images of enzymes.[22,23]

In vivo immunization of humans with either the individual or a mixture of the three immunogenic haptens (Chart 3), in a vaccine formulation, could produce catalytic antibodies that will stay in circulation and memory B and T lymphocytes capable of rapidly producing them upon future vaccine exposure. These antibodies when challenged with cocaine will bind it, hydrolyze it, relase the inactive metabolites and proceed to immediately attack other cocaine molecules. If enough catalytic antibodies are circulating, the rate of hydrolysis of cocaine will be such that the half-life of cocaine in the blood will be significantly shortened.

Chemistry

The synthesis of TSAs started with (1*R*)-cocaine (**1**). (1*R*)-cocaine hydrochloride was exhaustively hydrolyzed in the presence of 1.0 N aqueous HCl followed by esterification at C-2 position in methanol saturated with dry HCl gas. The overall yield from (1*R*)-cocaine hydrochloride to (1*R*)-ecgonine methyl ester was 85%. The (1*R*)-ecgonine methyl ester (**2**) thus obtained served as the starting material for the synthesis of **11**, **12** and **17**. The crucial phenylphosphonylation of **2** was achieved by using phenyl phosphonic acid and dicyclohexyl carbodiimide (DCC) in pyridine in a 65—72% yield. 4-*N*,*N*-Dimethyl aminopyridine (4-DMAP) was used as an acylation catalyst.[24] Thus, the reaction of *p*-nitrophenyl phosphonic acid prepared according to the reported procedure[25] with **2** yielded **9** in 62% yield which after hydrogenation in methanol using 10% Pd/C (10% by weight of the substrate) furnished TSA-I (**12**) in 98% yield (Chart 2). Although TSA-I (**12**) can be directly conjugated to diphtheria toxoid, TSA-Ic (**13**) TSA-Id (**14**) were prepared for future testing to study the effects of five or two carbon atom spacer arms on the production of catalytic antibodies, respectively. These were synthesized from TSA-I (**12**) when treated with glutaric anhydride or α-bromoacetyl bromide in the appropriate solvent in the presence of triethyl amine (Chart 4). TSA-III (**11**) was conveniently prepared by hydrolysis of TSA-II (**8**) in equimolar aqueous sodium hydroxide solution (final concentration 0.5 N). Attempts to synthesize TSA-IV (**10**) from TSA-II (**8**) by N-demethylation with α-chloroethyl chloroformate and subsequent reaction with 1,2-dibromethane were not successful possibly due to electrophilicity of the tertiary amine rather than the nucleophilicity because of zwitterion formation. Thus, a different approach was adopted. (1*R*)-Cocaine was first converted into norcocaine (**3**) using a known procedure[26] which after reaction with dibromoethane in benzene/sodium carbonate was hydrolyzed exhaustively to yield N-bromoethyl norecgonine. The N-bromoethyl group here served two purposes: one, it acted as a protective group for the amine and two, it could directly be used for the conjugation with the immunogenic protein with its two atom spacer arm (Chart 2). The phenylphosphonylation of N-bromoethyl norecgonine methyl ester (**5**) with **6** gave TSA-IV (**10**) in 54% yield. TSA-IIb (**17**) was obtained by hydrolysis of TSA-IIa (**16**) in water which in turn was

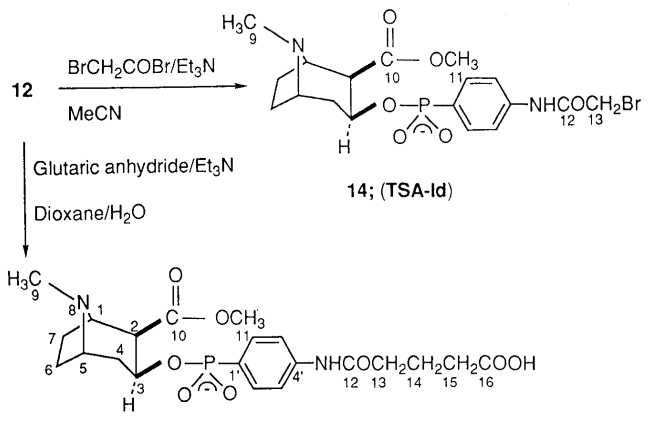


Chart 4

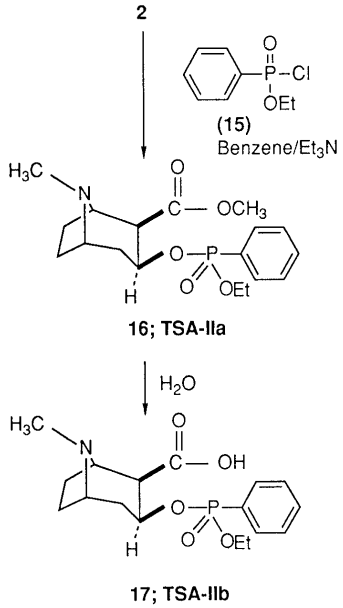


Chart 5

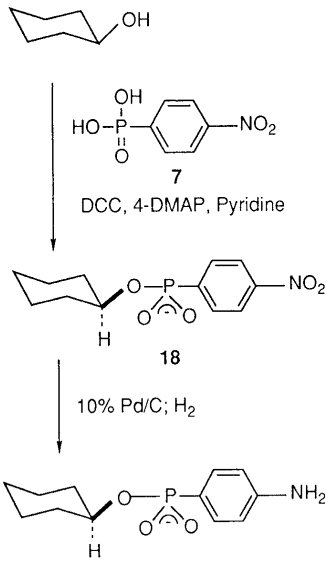


Chart 6

prepared from the reaction of ecgonine methyl ester (**2**) with phenyl phosphonic chloride ethyl ester (**15**) in the presence of triethyl amine (Chart 5). A non-tropane alcohol, cyclohexanol, was esterified with *p*-nitrophenyl phosphonic acid as above to produce (**18**) which upon hydrogenation yielded (**19**) (Chart 6).

Biological Results and Discussion

In our preliminary biological studies we immunized outbred mice (CF/1) using protocol 1, and inbred mice (C57BL/6J) using protocol 2 with TSA-I (**12**) and TSA-III (**11**) coupled to DT, respectively. The 4′-amino group of TSA-I (**12**) was used to conjugate to diphtheria toxoid using two methods: 1) diazotization and 2) glutaraldehyde aided conjugation. In addition, TSA-Ic (**13**), with a 5C spacer arm and TSA-III (**11**) were coupled with diphtheria toxoid after the activation of their free–COOH group with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The non-tropane TSA (**19**) was conjugated by diazotization to DT. The mice sera, at different time periods during the immunization protocols, were tested for the presence and titer of anti-TSA antibodies using an ELISA and the presence of polyclonal catalytic anti-cocaine antibodies using an enzyme assay (Figs. 1 and 2).

Immunization protocol 2 gave higher ELISA titers of anti-TSA antibodies in a shorter time period (data not shown). In addition, the anti-TSA antibodies present in the sera of mice immunized against DT-**20**, DT-**21**, DT-**13** and DT-**19** were tested for inhibition in an ELISA by TSA-I (**12**), TSA-II (**8**), TSA-III (**11**), cocaine (**1**), the metabolites of cocaine *e.g.* ecgonine methyl ester (EME), benzoic acid, atropine (another tropane alkaloid) and glycine, a negative control. The data is presented in Figs. 3A, 3B, and 3C.

The results show the specific affinity of the antibodies to the transition states of cocaine, ranging from 0—100% inhibition, rather than cocaine itself, ranging from 0—41%, and no affinity to the metabolites of cocaine, or atropine, a tropane alkaloid with a 3α-substituent at C-3 and glycine a negative control. The highest inhibitions were shown by the TSAs towards anti-DT-**20** (Fig. 3B). Some inhibition was seen at the highest concentration (0.28 mM) by cocaine towards all three anti-DT-TSAs tested and up to 41% against anti-DT-**20** at 0.48 mM. Atropine did not show any inhibition of the antibodies. No inhibition was detected by benzoic acid, EME showed up to 60% inhibition with anti-DT-**13** and 40% with anti-DT-**20**. No inhibition was detected by TSAs or the other inhibitors used above against sera containing anti-DT-**19** except with **19** of 32% and 43% at 0.048 and 0.48 mM concentrations.

The sera obtained from mice immunized with protocol 2 was shown to contain anticocaine catalytic antibodies with measurable enzymatic activity even in the presence of noncatalytic anti-TSA antibodies present in the polyclonal mixture (Fig. 2). There was no statistically significant increase in hydrolytic activity in the sera containing the different antibodies and also from sera obtained from bleed 1 to 3. No catalytic activity was detected in sera containing anti-DT-**19**, showing that the

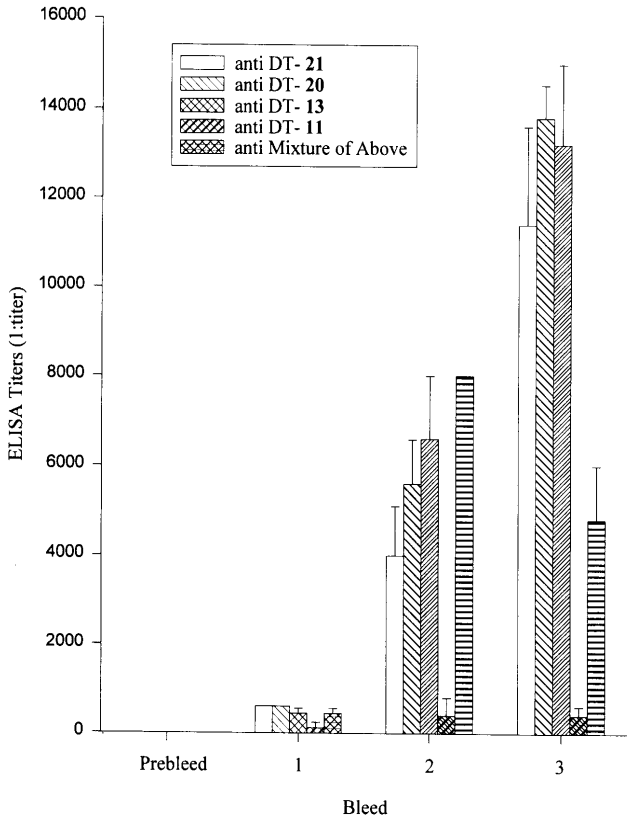


Fig. 1. ELISA Titers of the Various Antibodies Present in the Immunized Sera of C57BL/6J Mice at Bleed Times 10 d Post Boosting

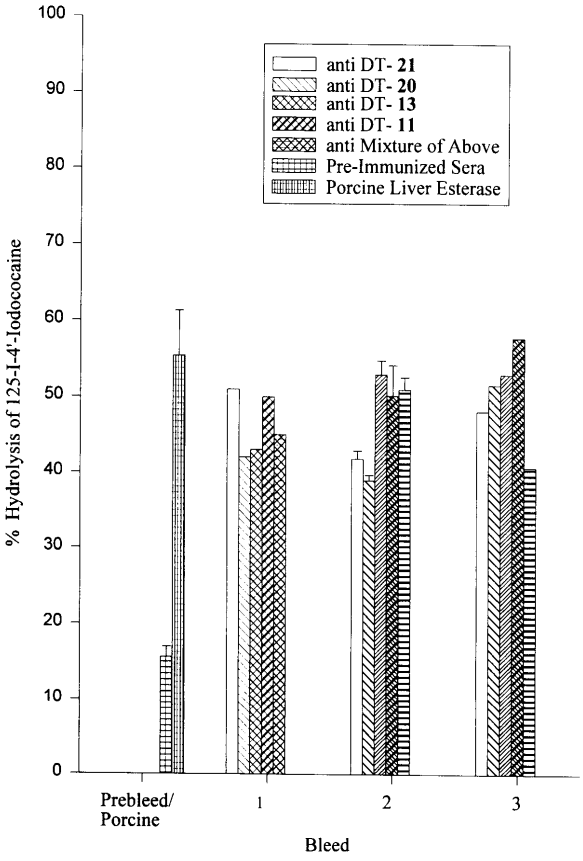
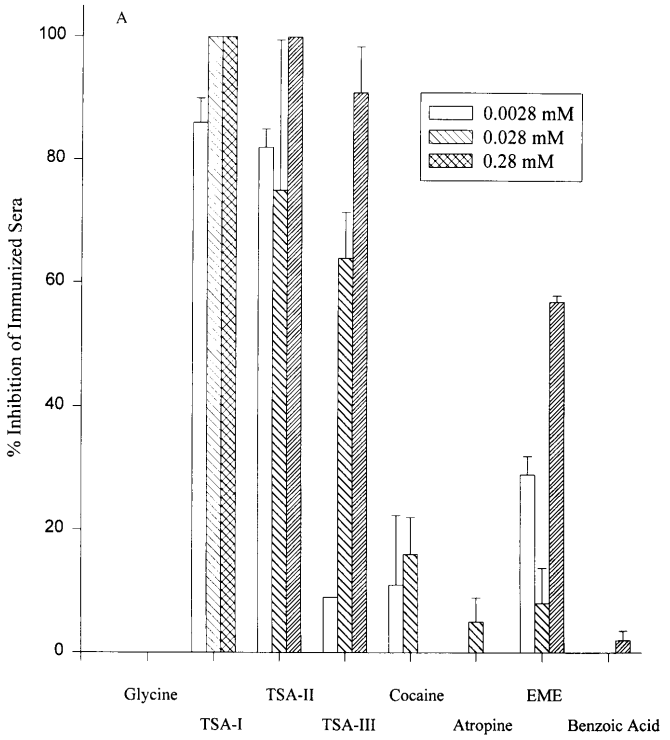
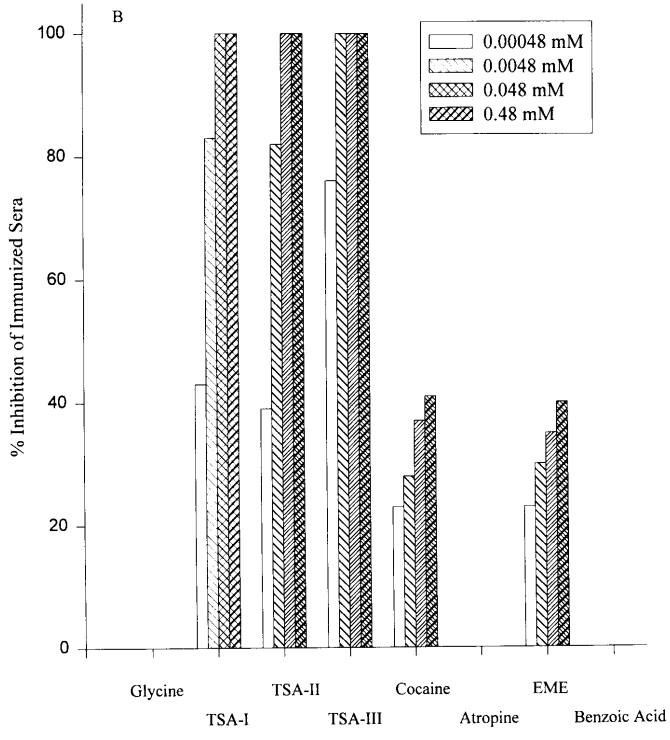


Fig. 2. Catalytic Activity Present in the Sera of Immunized C57BL/6J Mice Compared to Preimmunized Sera and Porcine Liver Esterase





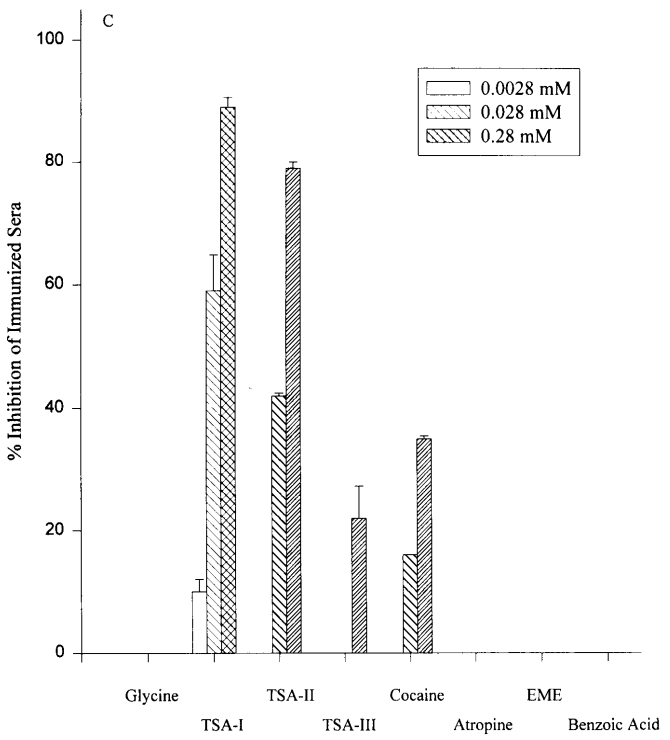


Fig. 3. Inhibitions of Anti-DT-**13** (A), Anti-DT-**20** (B) and Anti-DT-**21** (C) in Immunized Mice Sera with the Listed Inhibitors

(A) 1 : 10000 dilution of immunized C57BL/6J mice sera (third boost) was used in the ELISA. (B) 1 : 20000 dilution of immunized C57BL/6J mice sera (third boost) was used in the ELISA. (C) 1 : 30000 dilution of immunized C57BL/6J mice sera (third boost) was used in the ELISA.

The serum was preincubated with the inhibitors at the different concentration levels overnight at 4 °C and used in the ELISA. Each result represents a mean of 3 experiments.

alcohol portion of the TSA should be specific *i.e.* a tropane ring as present in cocaine.

The hydrolysis of ^{125}I-4′-iodococaine with pooled anti-sera from the C57BL/6J mice (bleed 3) immunized with DT-**20**, DT-**21**, DT-**13** and DT-**11** was inhibited by 37, 34, 54 and 54% respectively with a 0.28 mM TSA-II, corrected with inhibition observed in the preimmunized sera. Porcine liver esterase was inhibited by 45% corrected with inhibition observed in the incubation buffer. Although the inhibition by TSA-II of the hydrolysis of the radioactive substrate is lower than expected, this could be explained as below due to the presence of noncatalytic antibodies that are artificially depleting the amount of the inhibitor. The inhibition of the porcine liver esterase by TSA-II could be explained by the fact that TSA-II could be considered a nondescript phosphonate ester inhibitor of this type of enzyme.

No correlation was seen between ELISA titers (Fig. 1) and catalytic activity of the immunized sera (Fig. 2). The explanation of this observations is possibly due to the increase of non-catalytic anti-DT-TSA antibodies with each subsequent boost and the inhibitory effect of these non-catalytic antibodies toward the hydrolytic activity of the catalytic antibodies present in the mixture. We may be able to prove this theory if we are able to separate the catalytic from the non-catalytic antibodies present in the polyclonal mixtures using affinity chromatography techniques.

Conclusions

We have synthesized and completely characterized several TSAs of cocaine. We also ascertained the important fact that the TSAs retained the same stereochemistry at C-2 and C-3 as is present in the cocaine molecule. This is extremely important because the catalytic antibodies would then recognize the ground state substrate (−)-cocaine correctly.

These TSAs were designed to be conjugated to DT to expose the hapten in difierent conffgurations to the animal

immune system. We have successfully conjugated TSA-I, TSA-Ic and TSA-III to DT. TSA-I was conjugated using two different methods: by diazotization of the *p*-amino group on TSA-I and by glutaraldehyde spacer arm addition. Both methods gave us diphtheria conjugates that were analyzed by size exclusion HPLC and shown to be a single protein component with no free hapten. TSA-Ic and TSA-III were conjugated to DT using a carbodiimide technique. A non-tropane TSA (**19**) was also conjugated and tested.

We have presently immunized over 160 CF-1 (outbred) and C57BL/6J (inbred) mice. Two immunization protocols have been evaluated in search for the optimum one. The immunization protocol using Freund's Complete Adjuvant has been chosen to immunize mice with the other DT-TSA conjugates. The ELISA inhibition studies have clearly shown the production of anti-TSA antibodies with cross reactivity to the various TSAs of cocaine, less so to cocaine and some recognition of its metabolite, EME. Anti-cocaine catalytic antibodies have been shown to be present in the sera of all the immunized mice using either immunization protocol. This was determined using a simple, reproducible enzyme assay with a novel gamma-emitting labeled cocaine analog, ^{125}I-4′-iodococaine, as substrate.

Experimental

Methanol, pyridine, acetonitrile and ethyl acetate were dried over 4A type molecular sieves. Benzene and dioxane were dried over 5A type molecular sieves. Triethyl amine was distilled from lithium aluminum hydride (LAH). Impure cocaine hydrochloride was obtained from National Institute on Drug Abuse (NIDA). Unless otherwise stated all the starting materials were obtained from Aldrich (Milwaukee, WI) and were used without further purification. Glutaraldehyde, Freund's Complete Adjuvant (FCA), bovine serum albumin (BSA) and goat antimouse immunoglobulin (IgG) alkaline phosphatase were obtained from Sigma (St. Louis, MO). Diphtheria toxoid was obtained from Connaught Laboratories, Inc. (Swiftwater, PA). Sephadex G-25 was obtained from Pharmacia (Piscataway, NJ) and EDC was obtained from Bio-Rad (Hercules, CA). The outbred and inbred mice were obtained from Sasco Inc. (Omaha, NE) and Jackson Lab (Bar Harbor, ME), respectively.

The melting points (mp) were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Elemental analysis was performed by Midwest Microlab Ltd. Indianapolis, IN. The low and high resolution Fast atom bombardment mass spectrometry (FAB-MS) was carried out with a VG Instruments (Manchester, UK). ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-300 spectrometer. The chemical shifts are reported in parts per million (δ) with reference to TMS as internal standard; the multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). The assignment of the resonances is based on the selective decoupling experiments and by comparison.[27] Protein concentrations were measured using a Shimadzu, model UV160U, double beam spectrophotometer. Chromatographic separations were carried out with 230—400 mesh silica (Silica-60-Merck). The radioactivity was assayed with a Beckman 9000 gamma counter (Irvine, CA). HPLC was performed using a Beckman Gold System.

3-[(Benzoyl)oxy]-[1*R*-(*exo*,*exo*)]-8-bromoethyl-8-azabicyclo[3.2.1]-octane-2-carboxylic Acid Methyl Ester (4) To a solution of norcocaine[26] (**3**; 6.7 g, 23 mmol) in 100 ml of dry benzene were added 1,2-dibromoethane (26.1 g, 139 mmol) and sodium carbonate (14.7 g, 139 mmol). The resulting mixture was refluxed for 24 h, cooled and water added to dissolve the Na_2CO_3. The basic aqueous layer was separated and extracted with ether (3 × 50 ml). The combined organic extracts were dried over anhyd. $MgSO_4$ and concentrated to an oil. The oil was triturated with petroleum ether to obtain a yellowish solid which was recrystallized from petroleum ether–ether solvents to afford 6.8 g (74.5%) crystalline solid, mp 150—151 °C. ^{1}H-NMR ($CDCl_3$) δ: 8.02 (dd, J=1.5, 6.9 Hz, 2H, C2′,6′-H), 7.56—7.51 (m, 1H, C4′-H), 7.44—7.39 (m, 2H, C3′,5′-H), 5.28—5.21 (m, 1H, C3-H), 3.75—3.70 (m, 1H, C1-H), 3.70 (s, 3H, OCH_3), 3.40—3.34 (m, 1H, C2-H), 3.04—3.00 (m, 1H, C5-H), 2.40 (t, J=6.6 Hz, 2H, CH_2Br), 2.28—2.24 (m, 2H, C4-H_2), 2.07—2.04 (m, 2H, C7-H_2), 1.90—1.83 (m, 2H, C6-H_2), 1.73 (t, J=6.6 Hz, 2H, NCH_2). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 316 (M−Br, 35.95), 302 (M−CH_2Br, 74.9).

3-Hydroxy-[1*R*-(*exo*,*exo*)]-8-bromoethyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (5) Compound **4** (3.0 g, 7.6 mmol) as obtained above was dissolved in 20 ml of 1.0 N HCl and refluxed for 15 h. It was then cooled to room temperature to precipitate out benzoic acid which was filtered off and the filgrate was extracted with ether (3 × 30 ml) to remove traces of benzoic acid. The aqueous extract was evaporated to dryness and after removal of the traces of water by azeotrope with benzene a fluffy solid (2.3 g, 95.8%) was obtained as the hydrochloride salt, mp 230 °C (dec.). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 198 (M−Br, 14.5), 184 (M−CH_2Br, 10.1).

A solution of the above compound (2.2 g, 70 mmol) in 50 ml of dry MeOH was saturated with HCl gas and the solution stirred at room temperature for 16 h under anhydrous conditions. The solvent was removed and the residue dissolved in 50 ml of water and 10 ml of NH_4OH solution, extracted with $CHCl_3$ (4 × 25 ml). The pooled organic extracts were dried over anhyd. $MgSO_4$ and concentrated to a sticky solid which was purified by flash chromatography on silica gel (100 g, 90% EtOAc–MeOH) to afford 1.3 g (61.9%) of a white solid as free base, mp 155—156 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.88—3.74 (m, 1H, C3-H), 3.71 (s, 3H, OCH_3), 3.67—3.66 (m, 1H, C1-H), 3.21—3.18 (m, 1H, C2-H), 2.76—2.73 (m, 1H, C5-H), 2.32—2.19 (m, 2H, CH_2Br), 1.96—1.81 (m, 4H, C4,7-H_2), 1.70—1.50 (m, 4H, C6-H_2 and NCH_2). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 212 (M−Br, 53.7), 198 (M−CH_2Br, 74.0).

3-[(Phenylphosphonyl)oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo-[3.2.1]octane-2-carboxylic Acid Methyl Ester (TSA-II; 8) (1*R*)-Ecgonine methyl ester (**2**; 5.73 g, 28.8 mmol) free base prepared and purified from acid hydrolysis of (1*R*)-cocaine hydrochloride[28] followed by esterification with methanol in the presence of HCl gas, was dissolved in 45 ml of dry pyridine and 5.25 g (32.9 mmol) phenyl phosphonic acid was added in one lot with stirring. To this clear solution was added 4-DMAP (180 mg, 1.47 mmol) and DCC (1165 g, 56.5 mmol) all at once and stirred at room temperature overnight (16 h). After 10 min of the reaction a white precipitate of dicyclohexyl urea (DCU) started forming indicating the start of the reaction. The progress of the reaction was monitored by TLC (EtOAc–MeOH–NH_4OH, 80 : 3 : 3; I_2). The reaction was stopped and 10 ml of MeCN was added to precipitate out DCC which was filtered off and washed with MeCN (3 × 20 ml). The solvent was evaporated on a Rotavap under reduced pressure to yield 13.0 g of a crude yellow oil. It was then purified by chromatography on a silica gel column (70.0 g; 20% MeOH/EtOAc through 100% MeOH) to afford 7.1 g (72.7%) of a pure white solid, mp 83—85 °C. ^{1}H-NMR (D_2O) δ: 7.58, 7.54 (d, J=8.1 Hz, 1H and d, J=8.1 Hz, 1H, C5′,3′-H), 7.45—7.32 (m, 3H, C2′,4′,6′-H), 4.45 (dt, J=3.9, 10.8 Hz, 1H, C3-H), 3.92 (d, J=6.0 Hz, 1H, C1-H), 3.76 (m, 1H, C5-H), 3.60 (s, 3H, OCH_3), 3.02 (dd, J=1.5, 6.6 Hz, 1H, C2-H), 2.62 (s, 3H, NCH_3), 2.23—1.91 (m, 4H, C4, 7-H_2), 1.88—1.75 (m, 2H, C6-H_2). ^{13}C-NMR (D_2O) δ: 176.58 (C=O), 134.22 (C-4′), 134.18 (C-1′), 133.75 (C-2′), 133.62 (C-6′), 131.31 (C-3′), 131.13 (C-5′), 66.62 (C-3), 66.40 (C-1), 65.92 (C-5), 55.78 (OCH_3), 50.90 (C-2), 41.31 (NCH_3), 37.53 (C-4), 26.30 (C-7), 25.20 (C-6). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 340 (M+H$^+$, 99.3), 182 (100).[29] *Anal.* Calcd for $C_{16}H_{22}NO_5P \cdot H_2O$: C, 53.78; H, 6.72; N, 3.92; P, 8.68. Found: C, 53.59; H, 6.75; N, 3.92; P, 8.50.

3-[(Phenylphosphonyl)oxy]-[1*R*-(*exo*,*exo*)]-8-bromoethyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (TSA-IV; 10) Phenyl phosphonic acid (0.40 g, 3.0 mmol) was dissolved in 3 ml of absolute pyridine and 4-DMAP (0.02 g, 0.2 mmol) was added. To this clear solution was added a solution of **5** (0.30 g, 1 mmol) in 10 ml of absolute pyridine and followed immediately with solid DCC (2.40 g, 12 mmol). The resulting reaction mixture was stirred at room temperature overnight (18 h) and MeCN (20 ml) was added to precipitate out DCU which was filtered off and washed with MeCN. The filtrate was evaporated *in vacuo* to yield an oil which was purified by flash chromatography on a silica gel column (100 g, 20% MeOH–EtOAc through 100% MeOH) to obtain 0.30 g (65%) white solid, mp 205 °C (dec.). ^{1}H-NMR (D_2O) δ: 7.58, 7.53 (dd, J=1.5, 8.1 Hz, 1H and dd, J=1.8, 8.1 Hz, 1H, C2′,6′-H), 7.42—7.34 (m, 3H, C3′,4′,5′-H), 4.66—4.62 (m, 1H, C3-H), 4.07—4.05 (m, 1H, C1-H), 3.94—3.92 (m, 1H, C5-H),

3.60 (s, 3H, OCH_3), 3.32 (t, J=9.0 Hz, 2H, CH_2Br), 3.09—3.06 (m, 1H, C2-H), 2.11—1.98 (overlapping m, 6H, C4,6,7-H_2), 1.89 (t, J=9.0 Hz, 2H, NCH_2). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 352 (M−Br, 17.2), 338 (M−CH_2Br, 33.7). *Anal.* Calcd for $C_{17}H_{23}BrNO_5P \cdot 1/11CHCl_3$: C, 46.42; H, 5.23; Br, 28.38; N, 3.17; P, 7.02. Found: C, 46.54; H, 5.72; Br, 28.73; N, 3.45; P, 7.10.

3-[(4-Aminophenylphosphonyl)oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (TSA-I; 12) (1*R*)-Ecgonine methyl ester (**2**; 4.59 g, 23.06 mmol) free base was dissolved in 40 ml dry pyridine and *p*-nitrophenyl phosphonic acid[25] (4.87 g, 23.99 mmol) was added in one lot and stirred vigorously at room temperature. 4-DMAP (140 mg, 1.15 mmol) was added as a catalyst.[30] To this clear pale yellow solution under stirring was added DCC (9.47 g, 45.9 mmol) all at once at room temperature. Within 15 min of reaction a thick slurry resulted indicating the precipitation of DCU. After 4 h when the reaction was complete as monitored by TLC (EtOAc–MeOH–NH_4OH, 80:3:3; I_2), 10 ml acetonitrile was added to complete the precipitation of DCU which was filtered off and washed with acetonitrile (3×20 ml). The filtrate was concentrated on a Rotavap to furnish 10 g of a thick yellow oil which on trituration gave a tan powder. This was repeatedly washed with ether and then with ethyl acetate to remove traces of 4-DMAP and any unreacted DCC to obtain intermediate **9** (9.8 g) which was dissolved in 150 ml of dry MeOH and stirred with 400 mg of 10% Pd/C under hydrogen atmosphere for 24 h. The progress of reaction was monitored by TLC (EtOAc–MeOH, 1:1; 4-*N*,*N*-dimethyl aminobenzaldehyde as visualization reagent). TSA-1 (**12**) gave a bright yellow spot while the intermediate did not show any coloration when the TLC was sprayed with the visualization reagent. The catalyst was filtered off, solvent evaporated under vacuum and purified by column chromatography (SiO_2; 60 g; 20% MeOH/EtOAc through 100% MeOH to afford 4.3 g (53% yield based on EME) light yellow solid, mp 124—126 °C. ^{1}H-NMR (D_2O) δ: 7.30, 7.29 (d, J=8.4 Hz, 1H and d, J=8.4 Hz, 1H, C2′,6′-H), 6.66, 6.65 (d, J=8.4 Hz, 1H and d, J=8.4 Hz, 1H, C3′,5′-H), 4.42—4.28 (m, 1H, C3-H), 3.90—3.80 (m, 1H, C1-H), 3.75—3.65 (m, 1H, C5-H), 3.56 (s, 3H, OCH_3), 2.94—2.92 (m, 1H, C2-H), 2.56 (s, 3H, NCH_3), 2.17—1.91 (m, 4H, C4,7-H_2), 1.83—1.73 (m, 2H, C6-H_2). ^{13}C-NMR (D_2O) δ: 176.67 (C=O), 152.42 (C-4′), 135.54 (C-3′), 135.40 (C-5′), 131.27 (C-1′), 118.16 (C-2′), 117.96 (C-6′), 66.66 (C-3), 66.17 (C-1), 65.96 (C-5), 55.77 (OCH_3), 50.89 (C-2), 41.31 (NCH_3), 37.53 (C-4), 26.32 (C-7), 25.22 (C-6). FAB-MA (3-NBA matrix) *m/z* (rel. int. %): 355 ($M+H^+$, 63.6), 182 (100). *Anal.* Calcd for $C_{16}H_{23}N_2O_5P \cdot 1/2C_6H_6$: C, 58.15; H, 6.67; N, 7.13; P, 7.89. Found: C, 57.84; H, 6.81; N, 6.83; P, 7.89.

3-[(Phenylphosphonyl)oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo-[3.2.1]octane-2-carboxylic Acid (TSA-III; 11) TSA-II (**8**; 5.0 g, 14.74 mmol) was dissolved in 60 ml of water containing 1.4 g NaOH (0.5 N final concentration) and stirred at room temperature for a total of 26 h. After the reaction was complete as monitored by TLC (EtOAc–MeOH–NH_4OH, 80:3:3; I_2), the pH of the reaction solution was made acidic (pH *ca.* 4) with conc. hydrochloric acid and the water was removed on a Rotavap under reduced pressure to furnish 7.3 g of a solid. It was then dissolved in MeOH, insoluble NaCl filtered off and the filtrate concentrated to afford 4.8 g (98%) of a fluffy solid, mp 178—180 °C. ^{1}H-NMR (D_2O) δ: 7.60, 7.56 (d, J=7.8 Hz, 1H and d, J=8.1 Hz, 1H, C3′,5′-H), 7.40—7.31 (m, 3H, C2′,4′,6′-H), 4.36 (dt, J=3.9, 10.5 Hz, 1H, C3-H), 3.78 (d, J=3.3 Hz, 1H, C1-H), 3.67 (d, J=3.3 Hz, 1H, C5-H), 2.71 (d, J=5.4 Hz, 1H, C2-H), 2.57 (s, 3H, NCH_3), 2.19—2.04 (m, 4H, C4,7-H_2), 1.85—1.71 (m, 2H, C6-H_2). ^{13}C-NMR (D_2O) δ: 178.64 (C=O), 134.30 (C-4′), 133.92 (C-1′), 133.87 (C-2′), 133.79 (C-6′), 131.45 (C-3′), 131.26 (C-5′), 67.16 (C-3), 66.66 (C-1), 65.74 (C-5), 51.78 (C-2), 41.15 (NCH_3), 37.56 (C-4), 26.28 (C-7), 25.58 (C-6). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 326 ($M+H^+$, 40.9), 348 ($M+Na^+$, 63.8), 183 (25.5). *Anal.* Calcd for $C_{15}H_{20}NO_5P \cdot NaCl$: C, 46.95; H, 5.25; N, 3.64; P, 8.07. Found: C, 46.94; H, 5.58; N, 3.68; P, 7.76.

3-[{4-(4-Carboxybutyramido)phenylphosphonyl}oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (TSA-Ic; 13) TSA-I (**12**; 1 g, 2.82 mmol) was dissolved in 200 ml of dioxane containing 20 ml of water and Et_3N (7.8 ml, 56.4 mmol) was added followed by the addition of glutaric anhydride (3.14 g, 28.2 mmol). The reaction contents were stirred at room temperature overnight (16 h). After the reaction was complete as monitored by TLC (MeOH–EtOAc, 1:1; 4-*N*,*N*-dimethyl aminobenzaldehyde as visualization reagent) the solvent was removed on a rotavap and a thick yellow oil was obtained which was purified on a silica gel column (60 g, 10% MeOH–EtOAc through 100% MeOH). 1.20 g (90.9%) of a light yellow solid was obtained, mp 148—150 °C. ^{1}H-NMR (D_2O) δ: 7.53, 7.49 (d, J=8.7 Hz, 1H and d, J=8.7 Hz, 1H, C3′,5′-H), 7.38 (dd, J=8.7, 3.3 Hz, 2H, C2′,4′-H), 4.44—4.35 (m, 1H, C3-H), 3.90—3.85 (m, 1H, C1-H), 3.77—3.70 (m, 1H, C5-H), 3.56 (s, 3H, OCH_3), 3.02—2.99 (m, 1H, C2-H), 2.97 (t, J=7.2 Hz, 2H, C14-H_2), 2.58 (s, 3H, NCH_3), 2.69 (t, J=7.2 Hz, C12-H_2), 2.16—2.00 (m, 4H, C4,7-H_2), 1.95—1.72 (m, 4H, C6,13-H_2). ^{13}C-NMR (D_2O) δ: 183.52 (C-16), 177.84 (C-10), 176.56 (C-12), 142.56 (C-1′,4′), 134.84, 134.70 (C-3′,5′), 123.70, 123.51 (C-2′, 6′), 66.62 (C-3), 66.40 (C-1), 65.92 (C-5), 55.77 (C-11), 50.82 (C-2), 41.28 (C-9), 38.72 (C-15), 38.05 (C-13), 37.50 (C-4), 26.28 (C-7), 25.17 (C-6), 24.34 (C-14). FAB-MS (3-NBA matrix) *m/z*: 469.175 (Calcd for $C_{21}H_{30}N_2O_8P$: 469.1740). MS *m/z* (rel. int. %): 469 ($M+H^+$, 71), 182 (100).

3-[{4-(1-Bromoacetamido)phenylphosphonyl}oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (TSA-Id, 14) TSA-I (**12**: 1 g, 2.82 mmol) was dissolved in 100 ml of MeCN and 3.92 ml (28.2 mmol) Et_3N. α-Bromoacetyl bromide (2.46 ml; 28.2 mmol) was added and the mixture stirred at room temperature for 3 h. The progess of the reaction was monitored by TLC (EtOAc–MeOH, 1:1; 4-*N*,*N*-dimethyl aminobenzaldehyde as visualization reagent). After the reaction was complete the excess of bromoacetyl bromide was decomposed with MeOH and solvent mixture evaporated on rotavap under reduced pressure. A white solid precipitated out which was filtered off and discarded. The filtrate was then loaded on a silica gel column and purified (35 g, 10% MeOH–EtOAc through 100% MeOH) to afford 1.12 g (83.5%) of a light yellow solid, mp 206—208 °C (dec.). ^{1}H-NMR (D_2O) δ: 7.58, 7.54 (d, J=8.7 Hz, 1H and d, J=8.4 Hz, 1H, C3′,5′-H), 7.43 (dd, J=3.3, 8.7 Hz, 2H, C2′,6′-H), 4.52—4.39 (m, 1H, C3-H), 3.95—3.92 (m, 1H, C1-H), 3.91 (s, 2H, CH_2Br), 3.81—3.72 (m, 1H, C5-H), 3.59 (s, 3H, OCH_3), 3.03—3.00 (m, 1H, C2-H), 2.62 (s, 3H, NCH_3), 2.26—1.96 (m, 4H, C4,7-H_2), 1.86—1.76 (m, 2H, C6-H_2). ^{13}C-NMR (D_2O) δ: 176.39 (C-10), 171.11 (C-12), 134.90 (C-4′), 134.76 (C-1′), 123.62 (C-3′,5′), 123.44 (C-2′,6′), 66.78 (C-3), 66.62 (C-1), 65.91 (C-5), 55.84 (C-11), 50.82 (C-2), 41.38 (C-9), 37.50 (C-4), 31.69 (C-13), 26.31 (C-7), 25.19 (C-6). FAB-MS (3-NBA matrix) *m/z*: 475.0646 (Calcd for $C_{18}H_{25}{}^{79}BrN_2O_6P$: 475.0634), 477.0637 (Calcd for $C_{18}H_{25}{}^{81}BrN_2O_6P$: 477.0613). MS *m/z*: (rel. int. %): 475 ($^{79}BrMH^+$, 15.8), 477 ($^{81}BrMH^+$, 18).

3-[(Phenylphosphonyl ethyl ester)oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (TSA-IIA; 16) To a solution of ecgonine methyl ester (**2**; 0.5 g, 2.5 mmol) in 10 ml of dry benzene were added triethylamine (0.60 ml, 4 mmol) and phenyl phosphonic chloride ethyl ester[31] (**15**; 3.0 g, 14.7 mmol) in 10 ml of dry benzene under N_2 and the cloudy mixture was stirred at room temperature for 16 h. The slurry was taken in 20 ml of $CHCl_3$ and washed with 5% aq. Na_2CO_3 solution (3×15 ml), the combined organic layers dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford an oil which was purified by preparative TLC (EtOAc–MeOH–NH_4OH, 80:3:3) to obtain 0.5 g (55.6%) oil. The oil was converted to the tartrate salt to obtain a white fluffy solid, mp 96 °C. ^{1}H-NMR (D_2O) δ: 7.65—7.55 (m, 3H, C2′,4′,6′-H), 7.47—7.40 (m, 2H, C3′,5′-H), 4.87—4.76 (m, 1H, C3-H), 4.44—3.93 (m, 3H, C1-H and $OC\underline{H}_2CH_3$), 3.88—3.80 (m, 1H, C5-H), 3.43 (s, 3H, OCH_3), 3.23—3.19 (m, 1H, C2-H), 2.63 (s, 3H, NCH_3), 2.27—2.20 (m, 4H, C4,7-H_2), 1.90—1.80 (m, 2H, C6-H_2), 1.11 (t, 3H, J=6.9 Hz, $OCH_2C\underline{H}_3$). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 368 ($M+H^+$, 100), 182 (80.4). *Anal.* Calcd for $C_{22}H_{32}NO_{11}P \cdot 3/2H_2O$: C, 48.53; H, 6.43; N, 2.57; P, 5.70. Found: C, 48.19; H, 6.14; N, 2.47; P, 5.02.

3-[(Phenylphosphonyl ethyl ester)oxy]-[1*R*-(*exo*,*exo*)]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid (TSA-IIb; 17) 0.070 g (0.19 mmol) of **16** was dissolved in 5 ml of distilled water (pH 6.9) and refluxed for 18 h. The solution was evaporated to dryness under vacuum. A white solid (0.050 g) was obtained, mp 240 °C (dec.). ^{1}H-NMR (D_2O) δ: 7.69—7.52 (m, 3H, C2′,4′,6′-H), 7.46—7.39 (m, 2H, C3′,5′-H), 4.75—4.64 (m, 1H, C3-H), 3.98 (q, J=7.2 Hz, 2H, $OC\underline{H}_2CH_3$), 3.88—3.81 (m, 1H, C5-H), 2.80—2.74 (m, 1H, C2-H), 2.58 (s, 3H, NCH_3), 2.18—2.04 (m, 4H, C4,7-H_2), 1.88—1.69 (m, 2H, C6-H_2), 1.10 (t, 3H, J=6.9 Hz, $OCH_2C\underline{H}_3$). FAB-MS (3-NBA matrix) (rel. int. %): 354.1 ($M+H^+$, 9.2), 168 (43.9).

***p*-Aminophenylphosphonyloxy Cyclohexane (19)** Cyclohexanol (1.0 g, 0.01 mol), *p*-nitrophenylphosphonic acid (2.03 g, 0.01 mol), and 4-DMAP (61.0 mg, 0.5 mmol) were dissolved in 25 ml of dry pyridine at 45±2 °C. 4.12 g (0.02 mol) DCC was then added in one lot and the reaction mixture maintained with stirring at 45±2 °C for 24 h. The mixture was cooled

and 10 ml MeCN was added and filtered. The filtrate was evaporated to dryness under vacuum to give 3.4 g of a crude solid. This solid (**18**) without further purification was dissolved in 40 ml of dry MeOH and stirred with 200 mg of 10% Pd/C under H_2 for 24 h. The reaction was stopped, the catalyst was filtered off and the solvent removed under vacuum. The purification of the crude product on SiO_2 column (35 g; EtOAc–MeOH, 1 : 1) followed by crystallization from the same solvent system afforded 0.41 g of pure compound (**19**), mp 196 °C (dec.). ^{1}H-NMR (D_2O) δ: 7.70, 7.66 (d, J=8.4 Hz, 1H, and d, J=8.4 Hz, 1H, C3′,5′-H), 7.31 (dd, J=2.7, 8.4 Hz, 2H, C2′,6′-H), 3.98—3.86 (m, 1H, C1-H), 2.03 (s, 3H, NH_3^+), 1.63—1.59 (m, 2H, C2-H_2), 1.51—1.46 (m, 2H, C6-H_2), 1.31—1.12 (m, 3H, C3-H_2, C4-H), 1.11—0.94 (m, 3H, C5-H_2, C4-H). FAB-MS (3-NBA matrix) *m/z* (rel. int. %): 256.1 ($M+H^+$, 7.7), 255.1 (M^+, 3.4), 174.1 (67.7).

Conjugation of TSAs with DT DT-TSA-Ia (DT-**20**, Chart 7): 1) Diazotization Method: 1 ml of a 0.05 M aqueous sodium nitrite solution (50 μmol) was added to a solution of TSA-I (**12**) (17 mg; 50 μmol) in 150 μl of 0.3 N HCl (pH 2—3) and stirred at 0 °C for 10 min followed by the addition of 1 ml of the diphtheria toxoid solution (2.8 mg protein, 1000 LF units). The pH of the resulting solution was raised to 9.0 with 1.0 N aqueous NaOH solution and stirred for 20 min while maintaining the pH at 9.0 with the dropwise addition of 1.0 N NaOH. At this time a deep reddish-orange color developed. The reaction was stopped by adding 25 μl of 2 N urea in phosphate buffer (pH 7.4). In some experiments, the solution was passed through a 3 ml Sephadex G-25 column and washed with phosphate buffer saline (PBS). The first 3 ml of the effluent was collected, and filtered through a sterile 0.2 micron filter and stored at 4 °C. HPLC (on a TSK-250 SEC column eluted with 0.1 M phosphate buffer pH 7.0, 1 ml/min) showed absence of free TSA-I. In other experiments, the solution was dialyzed against PBS, pH 7.4 at 4 °C overnight using a Pierce Slide-A-Lyzer cassette. The solution was next filtered through a sterile 0.2 μm filter and stored at 4 °C.

DT-TSA-1b (DT-**21**, Chart 7): 2) Glutaraldehyde Method: 7.0 mg (20 μmol) of TSA-I (**12**), 0.50 ml of diphtheria toxoid solution (1.4 mg protein, 500 LF units) containing 0.25 ml of 0.066 M phosphate buffer and 10 μl of 25% glutaraldehyde were mixed together and stirred at 22± 2 °C for 1 h and at 4 °C for 16 h. 20 mg of glycine in 1 ml of PBS pH 7.4 was added to the yellow colored solution. The reaction mixture was treated as above.

DT-TSA-Ic (DT-**13**, Chart 7): To a solution of TSA-Ic (**13**; 3.26 mg, 7 μmol) in 1 ml of 0.066 M phosphate buffer (pH 7.4) was added a solution of 2.68 mg of EDC·HCl (13.6 μmol) in 1 ml of 0.066 M phosphate buffer

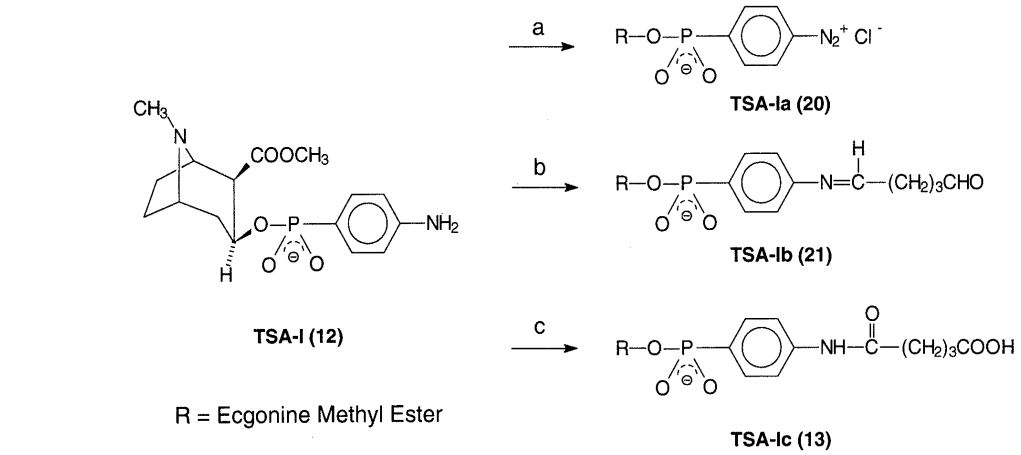


Chart 7

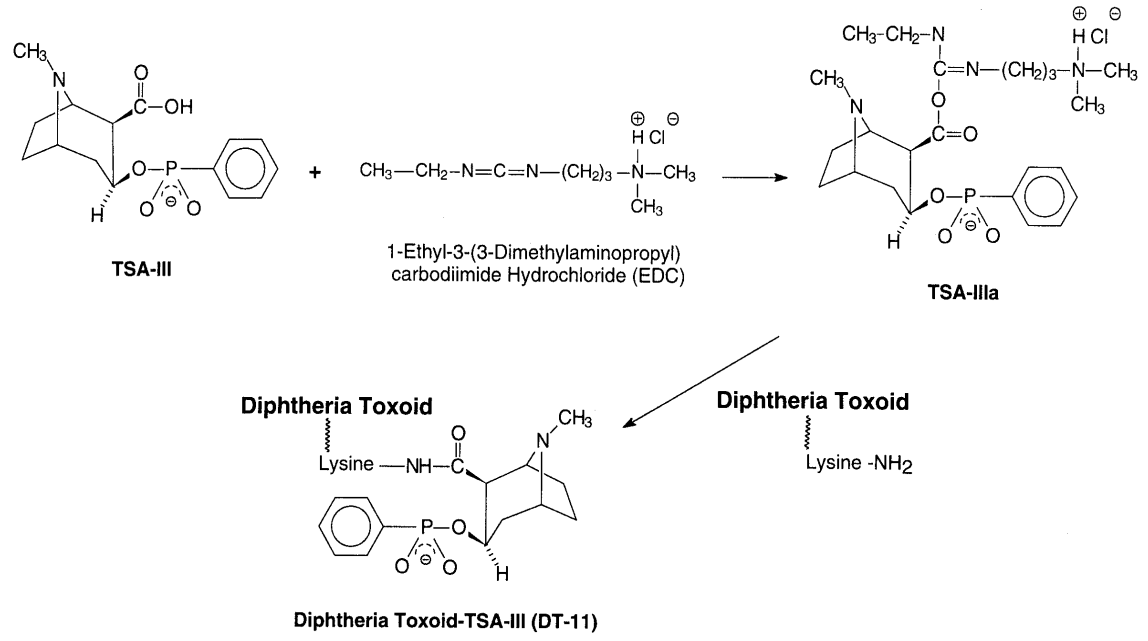


Chart 8

(pH 7.4) and stirred at 22±2 °C for 1 h. To this mixture was added 0.5 ml diphtheria toxoid (0.65 mg protein). The mixture was stirred at 22±2 °C for another 3 h. The solution was dialyzed against PBS, pH 7.4 at 4 °C overnight using a Pierce Slide-A-Lyzer cassette. The solution was next filtered through a sterile 0.2 μm filter and stored at 4 °C.

DT-TSA-III (DT-**11**, Chart 8): To a solution of TSA-III (**11**; 8 mg, 25 μmol) in 0.5 ml of 0.066 M phosphate buffer (pH 7.4) and 210 μl of diphtheria toxoid solution (0.6 mg protein) was added a solution of 9.4 mg of EDC·HCl in 0.5 ml of 0.066 M phosphate buffer (pH 7.4) and stirred at 22±2 °C for 4 h. The mixture was treated as above.

DT-**19** The same diazotization method as described under DT-**20** was used to conjugate **19** to DT.

The same conjugation methods were used to attach TSA-I, TSA-Ic, TSA-III and (**19**) to rabbit serum albumin (RSA) for use to coat ELISA plates.

Immunization of Mice Two immunization protocols were used.

Protocol 1: Immunization Protocol with DT and DT-Conjugates: Mice, CF/1 (outbred) were immunized first with a mixture of Diphtheria/Tetanus Toxoids (Diphtheria & Tetanus Toxoid Adsorbed (Aluminum Phosphate Adsorbed) Wyeth lot 4927502); 1.6 LF units/kg body weight of Diphtheria Toxoid and 0.8 LF units of Tetanus Toxoid, this was to "prime" the immune system of the animals with the carrier protein alone for subsequent exposure to the conjugates. One month later, the animals were given an equivalent dose of a boost. Twelve to fifteen days later, after DT titers were found to be adequate, the animals were divided into two groups (30 μl of blood was collected from each mouse by tail vein nick) and injected separately with a 0.029 LF units/kg body weight of the DT-**20** or DT-**11**. These doses are equivalent to human immunization doses. The animals were subsequently boosted twice with the same dose of the conjugates, at one month after the first immunization with the conjugate and three weeks after the first boost. The animals were bled by tail vein nick. The collected blood was centrifuged to obtain the serum.

Protocol 2: Immunization Protocol with DT-conjugates and Freund's Complete Adjuvant: Mice, C57BL/6J (inbred, $N=5$ per conjugate, age 7—8 weeks) were injected subcutaneously at a dorsal site with 100 μl of 10×PBS containing 10 μg protein of DT-**20**, DT-**21**, DT-**13**, DT-**11**, a mixture of all four DT-conjugates (containing equal protein concentrations), DT-**19** and 100 μl Freund's Complete Adjuvant. Before immunization, 30 μl of blood was collected from each mouse by tail vein nick. This was used as control in ELISA and catalytic antibody assay. The mice were boosted three times (5 μg protein without any adjuvant) at two weeks time intervals (boost 1 and 2) and 3 weeks interval after second boost. The mice were bled after 10 d of each boost. The collected blood was centrifuged to obtain the serum.

Detection of Antibodies. Screening for the Antibodies in Mice Sera The mouse sera were screened for antibody titer using ELISA. RSA-**20**, RSA-**21**, RSA-**13**, RSA-**11** or RSA-**19** (50 μl, 10 μg/ml) dissolved in carbonate-bicarbonate coating buffer, pH 9.6 were plated in a 96-well microtiter (Costar) overnight at 4 °C. After washing (0.05% Tween 20 in 0.1 M Tris/0.15 M NaCl, pH 8.4) and blocking of the unbound sites on the plates with Pierce SuperBlock. 100 μl of the test samples (various dilutions of the mouse sera) were added to the wells and incubated for 2 h at room temperature. Following additional washings with 0.1 M Tris/0.15 M NaCl-Tween solution, goat anti-mouse IgG conjugated with alkaline phosphatase (100 μl, 1 : 1500 dilution) was added to the well and allowed to react for 2 h at room temperature. The wells after washing were developed with *p*-nitrophenyl phosphate (pNPP) substrate (100 μl, 1 mg/ml in carbonate substrate buffer, pH 8.6). Serum from non-immunized mice was used as negative control. After 1 h incubation at room temperature in a dark chamber, the optical density was read at λ 405 nm in a Dynatech reader.

Inhibition Studies with Immune Sera The mouse sera from immunized and boosted animals at a 1 : 10000 to 1 : 40000 dilution was used to study the inhibition of binding of the following compounds: TSA-I (**12**), TSA-II (**8**), TSA-III (**11**), (**19**), cocaine (**1**), ecgonine methyl ester (**2**), benzoic acid, atropine and glycine at various concentrations. The serum was preincubated with the inhibitors at the different concentration levels overnight at 4 °C and then used in the ELISA test as described above.

Catalytic Antibody Assay: (*In Vitro* Assay for Esterase Activity) 5 μl of an alcoholic solution of ^{125}I-4′-iodococaine (5×10^4 dpm, S.A. 56.5 mCi/mmol)[32,33] was placed in a 13×75 mm tube and the alcohol was evaporated under a gentle stream of N_2. To the tube was added 10—50 μl of the test serum followed by 0.05 M phosphate buffer, pH 8.0 q.s. to 100 μl. This mixture was shaken for 10 s and incubated at room temperature for 15 min to 3 h at room temperature or 15—20 min at 36—37 °C.

The negative control tube contained serum from non-immunized mice. The positive control tube contained 10 μl of porcine liver esterase (EC 3.1.1.1) (5.5 μg protein/ml, S.A. = 230 units/mg).

At the end of the incubation periods, 2 μl of the incubate (in duplicate) was pipetted out and placed on a silica gel coated thin layer strip. The spot was dried under a stream of N_2 and the strip was developed with a solvent mixture consisting of EtOAc–MeOH–NH_4OH (80 : 3 : 3; v/v). After the development, the strip was cut at 1 cm above the origin and both pieces of the strip were placed in separate counting tubes and the radioactivity counted in a gamma counter. ^{125}I-4-iodobenzoic acid has an $Rf=0$ and ^{125}I-4′-iodococaine has an $Rf=0.8$ in this solvent system. The counts per minute in the lower and upper parts of the strip were added after the substraction of the background counts. This represented the total cpm. The % of ^{125}I-4-iodobenzoic acid formed was calculated by subtracting the background activity from the activity in the lower portion divided by the total cpm and multiplied by 100. The formation of ^{125}I-4-iodobenzoic acid was confirmed by HPLC using a cyano column eluted with MeOH : 0.015 M phosphate buffer pH 6.4 (70 : 30; v/v) at 1 ml/min using an in-line radioisotope detector (Beckman 170). No ^{125}I-4-iodobenzoyl ecgonine was detected by HPLC, confirming that the assay was only detecting the hydrolysis of the benzoyl ester group. Greater than 60% hydrolysis was detected with the porcine liver esterase within 15—20 min. This assay is fast and was shown to be reproducible with the porcine liver esterase as well as different sera from immunized mice.

Hydrolytic activity was calculated as % hydrolyzed substrate in 20 min. When affinity purified antibody fractions become available, the activity will be expressed as % hydrolyzed per μg antibody protein per 20 min incubation time.

Hydrolytic activity inhibition using TSA-II was performed by pre-incubating the preimmunized serum, porcine liver esterase and the combined sera from the various immunized mice for 30 min with 0.28 mM TSA-II prior to the addition of this incubate to the radioactive substrate. The incubation and analysis was continued as above.

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