Retro-Diels−Alder Reaction: Possible Involvement in the Metabolic Activation of 7-Oxabicyclo[2.2.1]hepta-2(3),5(6)-diene-2,3-dicarboxylates and a Phosphonate Analog

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A discontinuous structure−activity relationship signaled a change in mode of action and led to the discovery of a possible novel metabolic activation mechanism. The toxicity of the herbicide endothal (*exo,exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) to mice (ip LD_{50} = 14 mg/kg) is attributed to the inhibition of protein phosphatase 2A (PP2A) at the cantharidin binding site. The potency is reduced by the introduction of a 2,3- or 5,6-double bond. Surprisingly, high toxicity (ip LD_{50}'s = 15−50 mg/kg) is restored in oxabicyclohepta-2(3),5(6)-dienes substituted in the 2- and 3-positions with bis(methyl carboxylate), bis(ethyl carboxylate), and diethyl phosphonate/ethyl carboxylate, whereas the dicarboxylic acid, bis(*tert*-butyl carboxylate), and bis(dimethyl phosphonate) are inactive. The diene adducts do not inhibit the cantharidin binding site of PP2A. Two observations provided an alternative working hypothesis that the active but not the inactive diene adducts are protoxicants: GC analyses revealed that selected bicyclic dienes readily undergo thermal dissociation by retro-Diels−Alder reactions to liberate disubstituted acetylenes; the liberated acetylenes have mouse ip LD_{50}'s of 8−25 mg/kg. Apparent exceptions to this hypothesis are that bicyclic dienes with bis(*tert*-butyl carboxylate) and bis(dimethyl phosphonate) substituents are not toxic, yet their corresponding acetylenes are quite toxic. These apparent anomalies are resolved by finding that only the toxic bicyclic dienes readily react with albumin and 4-nitrobenzenethiol and that their low-toxicity analogs are much less reactive. Albumin can be replaced by hemoglobin but not by myoglobin or chymotrypsin in reaction with a bicyclic diene indicating the importance of the free thiol group. Diethyl oxabicycloheptadienedicarboxylate readily reacts with GSH to give two products, which are also formed from the corresponding acetylene, identified as the *cis* and *trans* isomers of the GSH−acetylene conjugate. This is the first proposal, to our knowledge, that a retro-Diels−Alder-type reaction is involved in the metabolic activation of a toxicant.

Introduction

exo,exo-7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid (endothal) (**1**) is an important herbicide (*1*) and is also toxic to mice (ip LD_{50} = 14 mg/kg) (*2, 3*), where it acts as an inhibitor of protein phosphatase 2A (PP2A)[1] (*4, 5*) at the [^{3}H]cantharidin binding site (*6, 7*). Further studies on endothal analogs as toxicants and inhibitors of the cantharidin site revealed a discontinuous structure−activity relationship (Table 1), suggesting a possible change in mode of action within the series. The toxicity and inhibitory activity are reduced by the introduction of a 2,3- or 5,6-double bond (*2, 3, 7*). It was therefore surprising to find that several 2,3-disubstituted 7-oxabicyclo[2.2.1]hepta-2(3),5(6)-dienes, with double bonds in both positions, are of high toxicity and do not act at the PP2A site (Table 1). Two observations led to a working hypothesis that the toxic bicyclic adducts are protoxicants. First, they undergo thermal dissociation under GC conditions to the corresponding disubstituted acetylenes by retro-Diels−Alder reaction (Figure 1). Second, the toxicity of dimethyl acetylenedicarboxylate (*8, 9*) approximates that of the corresponding diene adduct. The research described here indicates that several oxabicycloheptadienes derived from toxic disubstituted acetylenes undergo metabolic conversion back to the acetylene derivatives in a biologically catalyzed retro-Diels−Alder-type reaction and that this constitutes a new bioactivation mechanism.

Experimental Procedures

General. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, with a Bruker WM-300 spectrometer using tetramethylsilane as the internal reference. GC/chemical ionization mass spectrometry (CI-MS) with selected ion monitoring (SIM) involved a Hewlett-Packard 5890 gas chromatograph coupled to a 5971A mass spectrometer, a DB-5 fused-silica capillary column (30 m × 0.25 mm id, J and W Scientific, Folsom, CA), an injection port temperature of 175 °C (to prevent thermal decomposition for the oxabicycloheptadienes, which was complete at 250 °C), and a temperature program of 70 to 250 °C over 20 min. This method was modified in two ways for the analysis of furan: the column temperature was 50 °C, and the

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[1] Abbreviations: BSA, bovine serum albumin; CI-MS, chemical ionization mass spectrometry; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; FAB, fast atom bombardment; HRMS, high-resolution mass spectrometry; I_{50}, concentration for 50% inhibition; PP2A, protein phosphatase 2A; PSCP, 2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; SIM, selected ion monitoring.

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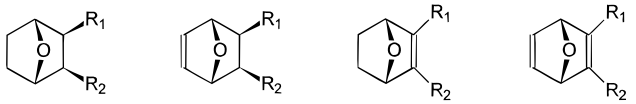
Table 1. Structure−Activity Relationships for Toxicity and Relative Binding Affinity for PP2A of Endothal and Related Compounds



1-3 4 5-7 8-10

	substituents			
no.	R_1	R_2	LD_{50}[a] (mg/kg)	PP2A IC_{50}[b] (μM)
	2,3-Disubstituted 7-Oxabicyclo[2.2.1]heptanes			
1[c]	C(O)OH	C(O)OH	14	0.59
2	C(O)OH	C(O)OEt	110	1.4
3	C(O)OEt	C(O)OEt	50	2.2
	2,3-Disubstituted 7-Oxabicyclo[2.2.1]hept-5(6)-ene			
4	C(O)OH	C(O)OH	55	4.1
	2,3-Disubstituted 7-Oxabicyclo[2.2.1]hept-2(3)-enes			
5	C(O)OH	C(O)OH	>250	>10
6	C(O)OMe	C(O)OMe	>250	>10
7	C(O)OEt	$P(O)(OEt)_2$	>250	>10
	2,3-Disubstituted 7-Oxabicyclo[2.2.1]hepta-2(3),5(6)-dienes			
8	C(O)OH	C(O)OH	>250	>10
9	C(O)OMe	C(O)OMe	15	>10
10	C(O)OEt	C(O)OEt	21	>10

[a] Mouse ip 72-h data for **1**−**5** from earlier studies (*2*, *3*) and for **6**−**10** from this study. [b] Inhibition of [^{3}H]cantharidin binding site of PP2A in mouse liver cytosol from this study. [c] The herbicide endothal.

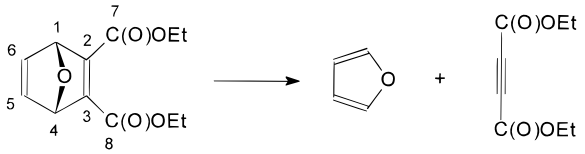
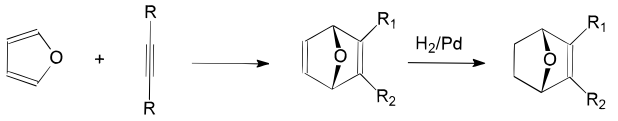


Figure 1. Retro-Diels−Alder reaction of diethyl 7-oxabicyclo-[2.2.1]hepta-2(3),5(6)-diene-2,3-dicarboxylate.



14-19 **8 - 13** **6,7**

Figure 2. Synthesis of 2,3-disubstituted 7-oxabicyclo[2.2.1]-hepta-2(3),5(6)-dienes (**8**−**13**) by Diels−Alder reaction of furan and disubstituted acetylenes (**14**−**19**) and partial reduction of two products to 2,3-disubstituted 7-oxabicyclo[2.2.1]hepta-2(3)-enes (**6, 7**).

sample for analysis was 10 μL withdrawn from the 8-mL head space above a reaction mixture in a vial sealed with a septum. Fast atom bombardment/high-resolution mass spectrometry (FAB/HRMS) was performed with a Kratos MS-50 instrument (Department of Chemistry, University of California at Berkeley). HPLC utilized a Merck LiChrospher 100 RP-18 (5-μm) reverse-phase column on a Waters Model 600E solvent delivery system coupled to a Model 994 photodiode array detector: 0.1% trifluoroacetic acid for 5 min, linear gradient of 0 to 60% methanol in water with constant 0.1% trifluoroacetic acid over 20 min, and finally a linear gradient to 100% methanol over an additional 5 min, each at 1.5 mL/min (monitoring at 220 nm).

Chemicals. Caution: *Dimethyl acetylenedicarboxylate, a widely used reactant solvent, is a lachrymator and primary skin irritant (8, 9). Care therefore should be taken in studies using this chemical and its analogs.* The oxabicycloheptadienes (**8**−**13**) and oxabicyclohept-2(3)-enes (**6** and **7**) were synthesized as shown in Figure 2 and detailed in the following. The purity of each of these compounds was estimated to be >97% on the basis of ^{1}H and ^{13}C NMR integration. Acetylene derivatives **14** (monopotassium salt) and **17** were from Aldrich Chemical Co. (Milwaukee, WI) and the others were synthesized by reported procedures, *i.e.*, **15** and **16** (*10*), **18** (*11*), and **19** (*12*).

Oxabicycloheptanes (1−3) and -heptenes (4−7). Compounds **1**−**5** were available from previous syntheses in this laboratory (*2*, *3*). Oxabicyclohept-2(3)-enes **6** and **7** were prepared by partial reduction of oxabicycloheptadienes **9** and **12**, respectively, described in the following. More specifically, **9** (10 mmol) in acetone (20 mL) was partially reduced by stirring for 18 h over 10% (w/w) palladium on carbon (210 mg) under 1 atm of hydrogen. Filtration and solvent evaporation gave an oily product, which was recrystallized from hexane [mp 51−52 °C, lit. mp 51−52 °C (*13*)] (71%). Compound **7** was synthesized in 82% yield from **12** by the same procedure, but using ethyl acetate.

Oxabicycloheptadienes (8−13) (Table 2). To prepare dicarboxylic acid **8**, furan (12 mmol) and bis(trimethylsilyl) acetylenedicarboxylate (10 mmol) were stirred at 100 °C overnight in a 3-oz., thick-walled glass pressure reaction apparatus (Lab Glass, Inc., Vineland, NJ). Dry ether was added, and after cooling to 0 °C and filtration, the clear solution was evaporated and the oil residue of bis(trimethylsilyl) oxabicycloheptadiene-dicarboxylate was hydrolyzed by the addition of methanol, which was evaporated to give **8** (42%). The general procedure to prepare **9** (*13*) was also used for **10** and **11**. Phosphonate **12** was made by treating ethyl (diethoxyphosphonyl)propynoate (**18**) (4 mmol) with excess furan (6 mL) at 120 °C under pressure as before for 36 h. Unreacted furan was removed by distillation, ether was added, and after filtration and solvent evaporation the oil was chromatographed on a silica gel column with *n*-hexane−ether (1:1) to obtain **12** (58%). A similar procedure converted **19** to **13** (39%).

Toxicity to Mice. LD_{50} values were determined 72 h after ip administration of the test compounds to male albino Swiss-Webster mice (18−23 g), using Me_2SO as the carrier vehicle (50 μL).

Competition for [^{3}H]Cantharidin Binding Site (*6, 7*). Mouse liver cytosol was prepared at 20% (w/v) equivalent in 50 mM imidazole hydrochloride (pH 7.0) containing 1 mM EDTA, 1 mM EGTA, 100 μM *N*-ethylmaleimide, and 10 μM 2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (PSCP) (an esterase inhibitor) (*14*). The competitive binding assays were carried out with cytosolic protein (250 μg) and 5 nM [^{3}H]cantharidin in the preceding buffer (500 μL); the inhibitors were added in buffer or acetone (<1% final concentration) and incubated for 120 min at 37 °C before filtration through poly(ethylenimine)-treated glass fiber filters. Nonspecific binding was determined with 10 μM unlabeled cantharidin.

Reactivity with 4-Nitrobenzenethiol. The relative reactivities were compared for the various bicyclic adducts and acetylene derivatives with 4-nitrobenzenethiol. The acetylene or bicyclic diene derivative (**8**−**19**) was added individually as an acetonitrile solution to 4-nitrobenzenethiol in 200 mM sodium phosphate (pH 7.4) buffer containing 0.1 mM EDTA (to minimize thiol autoxidation) at 25 °C. The final concentrations were 120 μM for the acetylene or diene derivative, 120 μM for nitrobenzenethiol, and 2% for acetonitrile. The extent of reaction was determined spectrophotometrically as the decrease in absorbance at 410 nm due to the disappearance of 4-nitrobenzenethiolate ion (*15*) after 200 s for the bicyclic dienes and after 10 s for the acetylene derivatives.

Products from Reactions with *N*-Acetylcysteine and GSH. The products from reactions of diethyl oxabicycloheptadienedicarboxylate and the corresponding acetylene with N-acetylcysteine were isolated and identified (Figure 3). A solution of **10** (or **16**) (20 mg) and *N*-acetylcysteine (1 molar equiv) in 100 mM phosphate buffer (pH 7.4, 2 mL) was incubated for 1 h at 37 °C and then analyzed by HPLC. The same two products were found in each case. The starting materials gave t_R values of 25.2 and 27.6 min for **10** and **16**, respectively, while the t_R's for the *N*-acetylcysteine conjugates (**20** and **21**) were 24.3 and 25.7 min, respectively (ratio 3:1). Conjugates **20** and **21** were isolated by HPLC in amounts of 3−8 mg each. NMR and MS data for the isomers are given for **20** followed by those for **21** in parentheses. NMR (CD_3OD): ^{1}H δ 1.25 (1.28) (3H, t, HC(O)-OCH_2CH_3), 1.32 (1.35) (3H, t, SC(O)OCH_2CH_3), 1.99 (1.97) (3H,

Table 2. ^{13}C and ^{1}H NMR Spectral Characteristics of 2,3-Disubstituted 7-Oxabicyclo[2.2.1]hepta-2(3),5(6)-dienes[a]

	substituents		^{13}C NMR (ppm)								
no.	R_1	R_2	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	other
8	C(O)OH	C(O)OH	86.1	157.5	157.5	86.1	144.2	144.2	166.3	166.3	
9	C(O)OMe	C(O)OMe	85.0	152.9	152.4	85.0	143.2	143.2	163.1	163.1	52.3 (2Me)
10	C(O)OEt	C(O)OEt	84.8	152.4	153.1	84.8	142.9	142.9	162.8	162.8	61.0, 13.9 (2Et)
11	C(O)O-*t*-Bu	C(O)O-*t*-Bu	85.0	153.1	153.1	85.0	143.1	143.1	163.2	163.2	82.3, 28.0 (2*t*-Bu)
12	C(O)OEt	$P(O)(OEt)_2$	84.7	153.4	158.8	86.9	142.9	142.9	162.4		61.2, 13.7 [C(O)OEt], 62.2, 15.8 [$P(O)(OEt)_2$]
13	$P(O)(OMe)_2$	$P(O)(OMe)_2$	87.3	158.2	158.2	87.3	143.1	143.1			53.0 (4Me)

	substituents		^{1}H NMR (ppm)				
no.	R_1	R_2	H_1	H_4	H_5	H_6	other
9	C(O)OMe	C(O)OMe	5.68 (s)	5.68 (s)	7.22 (s)	7.22 (s)	3.83 (s) (2Me)
10	C(O)OEt	C(O)OEt	5.68 (s)	5.68 (s)	7.22 (s)	7.22 (s)	4.29 (q) 1.3 (t) (2Et)
11	C(O)O-*t*-Bu	C(O)O-*t*-Bu	5.55 (s)	5.55 (s)	7.16 (s)	7.16 (s)	1.48 (s) (2*t*-Bu)
12	C(O)OEt	$P(O)(OEt)_2$	5.68 (s)	5.79 (s)	7.17 (d)	7.21 (d)	4.26 (q), 1.32 (t) [C(O)OEt], 4.15 (dq), 1.30 (t) [$P(O)(OEt)_2$]
13	$P(O)(OMe)_2$	$P(O)(OMe)_2$	5.81 (s)	5.81 (s)	7.14 (s)	7.14 (s)	3.83 (m) (4Me)

[a] $CDCl_3$ solutions in all cases except **8** in CD_3OD. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

s, $C(O)CH_3$), 3.14–3.42 (3.13–3.46) (2H, m, SCH_2), 4.14 (4.22) (2H, q, $HC(O)OCH_2$), 4.29 (4.32) (2H, q, $(SC(O)OCH_2)$, 4.64 (4.55) (1H, q, NCH), 5.96 (6.45) (1H, s, HC=C); ^{13}C δ 14.17 (14.32), 14.46 (14.52), 22.37 (22.43), 34.02 (34.95), 52.92 (54.06), 61.91 (61.97), 63.59 (63.92), 116.15 (122.95), 149.91 (148.38), 164.98 (165.40), 166.77 (166.44), 172.52 (172.73), 173.36 (173.24). FAB/HRMS [MH^+] calcd for $C_{13}H_{19}NO_7S$ 334.0960, found 334.0963 (334.0960).

The products from reaction of dimethyl oxabicycloheptadienedicarboxylate with GSH were isolated for characterization (Figure 3). Reaction of **9** (20 mg) with GSH (1 molar equiv) was carried out in 100 mM phosphate buffer (pH 7.4, 2 mL) for 1 h at 37 °C, with the isolation of two products (**22** and **23**) in a 3:1 ratio and analyses as before. Data for the conjugates are given for **22** followed by those for **23** in parentheses. t_R: 18.4 (19.5) min. NMR (CD_3OD): ^{1}H δ 2.17 (2.19) (2H, s, H_b), 2.57 (2.56) (2H, t, H_c), 3.08–3.39 (3.09–3.40) (2H, m, H_f), 3.69 (3.74) (3H, s, H_i), 3.83 (3.87) (3H, s, H_h), 3.92 (3.90) (2H, s, H_e), 4.03 (4.03) (1H, t, H_a), 4.56 (4.59) (1H, q, H_d), 6.03 (6.49) (1H, s, H_g); ^{13}C δ 26.93 (26.98), 32.35 (32.41), 34.14 (35.34), 41.84 (41.84), 52.38 (52.38), 53.28 (53.55), 53.52 (53.94), 53.73 (54.66), 115.82 (122.83), 149.94 (148.04), 165.55 (165.95), 167.34 (167.12), 171.42 (171.38), 171.84 (171.89), 172.61 (172.07), 174.41 (174.21). FAB/HRMS [MH^+] calcd for $C_{16}H_{23}N_3O_{10}S$ 450.1182, found 450.1182.

Reactivity in the Presence of Albumin and Other Proteins. The bovine serum albumin (BSA) used was fraction V from initial cold ethanol precipitation (96–99% pure) or heat shock (98–99% pure) (Sigma Chemical Co., St. Louis, MO). Incubation mixtures consisted of 100 mg (1.67 μmol) of BSA in 100 mM phosphate buffer (pH 7.4, 1 mL), to which was added the test compound (1.67 μmol unless indicated otherwise) in 10% methanol in buffer (1 mL) followed by incubation for 1 h at 37 °C. Analysis involved extraction with dichloromethane (2 mL) and GC/MS on a 1-μL aliquot. t_R values (minutes) for the test compounds and products were as follows: **9** (10.0), **10** (11.6), **11** (12.8), **15** (4.4), **16** (6.5), **17** (9.1), and furan (1.8; head-space analysis). Phosphonates **12** and **13** are unstable on GC, yielding their acetylene derivatives with t_R's (minutes) of 12.7 for **12** and **18** and 12.1 for **13** and **19**. Accordingly, the phosphonyl derivatives were analyzed by HPLC on a direct aliquot (10 μL) of the incubation mixture, resulting in t_R's (minutes) of **12** (25.1), **13** (18.4), **18** (26.5), and **19** (19.5).

In another set of experiments with bicyclic adduct **9**, BSA was compared with bovine hemoglobin, myoglobin, and α-chymotrypsin (1.67 μmol) in the presence and absence of the esterase inhibitor PSCP (*14*) (10 μmol, 10-min preincubation) added in Me_2SO (100 μL). The reaction and analytical conditions were as before except that the incubation time was 30 min.

Metabolism in Mice. Mice as before were administered a test compound (**9**–**11** and **15**–**17**) ip at 250 mg/kg, with Me_2SO as the vehicle (50 μL). At 5–60 min after treatment, the animals were sacrificed by cervical dislocation and the liver was homogenized in water (2 mL) at 5 °C. The homogenate was centrifuged (10000*g*, 30 min), the supernatant fraction was extracted with dichloromethane (2 mL), and a 1-μL aliquot was analyzed by GC/MS.

Table 3. Structure–Activity Relationships for Toxicity and Relative Binding Affinity for PP2A of 2,3-Disubstituted 7-Oxabicyclo[2.2.1]hepta-2(3),5(6)-dienes and Disubstituted Acetylenes

substituents		compound no.		LD_{50} (mg/kg)[a,b]	
R_1	R_2	bicyclic diene	acetylene deriv	bicyclic diene	acetylene deriv
C(O)OH	C(O)OH	**8**	**14**	>250	>250
C(O)OMe	C(O)OMe	**9**	**15**	15	8
C(O)OEt	C(O)OEt	**10**	**16**	21	12
C(O)O-*t*-Bu	C(O)O-*t*-Bu	**11**	**17**	>200	27
C(O)OEt	$P(O)(OEt)_2$	**12**	**18**	50	10
$P(O)(OMe)_2$	$P(O)(OMe)_2$	**13**	**19**	>200	25

[a] Mouse ip at 72 h. [b] The IC_{50} for each compound is >10 μM for the [^{3}H]cantharidin binding site of protein phosphatase 2A (PP2A) in mouse liver cytosol.

Results

Structure–Activity Relationships (Tables 1 and 3). The dicarboxylic acid endothal (**1**) is the most toxic of the oxabicycloheptanes examined and is also the most potent at the PP2A binding site. The toxicity and inhibitory potency are reduced upon conversion of **1** to its ethyl and diethyl esters (**2** and **3**). Introduction of a 5,6-double bond to give **4** reduces the potency, and introduction of a 2,3-double bond to yield **5**–**7** results in the loss of toxic and inhibitory properties. On an overall basis for compounds **1**–**7**, the toxic compounds interact and the nontoxic ones do not with the PP2A binding site.

The combination of 2,3- and 5,6-double bonds in the 2(3),5(6)-diene series alters the potency, but not always with the expected total loss of activity. The dicarboxylic acid **8** is neither a toxicant nor an inhibitor at the PP2A site, perhaps due to the 2,3-double bond. It therefore was surprising to find high toxicity for the corresponding dimethyl and diethyl esters (**9** and **10**). Further synthesis and testing established that the diethyl phosphonate/ethyl carboxylate **12** is toxic, but the bis(*tert*-butyl carboxylate) **11** and bis(dimethyl phosphonate) **13** are not, *i.e.*, an unusual structure–activity pattern.

Table 4. Substituent Effects on Relative Reactivity with 4-Nitrobenzenethiol and BSA of 2,3-Disubstituted 7-Oxabicyclo[2.2.1]hepta-2(3),5(6)-dienes and Disubstituted Acetylenes

substituents		compound no.		relative reactivity[a]			
				4-nitrobenzenethiol[b]		albumin[c]	
R_1	R_2	bicyclic diene	acetylene deriv	bicyclic diene	acetylene deriv	bicyclic diene	acetylene deriv
C(O)OH	C(O)OH	**8**	**14**	−	−	−[d]	
C(O)OMe	C(O)OMe	**9**	**15**	+++	+++	+++	+++
C(O)OEt	C(O)OEt	**10**	**16**	++	+++	+++	+++
C(O)O-*t*-Bu	C(O)O-*t*-Bu	**11**	**17**	−	++	−	−
C(O)OEt	$P(O)(OEt)_2$	**12**	**18**	+	++	+	+++
$P(O)(OMe)_2$	$P(O)(OMe)_2$	**13**	**19**	−	+	−	++

[a] Extent of reaction in pH 7.4 phosphate (%): +++, 90−100; ++, 60−80; +, 20−40; −, <5. [b] Reaction for 200 s with bicyclic dienes and for 10 s with acetylene derivatives at 25 °C. [c] Reaction for 1 h at 37 °C. Analysis for loss of **12** and **13** by HPLC and of the other compounds (except for dicarboxylic acids **8** and **14**) by GC/MS. [d] No furan was found in the head space above the reaction mixture.

Acetylene derivatives **14**−**19** corresponding to dienes **8**−**13** were also examined for toxicity and binding site interaction. Acetylenedicarboxylic acid **14** is much less toxic than the carboxylic and phosphonic acid ester derivatives **15**−**19**, which have ip LD_{50}'s of 8−27 mg/kg.

The toxicity of the bicyclic dienes (**8**−**13**) and acetylene derivatives (**14**−**19**) does not correlate with their potency at the PP2A binding site. Lethal doses of the oxabicycloheptanes and oxabicycloheptene (**1**−**4**) lead to hepatohemia (visually evident), whereas lethal doses of the oxabicycloheptadienes (**9**, **10**, and **12**) and acetylene derivatives (**15**−**19**) do not.

Reactivity with 4-Nitrobenzenethiol (Table 4). Nitrobenzenethiol was used as a convenient model for biological thiols since it combines high reactivity with ease of monitoring the reaction rates using a single method for all compounds. The acetylene derivatives (**15**−**19**) react with nitrobenzenethiol at least 20-fold faster than the corresponding bicyclic dienes (**9**−**13**). The reactivity pattern in each series follows the general order bis(methyl carboxylate) ≥ bis(ethyl carboxylate) > diethyl phosphonate/ethyl carboxylate > bis(*tert*-butyl carboxylate) > bis(dimethyl phosphonate) ≫ dicarboxylic acid.

Products from Reactions with *N*-Acetylcysteine and GSH (Figure 3). Bicyclic dienes such as the bis(methyl carboxylate) **9** and bis(ethyl carboxylate) **10** readily react with cysteine, *N*-acetylcysteine, and GSH, but not with serine, lysine, or *N*-acetyllysine on the basis of comparative losses analyzed by GC of these substrates in the organic extracts (data not given). The reaction of bicyclic diene **10** with *N*-acetylcysteine yields conjugates **20** and **21** in a 1:1 ratio, and furan is also detected by GC/MS. The reaction of the corresponding acetylene (**16**) with equimolar *N*-acetylcysteine also gives conjugates **20** and **21** (identified by coinjection on HPLC), but in a 1:3 ratio. Bicyclic diene **9** reacts with equimolar GSH at pH 7.4 to give not only furan but also conjugates **22** and **23** in a 3:1 ratio and a quantitative yield based on HPLC analysis. Geometrical isomer assignments for **20**−**23**, isolated by HPLC from the bicyclic diene reactions, are based on differences in the ^1H chemical shift for the olefinic proton, *i.e.*, this proton in the *cis* isomers (**20** and **22**) is shielded by about 0.5 ppm compared with that in the *trans* isomers (5.96 and 6.03 ppm for **20** and **22**, respectively, versus 6.45 and 6.49 ppm for **21** and **23**, respectively). In ^{13}C NMR, the olefinic carbon bonded to sulfur is shielded by 6 ppm in the *cis* isomers (**20** and **22**) compared with the *trans* isomers (**21** and **23**), *i.e.*, 116.15 and 115.82 ppm, respectively, versus 122.19 and 122.83 ppm, respectively. The mixture of GSH adducts derived from **9** is not toxic to mice ip at 250 mg/kg (equivalent to >120 mg/kg for the parent **9**).

Table 5. Relative Reactivities of BSA, Hemoglobin, Myoglobin, and Chymotrypsin with Dimethyl 7-Oxabicyclo[2.2.1]hepta-2(3),5(6)-dienedicarboxylate Alone and in the Presence of an Esterase Inhibitor

protein	relative reactivity[a]		proposed reactive site
	alone	PSCP	
albumin	+++	+++	cysteine
hemoglobin	+++	+++	cysteine
myoglobin	±	−	none
chymotrypsin	+++	−	serine

[a] Reaction for 30 min at 37 °C. Extent of reaction in pH 7.4 phosphate (%): +++, 80−90; ++, 60−70; ±, 10−15; −, <5.

Reactivity in the Presence of Albumin (Table 4). Upon incubation with BSA, bicyclic dienes **9** and **10** react rapidly, **12** reacts more slowly, and **11** and **13** do not react, paralleling their relative reactivities with nitrobenzenethiol. Acetylenes **15**, **16**, and **18** react rapidly with BSA, **19** reacts more slowly, and **17** does not react, following the pattern of the corresponding dienes, except for the bis(dimethyl phosphonate) **19**. Interestingly, bicyclic dienes **9**, **10**, and **12**, which react readily with BSA, are toxic, whereas adducts **11** and **13**, which do not react with BSA, are not toxic (Table 3).

Further information is available on the nature of the reaction of BSA with bicyclic dienes. The analogous oxabicyclohept-2(3)-enes (**6** and **7**) are not reactive (data not given). Furan is detected by head-space analysis from **9** and **10** with BSA, but not without BSA. The reaction of diene adducts **9** and **10** is complete in 1 h with equimolar compound to BSA and in 24 h with a 20:1 molar ratio. Acetylenes **15** and **16** (5 molar equiv) react completely with BSA in 1 h. In the reaction of bicyclic dienes **9**−**13** with BSA, the corresponding acetylene derivatives **15**−**19** are never observed. In each case, the substrate is stable for the designated time in the absence of BSA, and the two preparations of BSA examined do not differ in their reactivities.

Reactivity in the Presence of Other Proteins (Table 5). Albumin, hemoglobin, and chymotrypsin almost completely degrade bicyclic diene **9**, whereas myoglobin is much less active or inactive. Furan was detected with hemoglobin but not with myoglobin. Heat-denatured albumin and hemoglobin do not release furan from dienes. Chymotrypsin is no longer effective when treated with the esterase inhibitor PSCP.

Metabolism in Mice. The reactivities of the dimethyl (**9**) and diethyl (**10**) oxabicycloheptadienedicarboxylates and the analogous acetylenes (**15** and **16**, respectively) are established by the failure to recover them from the livers of mice 5−60 min after ip administration at 250 mg/kg or even from liver homogenates fortified as in a recovery analysis. The corresponding experiments with

the *tert*-butyl diene led to recovery of the parent compound (**11**) but not its acetylene derivative (**17**), whereas the acetylene was recovered on direct administration. Total loss of the bicyclic dienes and acetylenes with bis(methyl carboxylate) substituents (**9** and **15**) probably is not due to esteratic cleavage since a 30-min pretreatment with PSCP at 100 mg/kg does not lead to recovery of the administered esters.

Discussion

The discontinuous structure−activity relationships reveal two types of toxic bicyclic adducts. One set consists of the oxabicycloheptane derivatives and an oxabicyclohept-5(6)-ene analog, which probably poison as PP2A inhibitors. The second set contains selected 2,3-disubstituted oxabicycloheptadienes with markedly altered substituent requirements and toxicity not associated with PP2A inhibition. Attention therefore was focused on the oxabicycloheptadienes because their mode of action is unknown. GC analyses provided a clue to the difference between the two sets in that the first series of toxic adducts is stable on GC, whereas the bicyclic dienes readily undergo thermal dissociation by retro-Diels−Alder reaction to liberate the corresponding acetylenedicarboxylates.

Dimethyl acetylenedicarboxylate is of relatively high toxicity to rats with both oral and dermal treatments (*8*, *9*). The present study uses mice to compare the substituent effects on the toxicity of the bicyclic dienes and the corresponding acetylene derivatives. There is a clear relationship for LD_{50}'s of the diene adducts and acetylenes in three cases [bis(methyl carboxylate), bis(ethyl carboxylate), and diethyl phosphonate/ethyl carboxylate], and the dicarboxylic acids are nontoxic as tested in both series, leading to the hypothesis that toxic bicyclic dienes are protoxicants for the acetylene derivatives. An apparent anomaly, that oxabicycloheptadienes with bis(*tert*-butyl carboxylate) and bis(dimethyl phosphonate) substituents have low toxicity but their acetylene counterparts are very toxic, might be due to the structural specificity of a bioactivation site, for which BSA was examined as a possible model. Importantly, only the toxic bicyclic dienes readily react with BSA, and the low-toxicity bis(*tert*-butyl carboxylate) and bis(dimethyl phosphonate) do not. A similar relationship is indicated for reactions *in vivo* or with liver homogenate since toxic bicyclic dienes with bis(methyl carboxylate) and bis(ethyl carboxylate) substituents are very reactive, while the nontoxic bis(*tert*-butyl carboxylate) analog is more stable and does not yield detectable amounts of di-*tert*-butyl acetylenedicarboxylate (which, in contrast to its methyl and ethyl analogs, would be detected if it was formed). The protoxicant hypothesis thus applies to the bicyclic dienes rapidly reacting with bis(methyl carboxylate), bis(ethyl carboxylate), and diethyl phosphonate/ethyl carboxylate substituents, but not to the less reactive bis(*tert*-butyl carboxylate), bis(dimethyl phosphonate) and dicarboxylic acid analogs, with possible stabilization by steric and electronic effects.

Proposed reactions and products for the bicyclic dienes and acetylene derivatives with *N*-acetylcysteine and GSH involve nucleophilic attack of the sulfur on the double bond followed by the departure of furan (Figure 3). The nucleophilicity of the thiol group is important since cysteine, *N*-acetylcysteine, and GSH react readily, but serine, lysine, and *N*-acetyllysine do not. The driving

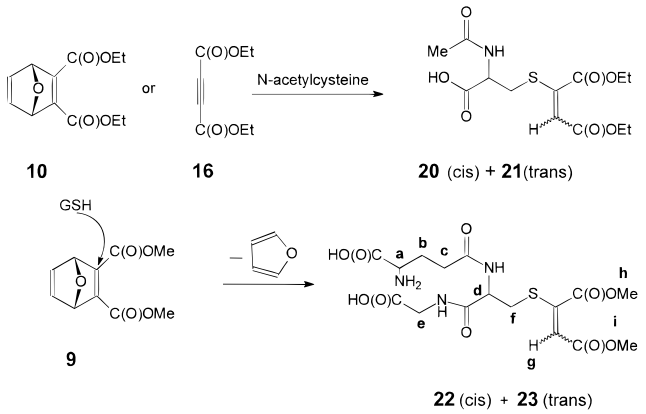


Figure 3. Reactions of diethyl 7-oxabicyclo[2.2.1]hepta-2(3),5-(6)-diene-2,3-dicarboxylate (**10**) and diethyl acetylenedicarboxylate (**16**) with *N*-acetylcysteine and of dimethyl 7-oxabicyclo-[2.2.1]hepta-2(3),5(6)-diene-2,3-dicarboxylate (**9**) with GSH. Dimethyl acetylenedicarboxylate (**15**) also reacts directly with GSH. Protons in structures **22** and **23** are designated as a−i.

force is the formation of the relatively stable leaving group (furan), since similar reactions do not occur for the 2,3-dehydro derivatives (**5**−**7**). Addition of *N*-acetylcysteine and GSH to the acetylenedicarboxylate is analogous to known additions of cysteine to acetylenedicarboxamide (an antibiotic produced by *Streptomyces chibaensis*, effective in the control of rice bacterial leaf blight) (*16*) and of GSH to the triple bond of 4-hydroxy-3-methyl-2-(2-propynyl)-cyclopent-2-en-1-one (an insecticide hydrolysis product) (*17*). Reactions of GSH with the bicyclic dienes and acetylene derivatives in this study may serve as detoxification mechanisms.

The bicyclic dienes are readily degraded by two proteins with free thiol substituents (albumin and hemoglobin), but not by two others lacking a free thiol (myoglobin and chymotrypsin) (*18*), provided that the latter two proteins are assayed with PSCP. The corresponding dimethyl oxabicyclohept-2(3)-enedicarboxylate and a diethyl phosphonate/ethyl carboxylate analog do not react with albumin, indicating that the loss upon incubation with this protein is not attributable to the carboxylic acid ester substituent, to the 2,3-double bond, or to nonspecific binding. Chymotrypsin readily degrades dimethyl oxabicycloheptadienedicarboxylate, but this reaction is blocked by PSCP, consistent with ester hydrolysis by this serine protease.

Earlier studies in this laboratory established the importance of albumin in the bioactivation of an organophosphorus delayed neurotoxicant (*19*), in "enzymatic" detoxification of the methylcarbamate insecticide carbaryl (*20*), and in reaction with the decachloro miticide dienochlor in which derivatization occurs (*21*). Albumin may also play a role in the action of the bicyclic dienes with methyl and ethyl carboxylate substituents. Thus, BSA at a concentration approximating that in plasma readily reacts with these dienes and the corresponding acetylene derivatives are never observed, consistent with the greater reactivity of the purported product than of the substrate. Albumin, which contains a single free thiol (*18*), degrades several equivalents of the bicyclic adducts and corresponding acetylenes so that the derivatized thiol site in the macromolecule may be regenerated, as with an enzymatic reaction, even though the derivatives are more stable with small molecules such as the GSH and *N*-acetylcysteine conjugates.

The target site(s) for the toxic oxabicycloheptadienes may prove to be as complex, and possibly the same, as

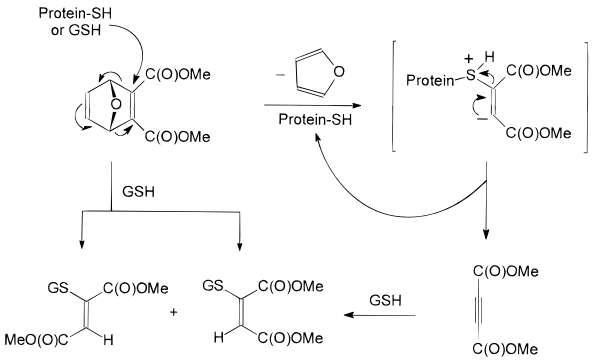


Figure 4. Retro-Diels−Alder-type reaction of dimethyl 7-oxabicyclo[2.2.1]hepta-2(3),5(6)-diene-2,3-dicarboxylate with a thiol-containing protein and addition of GSH to the bicyclic adduct and the liberated dimethyl acetylenedicarboxylate. The initial reaction with BSA, observed as substrate loss (and furan liberation in the case of the bis(methyl and bis(ethyl carboxylates)), also occurs for bicyclic adducts with 2,3-substituents of bis(ethyl carboxylate) and diethyl phosphonate/ethyl carboxylate, but not of dicarboxylic acid, bis(*tert*-butyl carboxylate) and bis(dimethyl phosphonate). The purported acetylene intermediates are not observed in reactions with BSA, presumably because they undergo further reaction faster than they are formed.

those for dimethyl acetylenedicarboxylate, including but not restricted to acetyl-CoA carboxylase and glucose-6-phosphate dehydrogenase (*9*). We propose that the toxicity of the oxabicycloheptadienedicarboxylates involves metabolic activation with liberation of the highly reactive acetylenedicarboxylates. This is the first case, to our knowledge, of a bioactivation mechanism involving a retro-Diels−Alder-type reaction (Figure 4).

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