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Design of novel bioisosteres of β-diketo acid inhibitors of HIV-1 integrase

Mario Sechi[1]*, Luciano Sannia[1], Fabrizio Carta[1], Michele Palomba[1], Roberto Dallocchio[2], Alessandro Dessì[2], Massimiliano Derudas[1], Zahrah Zawahir[3] and Nouri Neamati[3†]

[1]Dipartimento Farmaco Chimico Tossicologico, Università di Sassari, Sassari, Italy
[2]CNR-Istituto di Chimica Biomolecolare, sez. di Sassari, Li Punti, Italy
[3]Department of Pharmaceutical Sciences, University of Southern California, Los Angeles, CA, USA

Corresponding authors: *Tel: +39 079 228 753; Fax: +39 079 228 720; E-mail: mario.sechi@uniss.it
†Tel: +1 323 442 2341; Fax: +1 323 442 1390; E-mail: neamati@usc.edu

HIV-1 integrase (IN) is an attractive and validated target for the development of novel therapeutics against AIDS. Significant efforts have been devoted to the identification of IN inhibitors using various methods. In this context, through virtual screening of the NCI database and structure-based drug design strategies, we identified several pharmacophoric fragments and incorporated them on various aromatic or heteroaromatic rings. In addition, we designed and synthesized a series of 5-aryl(heteroaryl)-isoxazole-3-carboxylic acids as biological isosteric analogues of β-diketo acid containing inhibitors of HIV-1 IN and their derivatives. Further computational docking studies were performed to investigate the mode of interactions of the most active ligands with the IN active site. Results suggested that some of the tested compounds could be considered as lead compounds and suitable for further optimization.

Keywords: HIV-1 integrase inhibitors, lead compounds, drug discovery, high throughput docking, 3D-database, isoxazole carboxylic acids, bioisosterism

Introduction

Integration of viral DNA into host cell chromosomal DNA to form a provirus is an essential step in the viral life cycle (Brown, 1998). This process is mediated by integrase (IN), a 32 kDa viral enzyme, which catalyses two coordinated biochemical steps (Asante-Appiah *et al.*, 1997, 1999; Bushman *et al.*, 1991). Following reverse transcription in the cytoplasm of infected cells, IN first cleaves two nucleotides from the 3′-ends of the viral long-terminal repeats (‘3′-processing’). In the second step, after subsequent migration to the nucleus as a part of a large nucleoprotein complex, IN catalyses the insertion of the resulting shortened strands into a host chromosome by a direct transesterification reaction (‘strand transfer’). Because IN does not have a human homologue, it is considered a promising target for the development of new antiretroviral drugs and significant efforts have been devoted to identifying IN inhibitors (Pommier *et al.*, 1997, 2000; Neamati, 2001; Neamati *et al.*, 2000; d’Angelo *et al.*, 2001; De Clercq, 2000; Johnson *et al.*, 2004).

In recent years a plethora of IN inhibitors were identified by systematic screening of natural and synthetic products against purified enzyme (Neamati, 2002; Neamati *et al.*, 2001; Chen *et al.*, 2002; Dayam *et al.*, 2003). In particular, a class of compounds bearing a β-diketo acid moiety was independently discovered by the scientists from Shionogi & Co. and Merck as selective IN inhibitors (the structures of some representative compounds are shown in Figure 1; Hazuda *et al.*, 2000; Wai *et al.*, 2000; Pais *et al.*, 2002; Anthony, 2004; Sechi *et al.*, 2004). Several potent analogues were identified and two compounds, S-1360 (Shionogi & Co. Ltd; Billich, 2003) and L-870,810 (Merck & Co. Inc.; Hazuda *et al.*, 2004), are undergoing HIV-1 clinical trials.

In general, searching for IN inhibitors has involved testing compounds that inhibit other enzymes with similar mechanisms, conducting structure–activity relationships studies on known active compounds, performing database searches using pharmacophore models and high-throughput docking or random screening (Neamati *et al.*, 2002). In particular, three-dimensional structural database searching was successfully used to generate numerous lead compounds (Nicklaus *et al.*, 1997; Neamati *et al.*, 1997, 1998; Hong *et al.*, 1997, 1998; Chen *et al.*, 2000; Makhija *et al.*, 2004).

The aim of this work was to identify novel and/or unified pharmacophores required for activity in order to be

Figure 1. Structures of representative diketo acid-based IN inhibitors

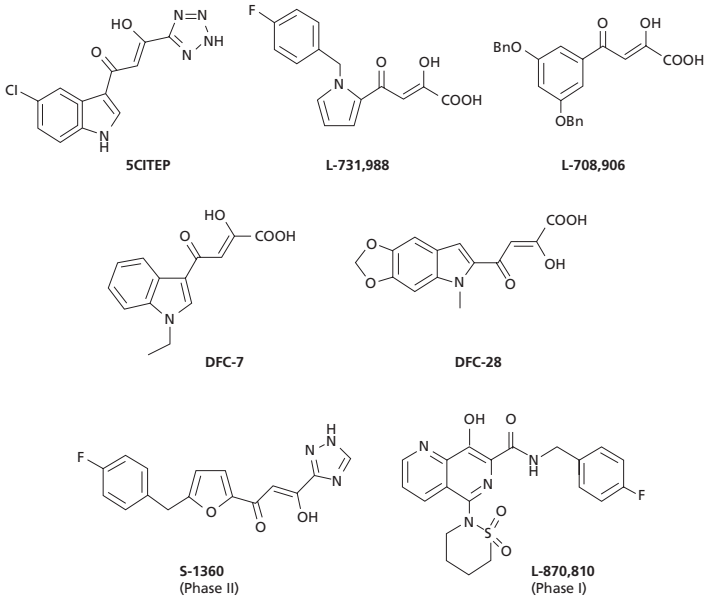


Figure 2. Design of target compounds

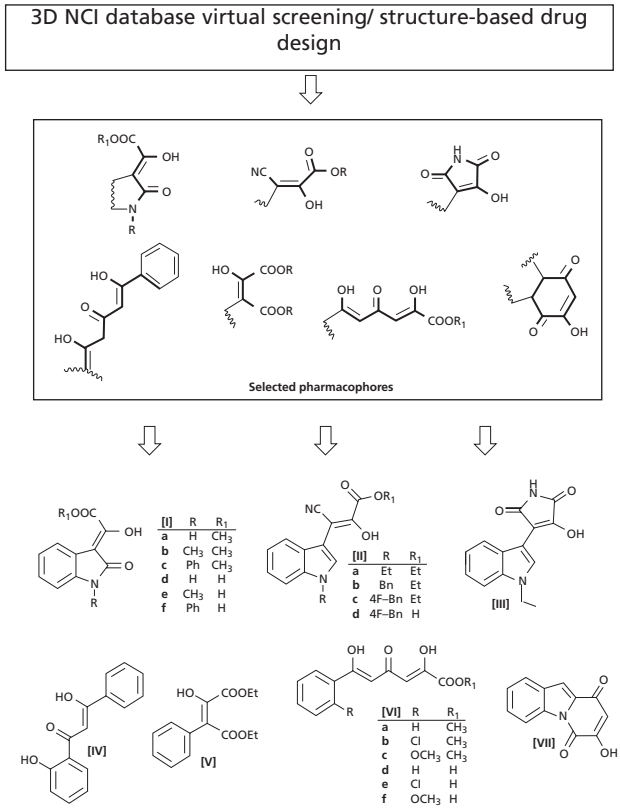
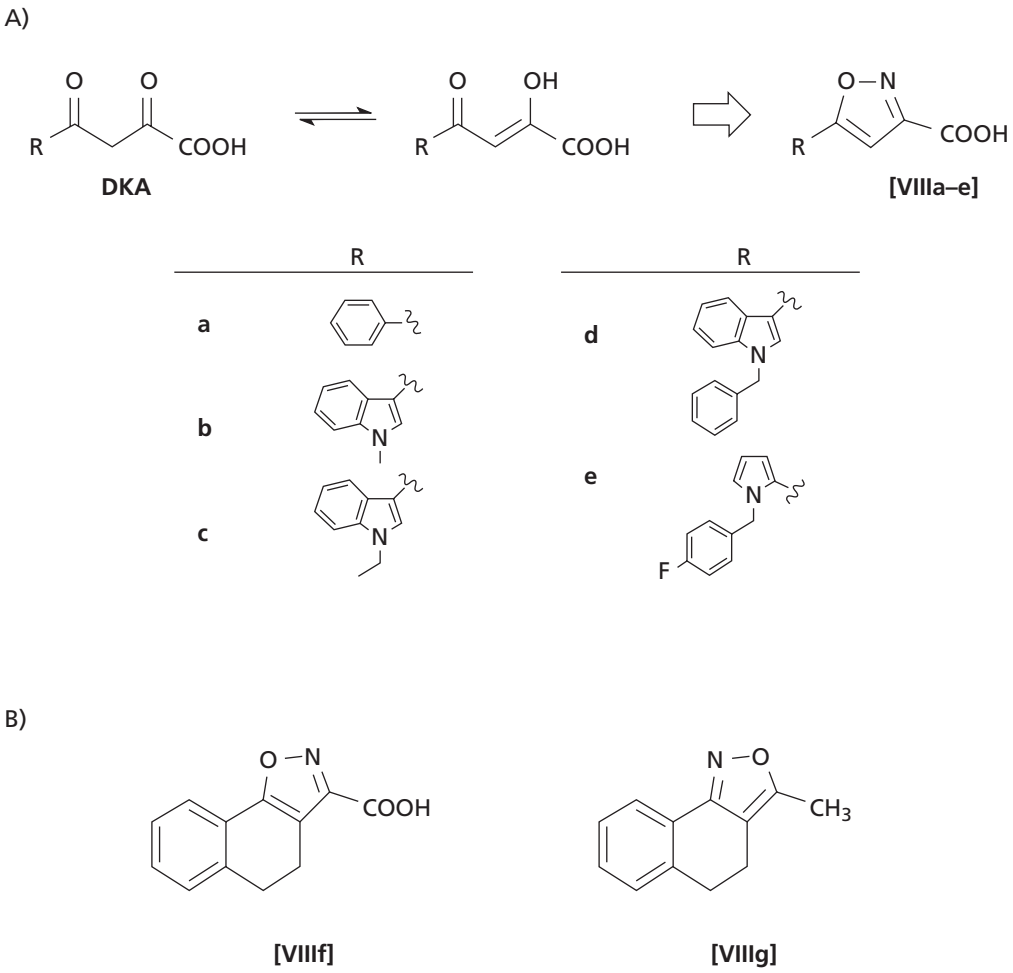


Figure 3. Design of compounds **[VIIIa–VIIIg]**



A, design of 5-aryl(heteroaryl)-isoxazole-3-carboxylic acids **[VIIIa–e]**; B, constrained compound **[VIIIf]** and its methyl-derivative isomer **[VIIIg]**.

implemented in potential lead compounds. Using the crystal structure of 5CITEP-IN complex (Goldgur *et al.*, 1999), we performed a computational 3D search in the nonproprietary, open part of the National Cancer Institute database (Milne *et al.*, 1994) to identify possible bioisosteres. Several structural platforms with potential as HIV-1 IN inhibitors were selected and their pharmacophoric fragments (Figure 2) incorporated into aromatic or heteroaromatic frameworks to give the general structures **[I–VII]**. Thus, target compounds were synthesized to evaluate their predicted potential activities. In order to obtain a consistent set of compounds, a series of derivatives of structure **[I]**, **[II]** and **[VI]** were also prepared.

Starting from the observation that the diketo acid functionality is not only responsible for antiviral activity, but also (unfortunately) contributes to cytotoxicity (Melek *et al.*, 2002), we thought that replacing the diketo group with a pertinent bioisostere (Patani *et al.*, 1996; Lipinski, 1986) endowed with reduced cytotoxicity was of paramount importance for discovering drugs targeting IN. In this context, as an extension of this work, we designed novel compounds by incorporating 1,3-diketo moiety (DKA) into a constrained isoxazole ring **[VIII]** and synthesized a series of 5-aryl(heteroaryl)-isoxazole-3-carboxylic acids **[VIIIa–e]** (Figure 3A). In addition, some modifications to the isoxazole framework were performed in order to establish a coherent structure–activity relationship among these compounds. In particular, we fixed the isoxazole ring into a coplanar tricyclic system and replaced the carboxylic function with an electron-donating group such as methyl to obtain compounds **[VIIIf]** and **[VIIIg]**, respectively (Figure 3B).

In this study, we present the design, synthesis and anti-IN activity of compounds **[I–VIII]** using a soluble mutant (F185KC280S). Moreover, we discuss the results of docking studies performed to determine the mode of binding between two of the more active compounds, **[IId]** and **[VIa]**, and some of the amino acid residues of the IN catalytic site.

Materials and methods

Chemistry

Anhydrous solvents and all reagents were purchased from Aldrich, Merck or Carlo Erba. Anhydrous diethyl ether was obtained by distillation from Na/benzophenone under nitrogen. All reactions involving air- or moisture-sensitive compounds were performed under nitrogen atmosphere using oven-dried glassware and syringe septa techniques to transfer solutions. Melting points (mp) were determined using an electrothermal melting point or a Köfler apparatus and are uncorrected. Infrared (IR) spectra were recorded as thin films or nujol mulls on NaCl plates with a Perkin-Elmer 781 IR spectrophotometer and are expressed in υ (cm^{-1}). Nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR, NOE difference and NOESY) spectra were determined in $CDCl_3$, DMSO or $CDCl_3$/DMSO (in ratio 3:1) and were recorded on a Varian XL–200 (200 MHz). Chemical shifts (δ scale) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) used as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double doublet. The assignment of changeable protons (O*H* and NH) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was done on Merck silica gel F–254 plates. For flash chromatography, Merck Silica gel 60 was used with a particle size 0.040–0.063 mm (230–400 mesh ASTM). Elemental analyses were performed on a Perkin-Elmer 2400 spectrometer at Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari (Italy), and were within ±0.4% of the theoretical values.

*General procedure for the preparation of (2Z)-hydroxy(2-oxo-1,2-dihydro-3H-indol-3-ylidene)carboxylic acids **[Id–If]**.* A mixture of the appropriate ester **[Ia–Ic]** (3 mmol) and 2N NaOH (5.5 eq) in methanol (10 ml) was stirred at room temperature until the reaction was complete (4–6 h). Then the solution was acidified with 1N HCl and the resulting precipitate was collected, washed with water and recrystallized from H_2O/EtOH for **[Id]** and **[If]** or from H_2O/MeOH for **[Ie]** to give yellow crystals.

*(2Z)-hydroxy(2-oxo-1,2-dihydro-3H-indol-3-ylidene)acetic acid **[Id]**.* Yield=38%; mp=275–277°C; IR (nujol) υ cm^{-1}=1640 (C=O amide), 1700 (C=O acid), 3360 (OH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 18.10 (brs, 1H, OH), 11.51 (brs, 1H, NH), 7.96 (d, 1H, Ar-H), 7.25 (t, 1H, Ar-H), 7.12–6.98 (m, 2H, Ar-H), 5.95-5.15 (brs, 1H, COOH). GC\MS *m/z* 205 (M^+). Anal. Calc. for $C_{10}H_7NO_4$: C, 58.54; H, 3.44; N, 6.83. Found: C, 58.58; H, 3.47; N, 6.80.

*(2Z)-hydroxy(1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)acetic acid **[Ie]**.* Yield=13%; mp=205–207°C; IR (nujol) υ cm^{-1}=1640 (C=O amide), 1700 (C=O acid), 3200 (OH). ^{1}H-NMR ($CDCl_3$) δ 17.95 (s, 1H, OH), 8.00 (d, 1H, Ar-H), 7.40-7.24 (m, 2H, Ar-H), 7.00 (d, 1H, Ar-H), 3.43 (s, 3H, N-CH_3), 1.82–1.40 (brs, 1H, COOH). GC\MS *m/z* 219 (M^+). Anal. Calc. for $C_{11}H_9NO_4$: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.41; H, 4.01; N, 6.53.

*(2Z)-hydroxy(2-oxo-1-phenyl-1,2-dihydro-3H-indol-3-ylidene)acetic acid **[If]**.* Yield=94%; mp=254–256°C; IR (nujol) υ cm^{-1}=1640 (C=O amide), 1700 (C=O acid), 3250 (OH). ^{1}H-NMR ($CDCl_3$) δ 17.60 (brs, 1H, OH), 8.06 (d, 1H, Ar-H), 7.70–7.21 (m, 7H, Ar-H), 6.90 (d, 1H, Ar-H). GC\MS *m/z* 281 (M^+). Anal. Calc. for $C_{16}H_{11}NO_4$: C, 68.32; H, 3.94; N, 4.98. Found: C, 68.57; H, 3.69; N, 5.14.

*General procedure for the preparation of methyl (2Z)-hydroxy(2-oxo-1,2-dihydro-3H-indol-3-ylidene)acetates **[Ia–Ic]**.* To a suspension of sodium methoxide (1 eq for **[Ia]** and **[Ib]** or 1.4 eq for **[Ic]**) in anhydrous methanol (50 ml) was added the appropriate oxindole **[3a–3c]** (1 eq) and dimethyloxalate (1.5 eq for **[Ia]**, 1 eq for **[Ib]** or 2 eq for **[Ic]**) and the mixture was stirred (at room temperature for **[Ia,Ib]** or reflux for **[Ic]**) under nitrogen atmosphere for 3 h (for **[Ia]**), 5 h (for **[Ib]**) or 3.5 h (for [**Ic**]). Then water was added and the mixture was acidified with 1N HCl. The resulting precipitate was filtered, washed with water and recrystallized from H_2O/EtOH (for **[Ia]** and **[Ib]**) or from H_2O/MeOH (for **[Ic]**) to give yellow crystals.

*Methyl (2Z)-hydroxy(2-oxo-1,2-dihydro-3H-indol-3-ylidene)acetate **[Ia]**.* Yield=36%; mp=268–270°C; IR (nujol) υ cm^{-1}=1635 (C=O amide), 1690 (C=O ester), 3220 (NH), 3410 (OH). ^{1}H-NMR ($CDCl_3$) δ 17.80 (brs, 1H, OH), 8.24 (d, 1H, Ar-H), 8.01 (brs, 1H, NH), 7.27 (t, 1H, Ar-H), 7.13 (t, 1H, Ar-H), 6.97 (s, 1H, Ar-H), 4.03 (s, 3H, OCH_3). GC\MS *m/z* 219 (M^+). Anal. Calc. for $C_{11}H_9NO_4$: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.39; H, 4.05; N, 6.48.

*Methyl (2Z)-hydroxy(1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene)acetate **[Ib]*** (Long *et al.*, 1978). Yield= 31%; mp=97–99°C; IR (nujol) υ cm^{-1}=1610 (C=O, amide),

1650 (C=O, ester), 2375 (OH). ^{1}H-NMR ($CDCl_3$) δ 17.91 (brs, 1H, OH) 8.26 (d, 1H, Ar-H), 7.32 (t, 1H, Ar-H), 7.14 (t, 1H, Ar-H), 6.95 (d, 1H, Ar-H), 4.02 (s, 3H, OCH_3), 3.38 (s, 3H, NCH_3). GC\MS *m/z* 233 (M^+). Anal. Calc. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.91; H, 4.50; N, 5.83.

Methyl (2Z)-hydroxy(2-oxo-1-phenyl-1,2-dihydro-3H-indol-3-ylidene)acetate ***[Ic]****.* Yield=37%; mp=110–112°C; IR (nujol) υ cm^{-1}=1655 (C=O amide), 1735 (C=O ester), 2220 (OH). ^{1}H-NMR ($CDCl_3$) δ 17.02 (brs, 1H, OH), 8.34 (d, 1H, Ar-H), 7.78-7.43 (m, 4H, Ar-H), 7.32–7.12 (m, 3H, Ar-H), 6.92 (d, 1H, Ar-H), 4.06 (s, 3H, OCH_3). GC\MS *m/z* 295 (M^+). Anal. Calc. for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.22; H, 4.25; N, 4.93.

Preparation of N-alkyl oxindoles ***[3b]*** *and* ***[3c]****.* The appropriate intermediate **[2b]** and **[2c]** (60 mmol) and alluminium chloride (2.0 eq for **[3b]** or 2.3 eq for **[3c]**) were mixed and introduced into a round-bottomed flask when the internal temperature reached 180–190°C. Heating was kept for 10 min. Melted mass was slightly cooled at room temperature and immediately worked up with crushed ice and 1N HCl. The brown product was washed with water and purified by EtOH recrystallization (for **[3b]**) or by flash chromatography using ethyl acetate as eluent (for **[3c]**).

1-methyl-1,3-dihydro-2H-indol-2-one ***[3b]*** *(Hennessy et al., 2003).* Yield=28%; mp=87–89°C; IR (nujol) υ cm^{-1}= 1690 (C=O amide). ^{1}H-NMR ($CDCl_3$) δ 7.26 (t, 2H, Ar-H), 7.04 (t, 1H, Ar-H), 6.82 (d, 1H, Ar-H), 3.53 (s, 2H, CH_2), 3.21(s, 3H, N-CH_3). GC\MS *m/z* 147 (M^+).

1-phenyl-1,3-dihydro-2H-indol-2-one ***[3c]*** *(Sarges et al., 1989).* Yield=56%; mp=116–118°C; IR (nujol) υ cm^{-1}=1710 (C=O amide). ^{1}H-NMR ($CDCl_3$) δ 7.68–7.05 (m, 8H, Ar-H), 6.79 (d, 1H, Ar-H), 3.73 (s, 2H, CH_2). GC\MS *m/z* 209 (M^+).

Preparation of 2-chloro-N-methyl-N-phenylacetamide ***[2b]*** *and 2-chloro-N,N-diphenylacetamide* ***[2c]****.* A solution of the appropriate *N*-phenylamine **[1b]** and **[1c]** (5 mmol) in anhydrous toluene (20 ml) and chloroacetylchloride (2 eq) was refluxed for 1–2 h under nitrogen atmosphere. Then the mixture was cooled and solvent removed *in vacuo*. The residue was recrystallized from water (for **[2b]**) or from ethanol (for **[2c]**).

2-chloro-N-methyl-N-phenylacetamide ***[2b]*** *(Chupp et al., 1967).* Yield=93%; mp=160–161°C; IR (nujol) υ cm^{-1}=1660 (C=O amide). ^{1}H-NMR ($CDCl_3$) δ 8.33 (s, 3H, N-CH_3), 7.28–7.17 (m, 3H, Ar-H), 7.25 (d, 2H, Ar-H), 3.86 (s, 2H, CH_2). GC\MS *m/z* 183 (M^+).

2-chloro-N,N-diphenylacetamide ***[2c]*** *(Sarges et al., 1989).* Yield=78%; mp=115–117°C; IR (nujol) υ cm^{-1}= 1665 (C=O amide). ^{1}H-NMR ($CDCl_3$) δ 7.50–7.26 (m, 10H, Ar-H), 4.03 (s, 2H, CH_2). GC\MS *m/z* 245 (M^+).

Preparation of ethyl [2E]-3-cyano-3-(1-alkyl-1H-indol-3-yl)-2-hydroxyacrylates ***[IIa–IIc].*** To a solution of the appropriate indole acetonitrile **[5a–5c]** (15 mmol) and 99% diethyloxalate (1.5 eq for **[IIa]** or 2 eq for **[IIb,IIc]**) in anhydrous DMF (30 ml for **[IIa]**) or anhydrous diethyl ether (70 ml for **[IIb, IIc]**), potassium *tert*-butoxyde (2 eq for **[IIa]**) or 60% NaH in mineral oil (1.5 eq for **[IIb,IIc]**) was added and the mixture was stirred at room temperature for 2 h **[IIa]** or for 15 h **[IIb,IIc]** under nitrogen atmosphere. Then water was added, the mixture was acidified with 2N HCl and the solution was extracted with diethyl ether. Finally, the organic layer was washed with water, dried and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography (eluents petroleum ether-ethyl acetate 6:4). The orange solid obtained was recrystallized from ethanol to provide the desired **[IIa–IIc]**.

Ethyl [2E]-3-cyano-3-(1-ethyl-1H-indol-3-yl)-2-hydroxyacrylate ***[IIa]****.* Yield=70%; mp=108–110°C; IR (nujol) υ cm^{-1}=1715 (C=O ester), 2200 (CN), 3240 (OH). ^{1}H-NMR ($CDCl_3$) δ 8.38 (d, 1H, Ar-H), 7.90 (s, 1H, Ar-H), 7.45–7.23 (m, 3H, Ar-H), 4.57 (q, 2H, OCH_2), 4.22 (q, 2H, NCH_2), 1.50 (t, 6H, CH_3×2). GC\MS *m/z* 284 (M^+). Anal. Calc. for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.72; H, 5.84; N, 9.61.

Ethyl [2E]-3-(1-benzyl-1H-indol-3-yl)-3-cyano-2-hydroxyacrylate ***[IIb]****.* Yield=72%; mp=110–112°C; IR (nujol) υ cm^{-1}=1720 (C=O ester), 2225 (CN), 3250 (OH). ^{1}H-NMR ($CDCl_3$) δ 8.40–8.35 (m, 1H, Ar-H); 7.93 (s, 1H, Ar-H), 7.37–7.08 (m, 8H, Ar-H), 5.36 (s, 2H, CH_2), 4.53 (q, 2H, OCH_2), 1.50 (t, 3H, CH_3). GC\MS *m/z* 346 (M^+). Anal. Calc. for $C_{21}H_{18}N_2O_3$:C, 72.82; H, 5.24; N, 8.09. Found: C, 72.66; H, 5.11; N, 7.85.

Ethyl [2E]-3-cyano-3-[1-(4-fluorobenzyl)-1H-indol-3-yl]-2-hydroxyacrylate ***[IIc]****.* Yield=64%; mp=130–132°C; IR (nujol) υ cm^{-1}=1730 (C=O ester), 2210 (CN), 3235 (OH). ^{1}H-NMR ($CDCl_3$) δ 8.42–8.35 (m, 1H, Ar-H), 7.33–7.22 (m, 2H, Ar-H), 7.15–6.96 (m+dd, 5H, Ar-H), 5.34 (s, 2H, CH_2), 4.54 (q, 2H, OCH_2), 1.51 (t, 3H, CH_3). GC\MS *m/z* 364 (M^+). Anal. Calc. for $C_{21}H_{17}FN_2O_3$: C, 69.22; H, 4.70; N, 7.69. Found: C, 69.45; H, 4.61; N, 7.77.

*Preparation of [2E]-3-cyano-3-[1-(4-fluorobenzyl)-1H-indol-2-yl]-2-hydroxyacrylic acid **[IId]**.* A solution of **[IIc]** (2.2 mmol) and 2N NaOH (4 eq) in methanol (30 ml) was stirred at room temperature for 5 h. After dilution with water the mixture was acidified with 1N HCl. The precipitate that formed was filtered off and purified by silica gel flash column chromatography (eluents petroleum ether-ethyl acetate 6:4) and then recrystallized from H_2O/EtOH to give orange crystals. Yield=54%; mp=101–102°C; IR (nujol) υ cm^{-1}=1695 (C=O ester), 2200 (CN), 3330 (COOH+OH). ^{1}H-NMR ($CDCl_3$) δ 8.40–8.33 (m, 1H, Ar-H), 7.91 (s, 1H, Ar-H), 7.33–7.22 (m, 2H, Ar-H), 7.15-6.95 (m+dd, 5H, Ar-H), 5.33 (s, 2H, CH_2). GC\MS *m/z* 336 (M^+). Anal. Calc. for $C_{19}H_{13}FN_2O_3$: C, 67.85; H, 3.90; N, 8.33. Found: C, 67.98; H, 3.77; N, 8.42.

*Preparation of ethyl [2E]-3-cyano-3-[1-(4-fluorobenzyl)-1H-indol-2-yl]-2-methoxyacrylate **[IIe]**.* Dimethylsulphate (1.2 eq) was added during 10 min, under nitrogen atmosphere, to a stirred solution of **[IIc]** (2 mmol) in dry acetone (8 ml) containing anhydrous potassium carbonate (1.15 eq), mantained under gentle reflux. The mixture was heated under reflux for 2 h, then cooled and filtered. Evaporation of the acetone left a residue which was purified by silica gel flash column chromatography (eluents petroleum ether-ethyl acetate 7:3) to give a yellow oil. Yield=58%; mp=oil at room temperature; IR (nujol) υ cm^{-1}=1720 (C=O ester), 2200 (CN). ^{1}H-NMR ($CDCl_3$) δ 8.23–8.18 (m, 1H, Ar-H), 7.72 (s, 1H, Ar-H), 7.30–7.18 (m, 2H, Ar-H), 7.13–6.96 (m+dd, 5H, Ar-H), 5.31 (s, 2H, CH_2), 4.46 (q, 2H, OCH_2), 3.83 (s, 3H, OCH_3), 1.46 (t, 3H, CH_3). GC\MS *m/z* 378 (M^+). Anal. Calc. for $C_{22}H_{19}FN_2O_3$: C, 69.83; H, 5.06; N, 7.40. Found: C, 70.04; H, 5.16; N, 7.22.

*Preparation of (1-alkyl-1H-indol-3-yl)acetonitriles **[5a–5c]**.* A suspension of crushed KOH pellets (4 eq) in DMSO (40 ml) was magnetically stirred for 5 min and then **[4]** was added (19 mmol). The mixture was stirred at room temperature for 45 min. Finally the appropriate alkyl bromide (1.1 eq; bromoetane for **[5a]**, benzylbromide for **[5b]** or 4-fluoro-benzylbromide for **[5c]** was added and stirring was continued for 30 min. When reaction was complete it was quenched by the addition of water and the solution was extracted with diethyl ether. The organic layers were then washed with water, dried over Na_2SO_4 and solvent was removed *in vacuo* to dryness. The residue was purified by flash chromatography (eluents petroleum ether-ethyl acetate 8:2) to give the desired **[5a–c]**.

*(1-ethyl-1H-indol-3-yl)acetonitrile **[5a]**.* Yield=90%; mp=oil at room temperature; IR (nujol) υ cm^{-1}=2200 (CN). ^{1}H-NMR ($CDCl_3$) δ 7.55 (d, 1H, Ar-H), 7.41–7.12 (m, 3H, Ar-H), 4.15 (q, 2H, CH_2), 3,79 (s, 2H, CH_2CN), 1.47 (t, 3H, CH_3). GC\MS *m/z* 184 (M^+).

*(1-benzyl-1H-indol-3-yl)acetonitrile **[5b]** (Jahangir et al., 1987).* Yield=50%; mp=93–95°C; IR (nujol) υ cm^{-1}=2235 (CN). ^{1}H-NMR ($CDCl_3$) δ 7.59 (d, 1H, Ar-H), 7.33–7.09 (m, 8H, Ar-H), 5.29 (s, 2H, CH_2), 3.83 (s, 2H, CH_2CN). GC\MS *m/z* 246 (M^+).

*[1-(4-fluorobenzyl)-1H-indol-3-yl]acetonitrile **[5c]**.* Yield=55%; mp=88–90°C; IR (nujol) υ cm^{-1}=2230 (CN). ^{1}H-NMR ($CDCl_3$) δ 7.60 (d, 1H, Ar-H), 7.29–6.95 (m+dd, 7H, Ar-H), 5.27 (s, 2H, CH_2), 3.84 (s, 2H, CH_2CN). GC\MS *m/z* 264 (M^+).

*Preparation of 3-(1-ethyl-1H-indol-3-yl)-4-hydroxy-1H-pyrrole-2,5-dione **[III]**.* A solution of **[IIa]** (3.5 mmol) and methansulphonic acid (79 mmol) was stirred at room temperature for 15 h. Then 80% ethanol (10 ml) was added and the mixture was allowed to stir at room temperature for 3 h longer. The solution was filtered and evaporated *in vacuo* to dryness. The residue was purified by flash chromatography (eluents petroleum ether-ethyl acetate 1:1) and recrystallized from H_2O/EtOH. Yield=20%; mp=94–95°C; IR (nujol) υ cm^{-1}=3300 (NH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 9.49–9.41 (brs, 1H, NH), 8.32 (d, 1H, Ar-H), 7.86 (s, 1H, Ar-H), 7.45–7.10 (m, 3H, Ar-H), 4.22 (q, 2H, CH_2), 1.52 (t, 3H, CH_3). GC\MS *m/z* 256 (M^+). Anal. Calc. for $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 65.77 ; H, 4.55; N, 11.08.

*Preparation of [2Z]-3-hydroxy-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one **[IV]** (Adam, 1993).* To a suspension of 60% NaH in mineral oil (1.0 eq) in anhydrous THF (10 ml), a solution of **[7]** (3.4 mmol) dissolved in anhydrous THF (5 ml) was added. The mixture was stirred at room temperature under nitrogen atmosphere for 30 min. When an internal temperature of 0°C was achieved, benzoylchloride (1 eq) was added and the mixture was stirred at the same temperature for 1 h. Then a solution of potassium *t*-butoxyde/*t*-butyl alcohol complex (2 eq) was added rapidly. After 15 h glacial acetic acid (2 eq) and water was added. The mixture was concentrated *in vacuo* and the solid residue purified by flash chromatography (eluents petroleum ether-ethyl acetate 4:6). Finally the solid was triturated with petroleum ether-diethyl ether to give a green powder. Yield=66%; mp=116–118°C. IR (nujol) υ cm^{-1}=1630 (ketone), 3340 (OH). ^{1}H-NMR ($CDCl_3$) δ 15.55 (s, 1H, OH), 12.11 (s, 1H, OH), 7.98 (d, 2H, Ar-H), 7.85 (d, 1H, Ar-H), 7.62–7.39 (m, 3H, Ar-H), 7.03–6.85 (m, 4H, Ar-H). GC\MS *m/z* 240 (M^+). Anal. Calc. for $C_{15}H_{12}O_3$: C, 74.99; H, 5.03. Found: C, 75.24; H, 5.32.

*Preparation of 1-(2-hydroxyphenyl)ethanone **[7]**. (Abilgaard et al., 1998).* A solution of **[6]** (1.0 eq) in anhydrous dichloromethane was stirred under nitrogen atmosphere at −30°C for 5 min. Then 1M BBr_3 solution in dichloromethane (1.05 eq) was added and the mixture was stirred at −30°C for 12 h. After additional 8 h of stirring at room temperature, the reaction was quenched by addition of methanol and washed three times with methanol. Finally solvent was removed *in vacuo* to dryness and the residue purified by flash chromatography (eluents petroleum ether-ethyl acetate 9:1). Yield=26%; mp=57–59°C; IR (nujol) υ cm^{-1}=1730 (ketone), 3330 (OH). ^{1}H-NMR ($CDCl_3$) δ 12.27 (s, 1H, OH), 7.72 (d, 1H, Ar-H), 7.48 (t, 1H, Ar-H), 7.00–6.87 (m, 2H, Ar-H), 2.04 (s, 1H, CH_3). GC\MS *m/z* 136 (M^+).

*Preparation of diethyl (2E)-2-hydroxy-3-phenylbut-2-enedioate **[V]** (House et al., 1968).* To a mixture of 60% NaH in mineral oil (2 eq), diethyloxalate (1 eq) and absolute ethanol (0.1 eq) in toluene (15 ml), was added **[8]** (12 mmol) dissolved in toluene (5 ml). The reaction was stirred at room temperature for 12 h. Then glacial acetic acid (4.5 eq) was added and the mixture was concentrated to dryness *in vacuo*. The residue was purified by flash chromatography (eluents petroleum ether-ethyl acetate 8:2) to give an oil. Yield=14%; mp=oil at room temperature; IR (nujol) υ cm^{-1}=1690 (ester), 1730 (ester), 3280 (OH). ^{1}H-NMR ($CDCl_3$) δ 12.83 (s, 1H, OH), 7.51–7.12 (m, 5H, Ar-H), 4.35-4.15 (m, 4H, OCH_2×2), 1.35–1.19 (m, 6H, CH_3 x2). GC\MS *m/z* 264 (M^+). Anal. Calc. for $C_{14}H_{16}O_5$: C, 63.63; H, 6.10. Found: C, 63.72; H, 5.88.

*General preparation of 6-aryl-2,4,6-trioxohexanoic acids **[VId–VIf]**.* The appropriate trioxohexanoic methyl ester **[VIa–VIc]** (1 mmol) was added to a solution of water (6 ml) and 37% HCl (3 ml). The mixture was heated at 55°C for 6–8 h under stirring. The resulting pale yellow solid was filtered and washed with acetone.

*(2Z,5Z)-2,6-dihydroxy-4-oxo-6-phenylhexa-2,5-dienoic acid **[VId]** (Stiles et al., 1991).* Yield=42%; mp= 225–226°C dec; IR (nujol) υ cm^{-1}=1630 (ketone), 1721 (acid). ^{1}H-NMR ($CDCl_3$) δ 7.90–7.87 (m, 2H, Ar-H), 7.54–7.51 (m, 3H, Ar-H), 7.11 (s, 1H, C=CH), 6.87 (s, 1H, CH=C). GC\MS *m/z* 234 (M^+). Anal. Calc. for $C_{12}H_{10}O_5$: C, 61.54; H, 4.30. Found: C, 61.25; H, 4.58.

*(2Z,5Z)-6-(2-chlorophenyl)-2,6-dihydroxy-4-oxohexa-2,5-dienoic acid **[VIe]**.* Yield=38%; mp=227–229°C; IR (nujol) υ cm^{-1}=1640 (ketone), 1728 (acid). ^{1}H-NMR ($CDCl_3$-DMSO) δ 7.65–7.34 (m, 4H, Ar-H), 7.16 (s, 1H, C=CH), 6.78 (s, 1H, CH=C). GC\MS *m/z* 268 (M^+). Anal. Calc. for $C_{12}H_9ClO_5$: C, 53.65; H, 3.38, Cl, 13.20. Found: C, 53.41; H, 3.19, Cl, 13.50.

*(2Z,5Z)-6-(2-methoxyphenyl)-2,6-dihydroxy-4-oxohexa-2,5-dienoic acid **[VIf]**.* Yield=60%; mp=234–235°C dec; IR (nujol) υ cm^{-1}=1638 (ketone), 1720 (acid). ^{1}H-NMR ($CDCl_3$-DMSO) δ 7.84 (d, 1H, Ar-H), 7.54 (t, 1H, Ar-H), 7.18–7.07 (m, 2H, Ar-H+C=CH), 6.93 (s, 1H, CH=C), 3.92 (s, 3H, OCH_3). GC\MS *m/z* 264 (M^+). Anal. Calc. for $C_{13}H_{12}O_6$: C, 59.09; H, 4.58. Found: C, 58.83; H, 4.33.

*General preparation of 6-aryl-2,4,6-trioxohexanoic methyl esters **[VIa–VIc]**.* To a solution of 2M methylmagnesium carbonate in DMF (38 eq), the appropriate phenylmethylketone **[10a–10c]** (4 mmol for **[10a]** and **[10c]** or 3 mmol for **[10b]**) and dimethyloxalate (1.6 eq) were added. The mixture was heated at 170°C for 3 h (for **[VIa]** and **[VIc]**) or at 160°C for 2.5 h (for **[VIb]**) in a Claisen apparatus. When reaction was complete the mixture was put on crushed ice (~30 g) and acidified with 37% HCl while stirring. The solid products were filtrated, washed with water and recrystallized from H_2O/EtOH to give yellow or pale yellow crystals.

*Methyl (2Z,5Z)-2,6-dihydroxy-4-oxo-6-phenylhexa-2,5-dienoate **[VIa]**.* Yield=36%; mp=184–186°C; IR (nujol) υ cm^{-1}=1610 (ketone), 1710 (ester). ^{1}H-NMR ($CDCl_3$) δ 15.12 (brs, 1H, C=C-O*H*), 13.27 (brs, 1H, OH), 7.89 (d, 2H, Ar-H), 7.51–7.47 (m, 3H, Ar-H), 6.33 (s, 1H, C=CH), 6.13 (s, 1H, CH=C), 3.92 (s, 3H, CO_2CH_3). GC\MS *m/z* 248 (M^+). Anal. Calc. for $C_{13}H_{12}O_5$: C, 62.90; H, 4.87. Found: C, 62.77; H, 4.82.

*Methyl (2Z,5Z)-6-(2-chlorophenyl)-2,6-dihydroxy-4-oxohexa-2,5-dienoate **[VIb]**.* Yield=33%; mp=144–145°C; IR (nujol) υ cm^{-1}=1625 (ketone), 1721 (ester). ^{1}H-NMR (DMSO) δ 7.63 (d, 1H, Ar-H), 7.56–7.39 (m, 3H, Ar-H), 6.30 (s, 1H, C=CH), 6.08 (s, 1H, CH=C), 3.91 (s, 3H, CO_2CH_3). GC\MS *m/z* 282 (M^+). Anal. Calc. for $C_{13}H_{11}ClO_5$: C, 55.24; H, 3.92; Cl, 12.54. Found: C, 55.45; H, 4.15; Cl, 12.35.

*Methyl (2Z,5Z)-6-(2-methoxyphenyl)-2,6-dihydroxy-4-oxohexa-2,5-dienoate **[VIc]**.* Yield=17%; mp=219–220°C dec; IR (nujol) υ cm^{-1}=1600 (ketone), 1710 (ester). ^{1}H-NMR (DMSO) δ 7.96 (d, 1H, Ar-H), 7.48 (t, 1H, Ar-H), 7.09–6.99 (m, 2H, Ar-H), 6.54 (s, 1H, C=CH), 6.32 (s, 1H, CH=C), 3.94 (s, 3H, CO_2CH_3), 3.91 (s, 3H, OCH_3). GC\MS *m/z* 278 (M^+). Anal. Calc. for $C_{14}H_{14}O_6$: C, 60.43; H, 5.07. Found: C, 60.62; H, 5.18.

*Preparation of phenylmethylketones **[10b]** and **[10c]**.* The appropriate ketone **[9b]** and **[9c]** (6.5 mmol) and anhydrous ethyl acetate (2 eq) were added to a suspension of 60% NaH in mineral oil (2 eq) in anhydrous diethyl

ether (20 ml) under nitrogen atmosphere. The mixture was stirred at room temperature for 48 h (for **[10b]**) or at 50°C for 3 h (for **[10c]**). The solution was acidified by dropwise addition of 1N HCl, then extracted with diethyl ether. Finally organic layer was washed with water, dried and concentrated *in vacuo*. The residue was purified by flash chromatography (eluents petroleum ether-ethyl acetate 9.5:0.5) to give a pale yellow oil.

(3Z)-4-(2-chlorophenyl)-4-hydroxybut-3-en-2-one ***[10b]***. Yield=34%; mp=oil at room temperature; IR (nujol) υ cm^{-1}=1720 (ketone), 2750 (OH). ^{1}H-NMR ($CDCl_3$) δ 7.61–7.52 (m, 1H, Ar-H), 7.48–7.21 (m, 3H, Ar-H), 6.05 (s, 1H, C=CH), 2.19 (s, 3H, $COCH_3$). GC/MS *m/z* 196 (M^+).

(3Z)-4-hydroxy-4-(2-methoxyphenyl)but-3-en-2-one ***[10c]***. Yield=59%; mp=oil at room temperature; IR (nujol) υ cm^{-1}=1715 (ketone), 2720 (OH). ^{1}H-NMR ($CDCl_3$) δ 16.22 (brs, 1H, OH), 7.88 (d, 1H, Ar-H), 7.44 (t, 1H, Ar-H), 7.07–6.94 (m, 2H, Ar-H), 6.43 (s, 1H, C=CH), 3.92 (s, 3H, OCH_3), 2.19 (s, 3H, $COCH_3$). GC/MS *m/z* 192 (M^+).

Preparation of 7-hydroxypyrido[1,2-a]indole-6,9-dione ***[VII]***. A solution of **[13]** (5 mmol) and diethyloxalate (2 eq), anhydrous methanol (10 ml) and sodium methoxide (4 eq, generated from sodium in methanol) in THF (10 ml) was refluxed and stirred under nitrogen atmosphere for 15 h. Then the solution was acidified with 1 N H_2SO_4 and the yellow precipitate was filtered and recrystallized from H_2O/EtOH. Yield=19%; mp=213–215°C; IR (nujol) υ cm^{-1}=1645 (CO amide), 1710 (ester), 3325 (OH). ^{1}H-NMR (DMSO) δ 8.30 (d, 1H, Ar-H), 7.80 (d, 1H, Ar-H), 7.40–7.33 (m, 2H, Ar-H), 6.00 (s, 1H, C=CH). GC/MS *m/z* 213 (M^+). Anal. Calc. for $C_{12}H_7NO_3$: C, 67.61; H, 3.31, N 6.57. Found: C, 67.81; H, 3.11, N 6.84.

Preparation of 1-(1H-indol-2-yl)ethanone ***[13]***. A solution of 1.4 M methyllithium in diethyl ether (0.5 eq) was slowly added under nitrogen atmosphere to a suspension of **[12]** (1 eq) in anhydrous diethyl ether (30 ml) cooled at 0°C and the mixture was refluxed for 1 h. An additional 1.4 M methyllithium (0.5 eq) was added and the mixture was refluxed for 5 h. The reaction was then quenched by addition of saturated aqueous ammonium chloride, diluted with diethyl ether, and extracted with diethyl ether. Solvent was removed *in vacuo* and the solid residue purified by flash chromatography (eluents petroleum ether-ethyl acetate 8.5:1.5). Yield=58%; mp=151–153°C; IR (nujol) υ cm^{-1}=1715 (ketone), 3240 (NH). ^{1}H-NMR ($CDCl_3$) δ 9.81 (brs, 1H, NH), 7.70 (d, 1H, Ar-H), 7.44 (d, 1H, Ar-H), 7.38–7.02 (m, 3H, Ar-H), 2.55 (s, 3H, $COCH_3$). GC/MS *m/z* 159 (M^+).

Preparation of 1H-indole-2-carboxylic acid ***[12]***. (Katritzky *et al*., 1985) A suspension of the ester **[11]** (5 mmol) in 12% KOH aqueous solution (50 ml) was refluxed for 1 h. The clear solution was poured into crushed ice and acidified with 2N HCl. The white precipitate was filtered and washed with water. Yield=93%; mp= 207–209°C; IR (nujol) υ cm^{-1}=3260 (NH), 3840 (COOH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 7.64 (d, 1H, Ar-H), 7.48 (d, 1H, Ar-H), 7.26 (t, 1H, Ar-H), 7.17 (s, 1H, Ar-H), 7.09 (t, 1H, Ar-H). GC/MS *m/z* 161 (M^+).

General procedure for the preparation of isoxazole-carboxylic acids ***[VIIIa–VIIIf]***. A mixture of the appropriate ester **[15a–15f]** (1 mmol) in 12% KOH (5.2 eq) was stirred under reflux for 1 h. Then water was added and the solution was acidified with 2N HCl. The white precipitate that formed was filtered, washed with water and recrystallized from H_2O/EtOH.

5-phenylisoxazole-3-carboxylic acid ***[VIIIa]*** (King *et al*., 1972). Yield=34%; mp=181–182°C; IR (nujol) υ cm^{-1}= 1610 (isoxazole), 1710 (COOH). ^{1}H-NMR ($CDCl_3$–DMSO) δ 7.83–7.81 (m, 2H, Ar-H), 7.51–7.49 (m, 3H, Ar-H), 6.97 (s, 1H, H-4 isoxazole). GC/MS *m/z* 189 (M^+). Anal. Calc. for $C_{10}H_7NO_3$: C, 63.49; H, 3.73; N, 7.40. Found: C, 63.24; H, 3.71; N, 7.37.

5-(1-methyl-1H-indol-3-yl)isoxazole-3-carboxylic acid ***[VIIIb]***. Yield=28%; mp=160–161°C; IR (nujol) υ cm^{-1}=1610 (isoxazole), 1710 (COOH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 8.12–7.95 (m, 1H, Ar-H), 7.55–7.22 (m, 4H, Ar-H), 6.84 (s, 1H, H-4 isoxazole), 4.45–3.20 (brs, 1H, COOH), 3.92 (s, 3H, NCH_3). GC/MS *m/z* 242 (M^+). Anal. Calc. for $C_{13}H_{10}N_2O_3$: C, 64.46; H, 4.16; N, 11.56. Found: C, 64.70; H, 4.28; N, 11.60.

5-(1-ethyl-1H-indol-3-yl)isoxazole-3-carboxylic acid ***[VIIIc]***. Yield=21%; mp=158-160°C; IR (nujol) υ cm^{-1}= 1610 (isoxazole), 1710 (COOH). ^{1}H-NMR ($CDCl_3$) δ 8.05–7.90 (m, 1H, Ar-H), 7.74 (s, 1H, Ar-H), 7.49–7.15 (m, 3H, Ar-H), 6.86 (s, 1H, H-4 isoxazole), 4.27 (q, 2H, NCH_2), 2.85–2.25 (brs, 1H, COOH), 1.55 (t, 3H, CH_3). GC/MS *m/z* 256 (M^+). Anal. Calc. for $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 65.40; H, 4.58; N, 10.75.

5-(1-benzyl-1H-indol-3-yl)isoxazole-3-carboxylic acid ***[VIIId]***. Yield=47%; mp=148–149°C; IR (nujol) υ cm^{-1}= 1615 (isoxazole), 1710 (COOH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 8.02-7.89 (m, 1H, Ar-H), 7.74 (s, 1H, Ar-H), 7.48–7.12 (m, 8H, Ar-H), 6.81 (s, 1H, H-4 isoxazole), 5.37 (s, 2H, CH_2), 4.35–3.25 (brs, 1H, COOH). GC/MS *m/z* 318 (M^+). Anal. Calc. for $C_{19}H_{14}N_2O_3$: C, 71.69; H, 4.43; N, 8.80. Found: C, 71.75; H, 4.62; N, 8.89.

*5-[1-(4-fluorobenzyl)-1H-pyrrol-2-yl]isoxazole-3-carboxylic acid **[VIIIe]**.* Yield=79%; mp=141–143°C; IR (nujol) υ cm^{-1}=1615 (isoxazole), 1710 (COOH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 7.01–6.91 (m, 4H, Ar-H), 6.85 (s, 1H, H-4 isoxazole), 6.75–6.72 (m, 1H, Ar-H), 6.48 (s, 1H, Ar-H), 6.30–6.27 (m, 1H, Ar-H), 5.32 (s, 2H, CH_2), 3.02 (brs, 1H, COOH). GC/MS *m/z* 286 (M^+). Anal. Calc. for $C_{15}H_{11}FN_2O_3$: C, 62.94; 3H, 3.87; N, 9.79. Found: C, 62.90; H, 3.81; N, 9.72.

*4,5-dihydronaphtho[2,1-d]isoxazole-3-carboxylic acid **[VIIIf]**.* Yield=88%; mp=176–177°C; IR (nujol) υ cm^{-1}= 1615 (isoxazole), 1705 (COOH). ^{1}H-NMR ($CDCl_3$-DMSO) δ 7.70–7.65 (m, 1H, Ar-H), 7.35–7.33 (m, 3H, Ar-H), 3.07–3.01 (m, 4H, CH_2x2). GC/MS *m/z* 215 (M^+). Anal. Calc. for $C_{12}H_9NO_3$: C, 66.97; H, 4.22; N, 6.51. Found: C, 66.70; H, 3.98; N, 6.48.

*Preparation of 3-methyl-4,5-dihydronaphtho[1,2-c]isoxazole **[VIIIg]**.* A mixture of commercially available 2-acetyltetralone **[16g]** (5.3 mmol) and hydroxylamine hydrochloride (3 eq) in methanol (25 ml) was refluxed for 22 h. After evaporation of the solvent, the solid obtained was purified by silica gel flash chromatography (eluents petroleum ether-ethyl acetate 9.5:0.5) to give a pale yellow solid that was recrystallized from H_2O/EtOH. Yield=82%; mp=56–57°C; IR (nujol) υ cm^{-1}=1375 (CH_3), 1625 (isoxazole). ^{1}H-NMR ($CDCl_3$) δ 7.65 (d, 1H, Ar-H), 7.35–7.23 (m, 3H, Ar-H), 3.04 (t, 2H, CH_2), 2.67 (t, 2H, CH_2), 2.29 (s, 3H, CH_3). GC/MS *m/z* 185 (M^+). Anal. Calc. for $C_{12}H_{11}NO$: C, 77.81; H, 5.99; N, 7.56. Found: C, 77.75; H, 5.86; N, 7.43.

*General procedure for the preparation of the isoxazolecarboxylic esters **[15a–15f]**.* A mixture of the appropriate β-diketo ester **[16a–16f]** (1.2 mmol) and hydroxylamine hydrochloride (3 eq) in methanol (10 ml) was refluxed for 1 h. After evaporation *in vacuo,* a yellow solid was obtained; it was purified by silica gel flash chromatography (eluents petroleum ether-ethyl acetate 8:2) to give a solid that was recrystallized from H_2O/EtOH.

*Methyl 5-phenylisoxazole-3-carboxylate **[15a]** (Tanaka et al., 1998).* Yield=29%; mp=81-82°C; IR (nujol) υ cm^{-1}=1610 (isoxazole), 1728 (ester). ^{1}H-NMR ($CDCl_3$) δ 7.83–7.79 (m, 2H, Ar-H), 7.51–7.48 (t, 3H, Ar-H), 6.94 (s, 1H, H-4 isoxazole), 4.01 (s, 3H, OCH_3). GC/MS *m/z* 203 (M^+). Anal. Calc. for $C_{11}H_9NO_3$: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.76; H, 4.14; N, 6.66.

*Methyl 5-(1-methyl-1H-indol-3-yl)isoxazole-3-carboxylate **[15b]**.* Yield=69%; mp=119–120°C; IR (nujol) υ cm^{-1}=1605 (isoxazole), 1730 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.00–7.91 (m, 1H, Ar-H), 7.66 (s, 1H, Ar-H), 7.47–7.25 (m, 3H, Ar-H), 6.82 (s, 1H, H-4 isoxazole), 4.00 (s, 3H, OCH_3), 3.89 (s, 3H, NCH_3). GC/MS *m/z* 256 (M^+). Anal. Calc. for $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 65.79; H, 4.83; N, 11.02.

*Methyl 5-(1-ethyl-1H-indol-3-yl)isoxazole-3-carboxylate **[15c]**.* Yield=77%; mp=104–105°C; IR (nujol) υ cm^{-1} =1600 (isoxazole), 1730 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.20–7.95 (m, 1H, Ar-H), 7.73 (s, 1H, Ar-H), 7.52–7.34 (m, 3H, Ar-H), 6.82 (s, 1H, H-4 isoxazole), 4.27 (q, 2H, NCH_2), 4.01 (s, 3H, OCH_3), 1.57 (t, 3H, CH_3). GC/MS *m/z* 270 (M^+). Anal. Calc. for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.45; H, 5.02; N, 10.15.

*Methyl 5-(1-benzyl-1H-indol-3-yl)isoxazole-3-carboxylate **[15d]**.* Yield=69%; mp=139–140°C; IR (nujol) υ cm^{-1} =1605 (isoxazole), 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.20–7.95 (m, 1H, Ar-H), 7.71 (s, 1H, Ar-H), 7.42–7.15 (m, 8H, Ar-H), 6.83 (s, 1H, H-4 isoxazole), 5.38 (s, 2H, CH_2), 4.01 (s, 3H, OCH_3). GC/MS *m/z* 332 (M^+). Anal. Calc. for $C_{20}H_{16}N_2O_3$: C, 72.28; H, 4.85; N, 8.43. Found: C, 72.41; H, 4.92; N, 8.55.

*Methyl 5-[1-(4-fluorobenzyl)-1H-pyrrol-2-yl]isoxazole-3-carboxylate **[15e]**.* Yield=74%; mp=80–82°C; IR (nujol) υ cm^{-1}=1610 (isoxazole), 1735 (ester). ^{1}H-NMR ($CDCl_3$) δ 7.08–6.87 (m, 2H, Ar-H), 6.77 (s, 1H, H-4 isoxazole), 6.50 (s, 1H, Ar-H), 6.32–6.29 (m, 1H, Ar-H), 5.37 (s, 2H, CH_2), 3.96 (s, 2H, OCH_3). GC/MS *m/z* 300 (M^+). Anal. Calc. for $C_{16}H_{13}FN_2O_3$: C, 64.00; H, 4.36; N, 9.33. Found: C, 63.96; H, 4.23; N, 9.11.

*Methyl 4,5-dihydronaphtho[2,1-d]isoxazole-3-carboxylate **[15f]**.* Yield=94%; mp=129–130°C; IR (nujol) υ cm^{-1}=1610 (isoxazole), 1715 (ester). ^{1}H-NMR ($CDCl_3$) δ 7.79–7.65 (m, 1H, Ar-H), 7.37–7.30 (m, 3H, Ar-H), 4.00 (s, 3H, OCH_3), 3.07–3.03 (m, 4H, CH_2x2). GC/MS *m/z* 229 (M^+). Anal. Calc. for $C_{13}H_{11}NO_3$: C, 68.11; H, 4.84; N, 6.11. Found: C, 67.84; H, 4.62; N, 5.99.

*General procedure for the preparation of β-diketoesters **[16a–16f]**.* A mixture of the appropriate ketone (**[17a–17e]** and **[18]**) (2.5 mmol) and diethyloxalate (1.5 eq) in anhydrous methanol (25 ml) was added to a solution of sodium methoxide (3.4 eq, generated from sodium in anhydrous methanol). The mixture was refluxed under nitrogen atmosphere for 4 h. After dilution with water the solution was acidified with 1N HCl. The yellow precipitate formed was purified by silica gel flash chromatography (eluents petroleum ether-ethyl acetate 8:2) and then recrystallized from H_2O/EtOH.

Methyl 2-hydroxy-4-oxo-4-phenylbut-2-enoate ***[16a]*** *(Penning et al., 1997).* Yield=33%; mp=55–57°C; IR (nujol) υ cm^{-1}=1640 (ketone), 1729 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.00 (d, 2H, Ar-H), 7.62–7.50 (m, 4H, Ar-H), 7.09 (s, 1H, CH=C), 3.95 (s, 3H, OCH_3). GC/MS *m/z* 206 (M^+).

Methyl 2-hydroxy-4-(1-methyl-1H-indol-3-yl)-4-oxo-but-2-enoate ***[16b]*** *(Sechi et al., 2004).* Yield=81%; mp=174–175°C; IR (nujol) υ cm^{-1}=1610 (ketone), 1745 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.42–8.34 (m, 1H, Ar-H), 7.86 (s, 1H, Ar-H), 7.42–7.30 (m, 3H, Ar-H), 6.85 (s, 1H, CH=C), 3.93 (s, 3H, OCH_3), 3.88 (s, 3H, NCH_3). GC/MS *m/z* 259 (M^+).

Methyl 2-hydroxy-4-(1-ethyl-1H-indol-3-yl)-4-oxo-but-2-enoate ***[16c]*** *(Sechi et al., 2004).* Yield=59%; mp=159–161°C; IR (nujol) υ cm^{-1}=1610 (ketone), 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.41–8.36 (m, 1H, Ar-H), 7.93 (s, 1H, Ar-H), 7.58–7.28 (m, 3H, Ar-H), 6.87 (s, 1H, CH=C), 4.28 (q, 2H, NCH_2), 3.94 (s, 3H, OCH_3), 1.57 (t, 3H, CH_3). GC/MS *m/z* 273 (M^+).

Methyl 2-hydroxy-4-(1-benzyl-1H-indol-3-yl)-4-oxo-but-2-enoate ***[16d]*** *(Sechi et al., 2004).* Yield=37%; mp =178–179°C; IR (nujol) υ cm^{-1}=1610 (ketone) 1730 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.49–8.42 (m, 1H, Ar-H), 7.91 (s, 1H, Ar-H), 7.40–7.32 (m, 8H, Ar-H), 6.84 (s, 1H, CH=C), 5.39 (s, 2H, CH_2), 3.92 (s, 3H, OCH_3), 1.80–1.55 (bs, 1H, OH). GC/MS *m/z* 335 (M^+).

Methyl 4-[1-(4-fluorobenzyl)-1H-pyrrol-2-yl]-2-hydroxy-4-oxobut-2-enoate ***[16e]***. Yield=67%; mp=103–105°C; IR (nujol) υ cm^{-1}=1620 (ketone), 1720 (ester). ^{1}H-NMR ($CDCl_3$) δ 7.17–6.94 (m, 6H, Ar-H), 6.85 (s, 1H, CH=C), 6.30–6.27 (m, 1H, Ar-H), 5.60 (s, 2H, CH_2), 3.91 (s, 3H, OCH_3). GC/MS *m/z* 303 (M^+).

Methyl hydroxy(1-oxo-3,4-dihydronaphthalen-2(1H)-ylidene)acetate ***[16f]*** *(Emerson et al., 1991).* Yield= 59%; mp=64–65°C; IR (nujol) υ cm^{-1}=1630 (ketone), 1720 (ester). ^{1}H-NMR ($CDCl_3$) δ 8.03 (d, 1H, Ar-H), 7.50 (t, 1H, Ar-H), 7.36 (t, 1H, Ar-H), 7.27 (d, 1H, Ar-H), 3.93 (s, 3H, OCH_3), 2.98–2.86 (m, 4H, CH_2x2). GC/MS *m/z* 232 (M^+).

Preparation of 1-[1-(4-fluorobenzyl)-1H-pyrrol-2-yl]ethanone ***[17e]***. A mixture of KOH (crushed pellets) (4 eq) in DMSO (18 ml) was stirred at room temperature for 5 min. 2-acethylpyrrole **[7]** (9.0 mmol) was added and the mixture was stirred for 45 min. After this time, 4-fluoro-benzylbromide was added (2 eq) and the reaction mixture was stirred at room temperature for a further 45 min. Then reaction was quenched by addition of water and the solution was extracted three times with diethyl ether. The organic layer was washed with water and dried over Na_2SO_4. After evaporation of the solvent, the crude product was purified by silica gel flash chromatography (eluents petroleum ether-ethyl acetate 9:1) to give a yellow oil. Yield=94%; mp=oil at room temperature; IR (nujol) υ cm^{-1}=1640 (ketone). ^{1}H-NMR ($CDCl_3$) δ 7.36-7.28 (m, 2H, Ar-H), 7.11–6.83 (m, 4H, Ar-H), 6.22–6.18 (m, 1H, Ar-H), 5.53 (s, 2H, CH_2), 2.41 (s, 3H, $COCH_3$). GC/MS *m/z* 217 (M^+).

Preparation of 1-(1H-pyrrol-2-yl)ethanone ***[19]*** *(Garrido et al., 1984).* A solution of 2-acetylfurane **[20]** (45 mmol) and 30% NH_3 (19 eq) in 96% ethanol (30 ml) was heated in a sealed tube at 150°C for 12 h. After cooling, the solution was filtered and concentrated *in vacuo* to give a brown solid. The crude product was purified by silica gel flash chromatography (eluents petroleum ether-ethyl acetate 8:2) to give a yellow solid that was recrystallized from H_2O/EtOH. Yield=30%; mp=88–89°C; IR (nujol) υ cm^{-1}=1640 (ketone), 3260 (NH). ^{1}H-NMR ($CDCl_3$) δ 10.50–9.80 (brs, 1H, NH), 7.06–7.04 (m, 1H, Ar-H), 6.93–6.91 (m, 1H, Ar-H), 6.28–6.26 (m, 1H, Ar-H), 2.45 (s, 3H, $COCH_3$). GC/MS *m/z* 109 (M^+).

General procedure for the preparation of tetrafluoroborate isoxazolium salts ***[21c]***, ***[21e]*** *and* ***[21g]***. A mixture of the appropriate isoxazole (**[15c],[15e]** and **[VIIIg]**) (2 mmol) and dimethylsulphate (2.2 eq) in anhydrous toluene (5 ml) was refluxed under nitrogen atmosphere for 24 h (for **[15c]**), 30 h (for **[VIIIg]**) or 48 h (for **[15e]**). Subsequently the toluene layer was decanted, the oil residue was dissolved in water, and it was washed with ethyl acetate. To this aqueous solution was added a solution of sodium tetrafluoroborate (4 eq) in water and, after cooling with ice, a yellow solid was separated.

5-(1-ethyl-1H-indol-3-yl)-3-(methoxycarbonyl)-2-methylisoxazol-2-ium tetrafluoroborate ***[21c]***. Yield= 69%; mp=159–162°C; IR (nujol) υ cm^{-1}=1060 (BF_4^-), 1370 (CH_3), 1745 (ester). ^{1}H-NMR (DMSO) δ 8.81 (s, 1H, Ar-H), 8.19–8.07 (m, 1H, Ar-H), 7.91 (s, 1H, H-4 isoxazole), 7.83–7.72 (m, 1H, Ar-H), 7.46–7.38 (m, 2H, Ar-H), 4.53 (s, 3H, NCH_3), 4.38 (q, 2H, NCH_2), 4.07 (s, 3H, OCH_3), 1.49 (t, 3H, CH_3). GC/MS *m/z* 285 (M^+, cation). Anal. Calc. for $C_{16}H_{17}N_2O_3\,BF_4$: C, 51.64; H, 4.60; N, 7.53. Found: C, 51.33; H, 4.42; N, 7.36.

5-[1-(4-fluorobenzyl)-1H-pyrrol-2-yl]-3-(methoxycarbonyl)-2-methylisoxazol-2-ium tetrafluoroborate ***[21e]***. Yield=52%; mp=168–170°C; IR (nujol) υ cm^{-1}=1060 (BF_4^-), 1375 (CH_3), 1740 (ester). ^{1}H-NMR (DMSO) δ

7.90 (s, 1H, H-4 isoxazole), 7.67–7.64 (m, 1H, Ar-H), 7.52–7.47 (m, 1H, Ar-H), 7.25–7.18 (m, 5H, Ar-H), 5.51 (s, 2H, CH_2), 4.40 (s, 3H, NCH_3), 3.99 (s, 3H, OCH_3). GC/MS *m/z* 315 (M^+, cation). Anal. Calc. for $C_{17}H_{16}FN_2O_3BF_4$: C, 50.78; H, 4.01; N, 6.97. Found: C, 50.89; H, 4.22; N, 7.11.

*1,3-Dimethyl-4,5-dihydronaphtho[1,2-c]isoxazol-1-ium tetrafluoroborate **[21g]**.* Yield=29%; mp= 145–147°C; IR (nujol) υ cm^{-1}=1060 (BF_4^-), 1375 (CH_3). ^{1}H-NMR (DMSO) δ 7.65 (d, 1H, Ar-H), 7.52 (t, 1H, Ar-H), 7.39–7.33 (m, 2H, Ar-H), 4.29 (s, 3H, NCH_3), 3.15 (t, 2H, CH_2), 2.87 (t, 2H, CH_2), 2.65 (s, 3H, CH_3). GC/MS *m/z* 200 (M^+, cation). Anal. Calc. for $C_{13}H_{14}NOBF_4$: C, 54.39; H, 4.92; N, 4.88. Found: C, 54.25; H, 4.87; N, 4.69.

Biology

Materials, chemicals and enzymes. All compounds were dissolved in DMSO and the stock solutions were stored at –20°C. The γ[^{32}P]-ATP was purchased from either Amersham Biosciences or ICN. The expression systems for the wild-type IN and soluble mutant $IN^{F185KC280S}$ were generous gifts of Dr Robert Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD, USA.

Preparation of oligonucleotide substrates. The oligonucleotides 19top, 5′-GTGTGGAAAATCTCTAG-CA-3′ and 21bot, 5′-ACTGCTAGAGATTTTCCA-CAC-3′ were purchased from Norris Cancer Center Microsequencing Core Facility (University of Southern California) and purified by UV shadowing on polyacrylamide gel. To analyse the extent of strand transfer using 5′-end labelled substrates, 19top was 5′-end labelled using T4 polynucleotide kinase (Epicentre, Madison, WI, USA) and γ [^{32}P]-ATP (Amersham Biosciences or ICN). The kinase was heat-inactivated and 21bot was added in 1.5-molar excess. The mixture was heated at 95°C, allowed to cool slowly to room temperature, and run through a spin 25 mini-column (USA Scientific) to separate annealed double-stranded oligonucleotide from unincorporated material.

Integrase assays. To determine the extent of strand transfer, wild-type IN was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulphoxide, and 25 mM MOPS, pH 7.2) at 30°C for 30 min. Then, 20 nM of the 5′-end ^{32}P-labelled linear oligonucleotide substrate was added, and incubation was continued for an additional 1 h. Reactions were quenched by the addition of an equal volume (16 μl) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M tris-borate pH 8.3, 2 mM EDTA, 20% acrylamide, 8M urea).

Gels were dried, exposed in a PhosphorImager cassette, and analysed using a Typhoon 8610 Variable Mode Imager (Amersham Biosciences) and quantitated using ImageQuant 5.2. Percent inhibition (% I) was calculated using the following equation:

$$\% I = 100\ X\ [1 - (D - C)/(N - C)]$$

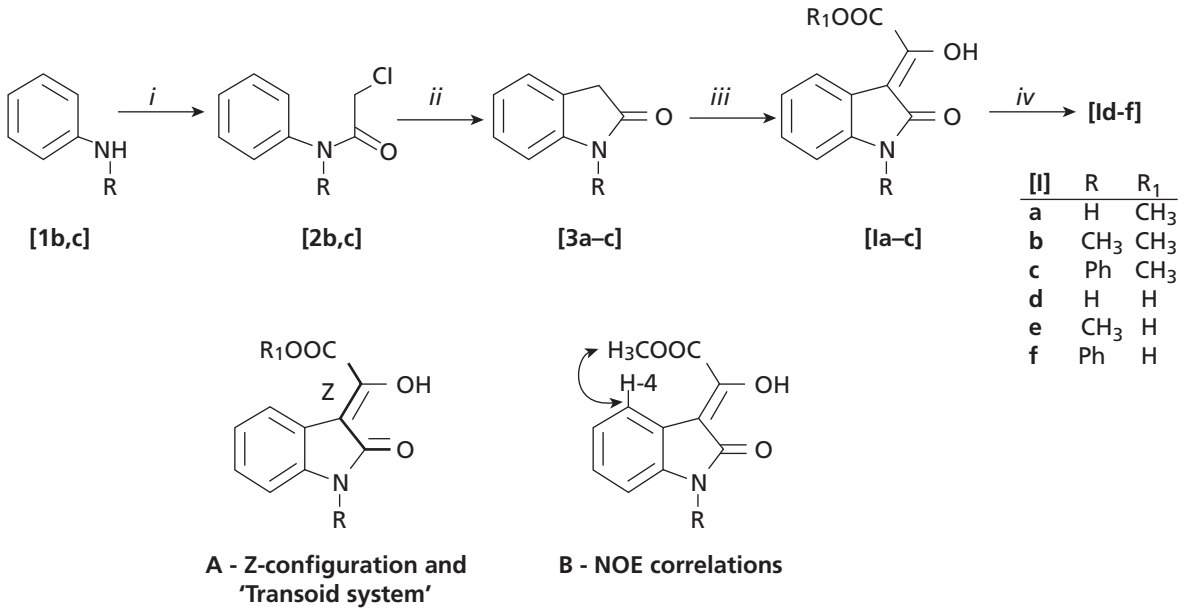
where *C*, *N* and *D* are the fractions of 21-mer substrate converted to strand transfer products for DNA alone, DNA plus IN, and IN plus drug, respectively. The IC_{50} values were determined by plotting the logarithm of drug concentration *versus* percent inhibition to obtain concentration that produced 50% inhibition.

Molecular modelling

Model compounds **[IId]** and **[VIa]** were constructed with standard bond lengths and angles from the fragment database with MacroModel 6.0 (1997) using a Silicon Graphics O2 workstation running on IRIX 6.3. Sybyl 6.2 (2001) was used as graphic platform. The atomic charges were assigned using the Gasteiger-Marsili method (Gasteiger *et al.*, 1980). Minimization of structures was performed with the MacroModel/BachMin 6.0 program using the Amber force field. Extensive conformational search was carried out using the Monte Carlo/Energy minimization (Chang *et al.*, 1989) for all the compounds considered in the study (*Ei-E*min <5 kcal/mol, energy difference between the generated conformation and the current minimum). Representative minimum energy conformations of each compounds were optimized using the *ab initio* quantum chemistry program Gaussian 98 with UHF/6-31* basis set. Docking calculations were performed on HP Exemplar Parallel Server V2200 running HP UX 11.0.

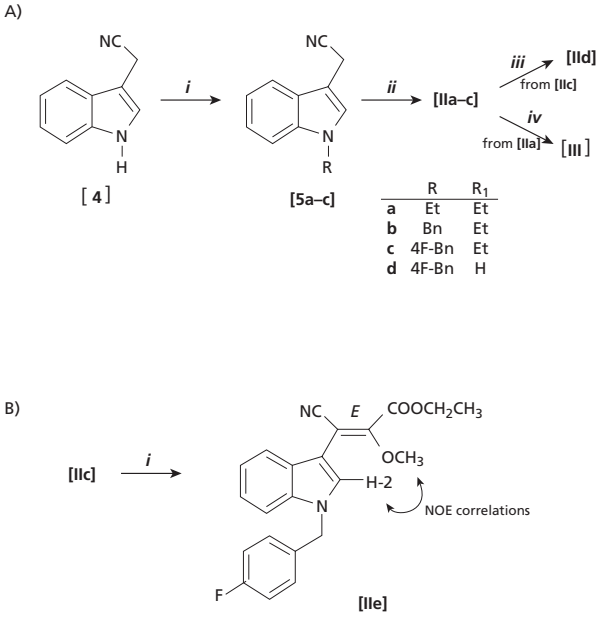
Subunit A of IN core domain in complex with 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2*H*-tetrazol-5-yl-propenone) (5CITEP; PDB 1QS4) was used for all docking studies. The missing residues at positions 141–144 in this subunit were incorporated from monomer B of the IN structure PDB 1BIS after superimposition of the backbones of residues 135–140 and 145–150, as previously reported (Sotriffer *et al.*, 2000). Docking was performed with AutoDock version 3.05 (Morris *et al.*, 1998) using the new empirical free energy function and the Lamarckian protocol (Morris *et al.*, 1996). Mass-centered grid maps were generated with 80 grid points for every direction and with 0.375 Angstroms spacing by the AutoGrid program for the whole protein target. Random starting position on

Figure 4. Experimental conditions for the preparation of **[Ia–f]**



i) chloroacetylchloride, toluene, reflux for 1–2 h, N_2; ii) $AlCl_3$, 180–190°C for 10 min; iii) dimethyloxalate, CH_3ONa, anhydr. MeOH, rt for 3 h **[Ia]** or 5 h **[Ib]**, or reflux for 3.5 h **[Ic]**, N_2; iv) 2N NaOH, MeOH, rt for 4–6 h, then 1N HCl.

Figure 5. Experimental conditions for the preparation of **[IIa–IIe]** and **[III]**



A, i) KOH pellets, appropriate alkylbromide, DMSO, rt for 30 min; ii) diethyloxalate, t-ButOK, DMF, rt for 2 h **[IIa]** or diethyloxalate, 60% NaH in mineral oil, anhydr. Et_2O, rt for 15 h **[IIb–c]**; iii) 2N NaOH, MeOH, rt for 5 h, then 1N HCl; iv) $MeSO_3H$, rt for 15 h, then 80% EtOH, rt for 3 h. (B) i) dimethylsulphate, anhydr. K_2CO_3, dry acetone, reflux for 2 h.

the entire protein surface, random orientations and torsions were used for the ligands. The distance-dependent dieletric permittivity of Mehler and Solmajer was used for the calculation of the electrostatic grid-maps. Fifty independent docking runs were carried out for each ligands. The cluster analyses were computed with a cluster tolerance by less than 1.5 Å in positional root-mean-square deviation.

Results

Chemistry

The synthesis of target compounds **[I–VIII]** are depicted in Figures 4–8. The oxindole-2-carboxylic acids **[Id–If]** were synthesized by treating under alkaline conditions the respective esters **[Ia–Ic]**, which were easily obtained by oxalylation of the appropriate 2-oxindoles **[3a–3c]** in the presence of sodium methoxide dissolved in methanol under reflux (Figure 4). The intermediate **[3a]** was commercially available and **[3b]** and **[3c]** were prepared as previously described (Sarges *et al*., 1989). Interestingly, the disubstituted 2-oxindole-ylidene derivatives, generated from the enolic form of C-2 carbonyl, were stereoselectively obtained in the Z-configuration. This assumption was supported by the marked deshielding effect of the H-4 proton of the oxindole nucleus due to the carboxylate group located on the ylidene moiety, which forms a 'transoid system' (Long *et al*., 1978, Autrey *et al*., 1967) with the oxindole carbonyl (Figure 4, A). These observations were unambigously confirmed on the basis of NOE difference and NOESY experiments data, which showed NOE correlations between H-4 and the methyl ester group of **[Ia-Ic]** (Figure 4-B).

The synthetic route to obtain sequentially the 3-ciano-2-ketoesters **[IIa–IIc]**, the correspondent acid **[IId]** (from **[IIc]**) and the 3-hydroxy-1*H*-pyrrole-2,5-dione derivative **[III]** (from **[IIa]**) is shown in Figure 5-A. The reaction of indole-3-acetonitriles **[5a–5c]** and diethyloxalate and *t*-ButOK in DMF gave **[IIa–IIc]** in good yields. Alkalyne hydrolysis of the ester **[IIc]** provided the corresponding acid **[IId]**. The analysis of ^{1}H-NMR and IR for **[IIa-IIc]** revealed the presence of enolic form in solution with consequent formation of a double bond. In order to establish the exact configuration of **[IIa–IId]**, we methylated the enol group of **[IIc]** to obtain its methoxy derivative **[IIe]** (Figure 5-B), which could be suitable for NMR investigations. NOE experiments conducted using this model compound showed an *E*-configuration of the cyanoketo ester moiety and demonstrated the stereoselectivity of the above-mentioned reaction (Figure 5-B). The ester **[IIa]** was then converted into a pyrrole ring system, **[III]** (Figure 5-A) by treating with methanesulphonic acid followed by quenching in ethanol (Rooney *et al*., 1983). Alkylation of **[4]** with an appropriate alkylbromide and solid KOH in DMSO gave the intermediate *N*-alkylindoles **[5a–5c]** (Figure 5-A).

The β-diketone **[IV]** was synthesized by an intramolecular acyl transfer reaction (Baker–Venkataraman–like reaction) by reacting the 2-hydroxyacetophenone **[7]** with benzoylchloride using NaH in THF and *t*-ButOH / *t*-ButOK complex (Kraus *et al*., 1984). Deprotection of the methoxy group of **[6]** to give the intermediate **[7]** was carried out with boron tribromide in dichloromethane at –40°C (Figure 6-A).

Claisen condensation of ethyl phenylacetate **[8]** with diethyloxalate and NaH dissolved in toluene (House *et al*., 1968) afforded the diester **[V]** in the enolic form (Figure 6-B).

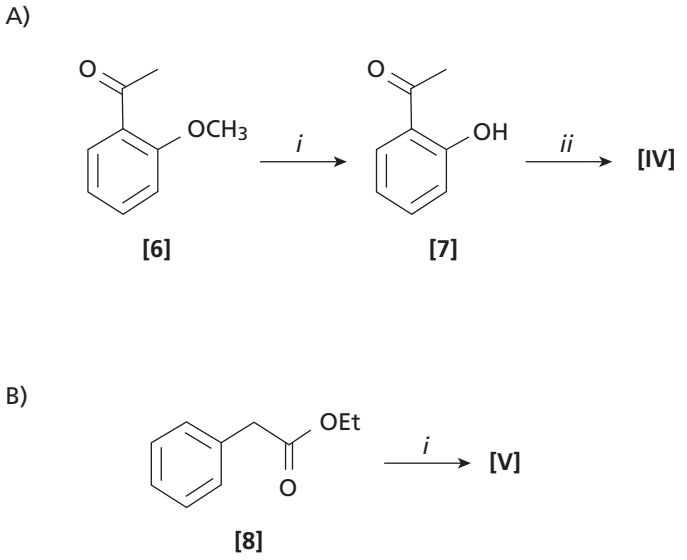
The 6-aryl-2,4,6-trioxohexanoic esters **[VIa–VIc]** were obtained by treatment of benzoylacetones **[10a–10c]** with dimethyloxalate in the presence of magnesium methyl carbonate (Stiles reagent; Stiles *et al*., 1991); the successive acid hydrolysis gave the corresponding acids **[VId-VIf]** in good yields. While the diketone **[10a]** was commercially available, the intermediates **[10b]** and **[10c]** were easily prepared by condensation of the appropriate acetophenones **[9b]** and **[9c]** with anhydrous ethyl acetate and NaH (Figure 7-A).

The tricycle **[VII]** was prepared following a tandem-reaction through a direct oxalylation of 2-acetylindole **[13]** using 4 eq of sodium methoxide under reflux conditions for 15 h to obtain β-diketo ester **[14]** (intermediated not isolated) as a first step. These reaction conditions favoured an intramolecular amination (second step) to give the desired **[VII]** (Figure 7-B). The intermediate **[13]** was prepared from the acid **[12]** and methyllithium. The latter was obtained by alkaline hydrolysis of the commercially available ester **[11]**.

The aryl(heteroaryl)-isoxazole carboxylic acids **[VIIIa–VIIIf]** were prepared by treating under alkaline conditions the respective esters **[15a–15f]**, which were synthesized through a [3+2] synthetic route by cyclocondensation of β–diketoesters **[16a–16f]** with hydroxylamine hydrochloride (Figures 8-A,B). These intermediates were easily obtained by oxalylation of the aryl(heteroaryl)-ketones **[17a–17f], [18]** in the presence of sodium methoxide dissolved in CH_3OH under reflux conditions. Finally, the methyl-derivative **[VIIIg]** was prepared by treating 2-acetyl-3,4-dihydronaphthalen-1(2*H*)-one [**16g**] with hydroxylamine hydrochloride (Figure 8-B). The starting materials [**17a–17e], [18]** were commercially available ([**17a], [18]**), previously described (**[17b–17d]**; Sechi *et al*., 2004) or prepared through a new synthesis (**[17e]**, Figure 9-A).

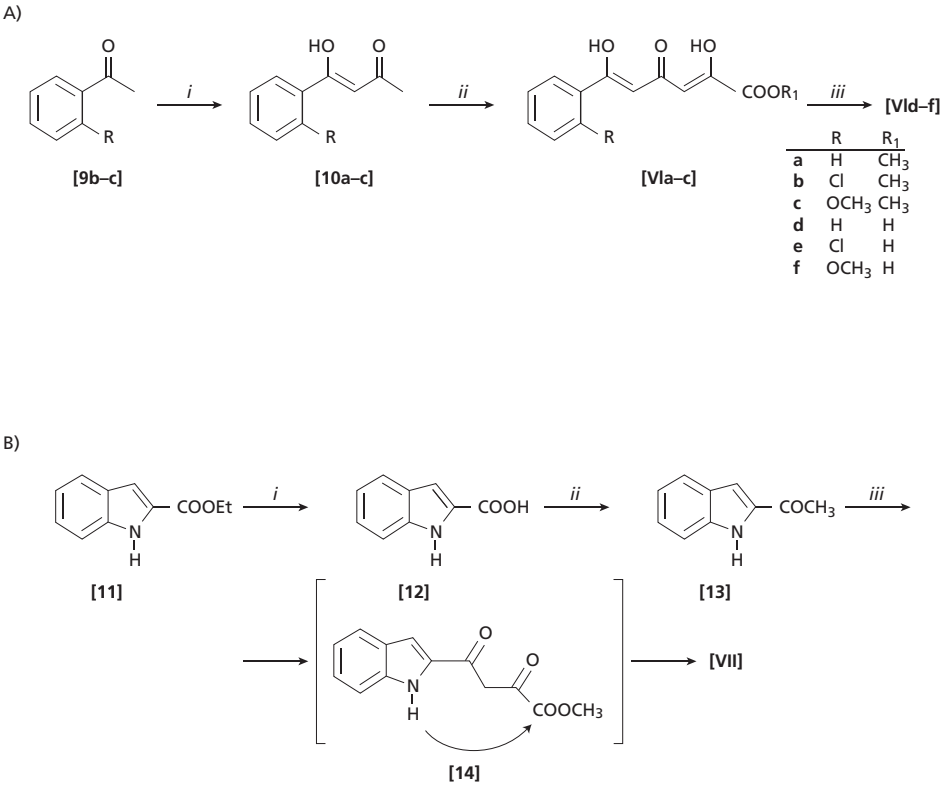
Several chemical aspects of reaction of aryl and heteroaryl β-diketoalkanoates with hydroxylamine hydrochloride, as well as methods to distinguish regioisomers, were recently described (Battaglia *et al*. 1970;

Figure 6. Experimental conditions for the preparation of **[IV]** and **[V]**



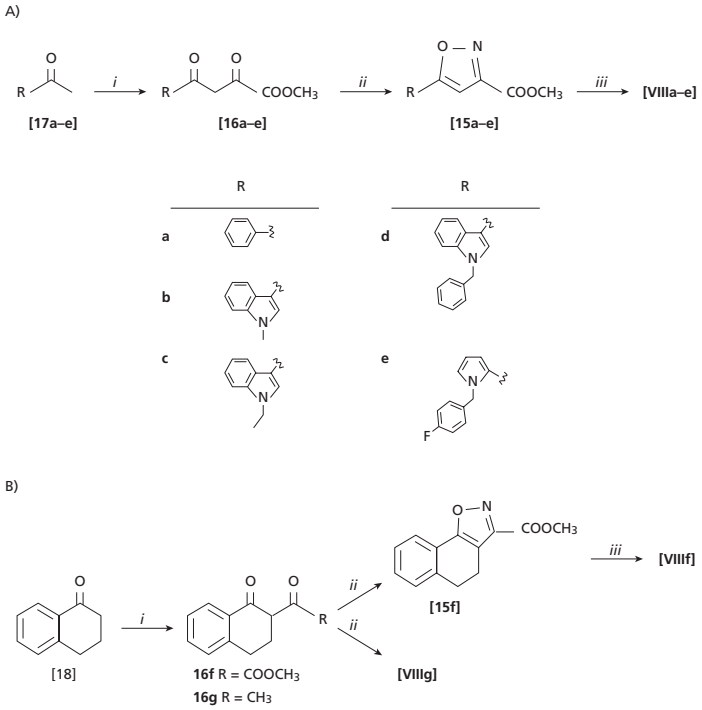
A, i) 1M BBr_3 in CH_2Cl_2, anhydr. CH_2Cl_2, –30°C for 12 h, then rt for 8 h; ii) 60% NaH in mineral oil, anhydr. THF, rt for 30 min, benzoylchloride, 0°C for 1 h, then *t*-ButOK/*t*-ButOH, 0°C for 15 min, then CH_3COOH/H_2O. B, i) diethyloxalate, 60% NaH in oil, abs. ethanol, toluene, rt for 12 h, then glacial CH_3COOH.

Figure 7. Experimental conditions for the preparation of **[VIa–VIf]** and **[VII]**



A, i) anhydr. ethyl acetate, 60% NaH in mineral oil, anhydr. Et_2O, rt for 48 h **[10 b]** or 50°C for 3 h **[10c]**; ii) dimethyloxalate, 2M methyl magnesium carbonate in DMF, 170°C for 2.5 h **[VIa, VIc]** or 160°C for 3 h **[VIb]**, then 2N HCl; iii) 37% HCl/H_2O 1:2, 55°C for 6–8 h. B, i) 12% KOH, H_2O, reflux for 1 h, then 2N HCl; ii) 1.4M MeLi in Et_2O, anhydr. Et_2O, reflux for 6 h; iii) diethyloxalate, CH_3ONa, anhydr. MeOH/THF, reflux for 15 h, then 1N H_2SO_4.

Figure 8. Experimental conditions for the preparation of **[VIIIa–VIIIg]**



A, i) diethyloxalate, CH_3ONa, CH_3OH reflux for 4 h; ii) NH_2OH HCl, MeOH, reflux for 1 h; iii) 12% KOH reflux for 1 h, then 2N HCl.
B, i) (for **[16f]**) diethyloxalate, CH_3ONa, CH_3OH reflux for 4 h; ii) NH_2OH HCl, MeOH, reflux for 1 h for **[15f]** or 22 h for **[VIIIg]**; iii) 12% KOH reflux for 1 h, then 2N HCl.

Baumstark *et al.*, 1980; Sechi *et al.*, 2003). Interestingly, we observed that compounds **[15a–15f]** and **[VIIIg]** were obtained as single regioisomers. As far as the regiochemistry is concerned, the only regioisomers obtained from the above-mentioned reaction were consistent with the structure of 5-aryl(heteroaryl)-isoxazole-3-carboxylic acids **[VIIIa–VIIIf]** and 3-methyl-4,5-dithydronaphtho[1,2-*c*]isoxazole **[VIIIg]**. The regioselectivity observed under our reaction condition was postulated on the basis of previous studies (Sechi *et al.*, 2003) and confirmed by NOE difference and NOESY experiments. We unambigously assigned their structures on the basis of NOE difference and NOESY data of corresponding *N*-methyl isoxazolium salts **[21c]**, **[21e]** and **[21g]**, chosen as representative compounds for the series, where the position of their *N*-methyl groups were detectable by NOE experiments. Both experiments showed NOE between N^+-CH_3 and methyl ester group in position 3 of the isoxazole rings of compounds **[21c]** and **[21e],** whereas no NOE enhancement of the signal between N^+-CH_3 and methyl group in position 3 was observed for **[21g]** (Figure 9-B). Accordingly, no NOE interactions between *N*-methyl isoxazolium group and another aromatic proton was observed for compounds **[21c]** and **[21e]**, while NOE enhancement was observed for regioisomer **[21g]**. It is important to note that the attack of hydroxylamine on the more electrophilic centre of the β-diketo moiety is an important governing factor for the subsequent ring closure to isoxazole.

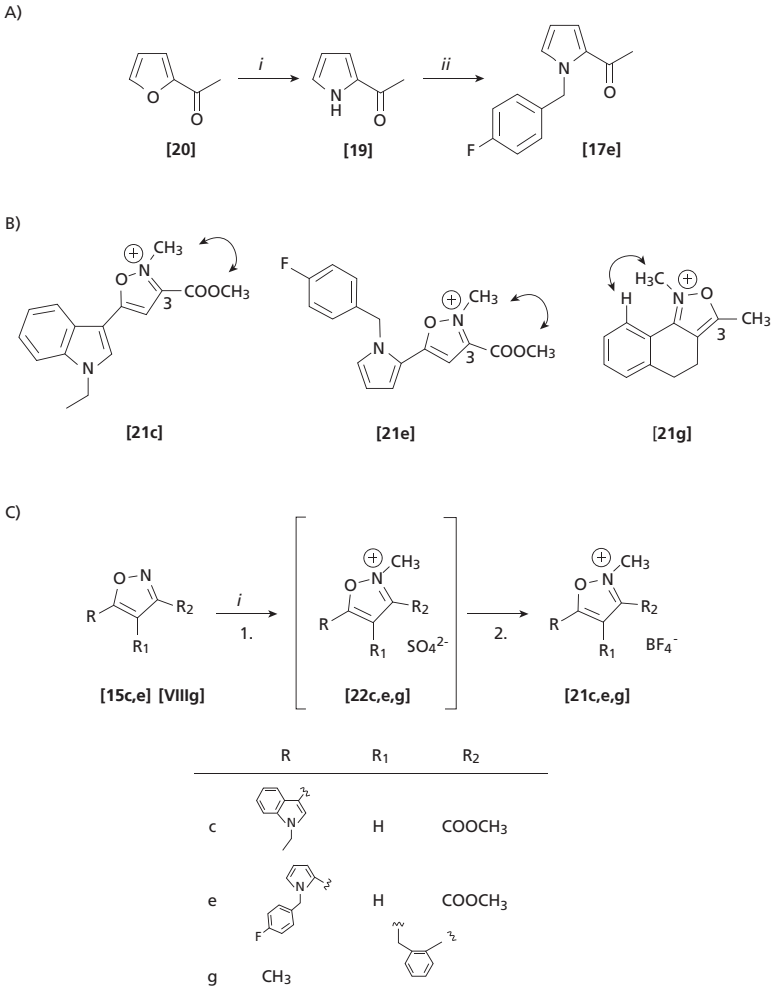
The isoxazolium salts **[21c]**, **[21e]** and **[21g]** necessary for NOE experiments were prepared by heating in toluene the corresponding isoxazole derivatives **[15c]**, **[15e]** and **[VIIIg]** with dimethylsulphate. The *N*-methyl-isoxazolium sulphate intermediates **[22c]**, **[22e]** and **[22g]** were then converted to the respective tetrafluroborates according to the reaction of Figure 9-C.

Discussion

Inhibition of HIV-1 IN catalytic activities

The top-ranking compounds **[IId]** and **[VIa]** inhibited strand transfer activity of purified IN with IC_{50} values ranging from 10–80 μM (Table 1). Interestingly, the cyanoketo acid **[IId]** proved to be the most potent compound (IC_{50}=10±4 μM). A preliminary observation can be made considering that substitution of the ester carbonyl with a carboxylate functionality led to a significant increase in activity (compare **[IIc]** and **[IId]**). Of the triketo derivatives, only the ester **[VIa]** was active against strand-transfer process of purified HIV-IN in soluble mutant (IC_{50}=80±20 μM). Moreover, we showed that compounds

Figure 9. Experimental conditions for the preparation of **[17e]**, **[21c]**, **[21e]** and **[21g]**



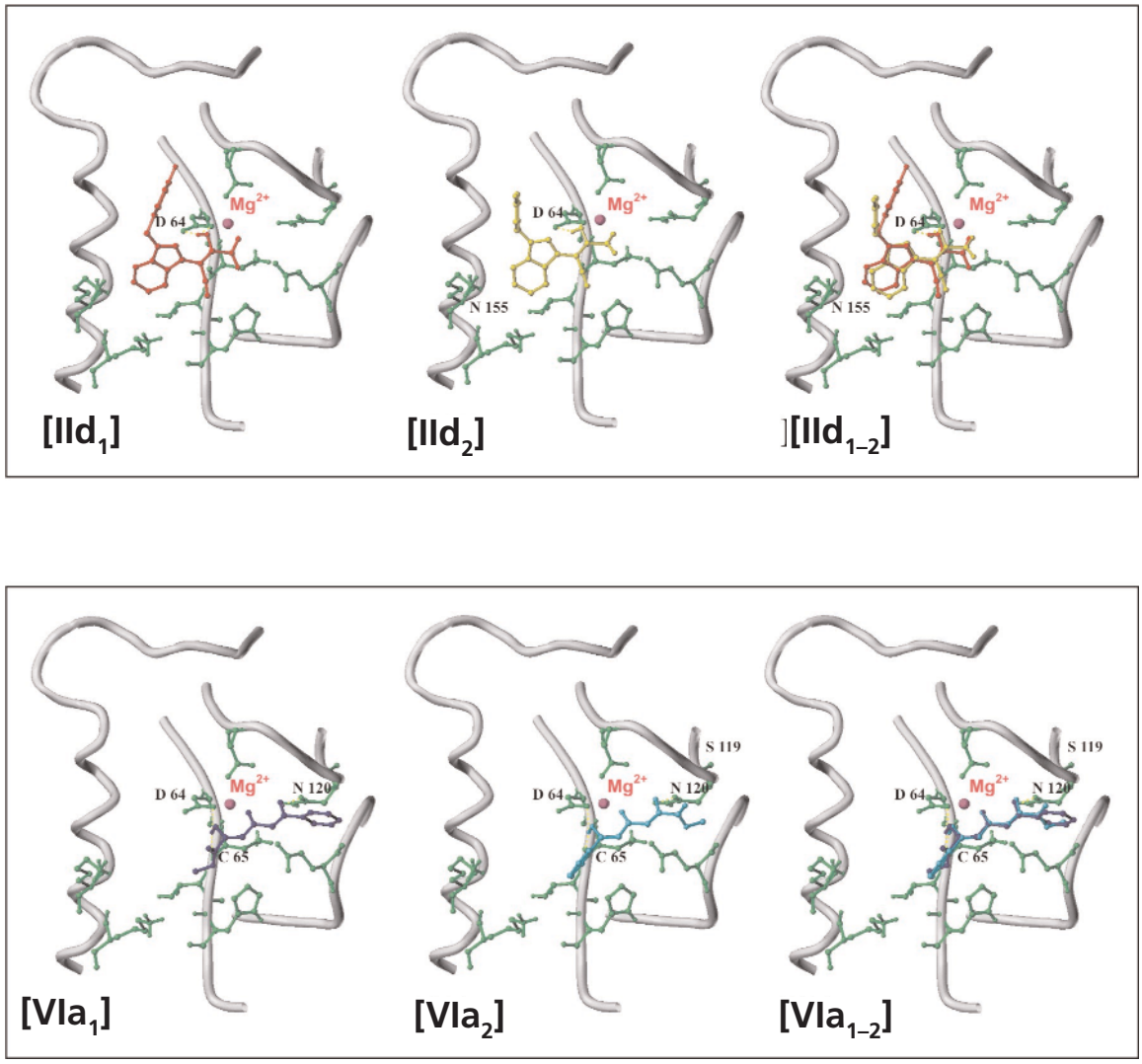
A, i) 30% NH_3, 96% EtOH, sealed tube, 150°C for 12 h; ii) p-fluoro-benzylbromide, KOH pellets, DMSO, rt for 45 min. B, NOE correlations for compounds **[21c, 21e, 21g]**. C, i) 1. dimethylsulphate, anhydr. toluene, reflux for 24 h for **[15c]**, 30 h for **[VIIIg]** or 48 h for **[15e]**; 2. $NaBF_4$, H_2O, 0–5°C.

[Ia], **[Ic–If]**, **[IIa–IIc]**, **[III]**, **[IV]**, **[VIc–VIf]** and **[VIIIa–VIIIe]** inhibited IN at a high micromolar concentration range (IC_{50s}=160–660 μM), while **[V]** and **[15a], [15c–15f]** were essentially inactive (IC_{50}=>1000 μM). One of the important achievements of this study was confirming that the diketo acid system was necessary for this activity. All isoxazole derivatives tested were inactive. Although the isoxazoles can be considered as bioisostere of diketo acids, the mechanism of IN inhibition by DKAs related compounds may involve chelation of divalent metals.

Molecular modelling

Several computational docking studies using the IN-5CITEP co-crystal structure (Goldgur *et al.*, 1999) have recently been reported (Sotriffer *et al.*, 2000; Ni *et al.*, 2001; Buolamwini *et al.*, 2002; Barreca *et al.*, 2003; Schames *et al.*, 2004). Unfortunately, many details of the protein–ligand interaction remain uncertain because the binding modes are significantly influenced by the crystal packing effect. As a 3D structure of the full-length IN complexed with DNA is not available, this is the only known structure that provided sufficient information of the IN active site in the presence of a bound inhibitor (Parril, 2003). It is also important to note that this model provides information of the mode of binding a ligand in an early step of the integration processes, in particular before the formation of the complex with donor DNA substrate. In order to investigate the binding-site of the most active compounds, **[IId]** and **[VIa]**, we performed computational docking studies on the IN-5CITEP complex (PDB 1QS4) as described (Sotriffer *at al.*, 2000; Sechi *et al.*, 2004). Based on the structure of

Figure 10. Graphical representation of hypothetical disposition of **[IId]** and **[VIa]** showing the interacting amino acid residues on the HIV-1 IN active site core domain*



***[IId$_1$]** (red) and **[IId$_2$]** (yellow) as well as **[VIa$_1$]** (blue) and **[VIa$_2$]** (cyan) represent the top and the second conformation derived from the docking results for each monomers, respectively. **[IId$_{1-2}$]** and **[VIa$_{1-2}$]** displayed the overlap of both conformers.

Table 1. Inhibition of strand transfer activity of title compounds

Compound	Strand transfer IC_{50} (μM)
[*DFC-7]	3 ±1
[*DFC-28]	2 ±1
[Ia]	400
[Ic]	273 ±50
[Id]	600
[Ie]	160
[If]	660
[IIa]	225
[IIb]	>100
[IIc]	>100
[IId]	10 ±4
[III]	300
[IV]	333
[V]	>1000
[VIa]	80 ±20
[VIc]	240
[VId]	600
[VIe]	220
[VIf]	190
[VIIIa]	200
[VIIIb]	475 ±136
[VIIIc]	498 ±200
[VIIId]	200
[VIIIe]	475 ±152
[15a]	>1000
[15c]	>1000
[15d]	>1000
[15e]	>1000
[15f]	>1000

Values are from average of two or three independent experiments. *From Sechi *et al*., 2004.

the above-mentioned compounds, we built compounds **[IId]** and **[VIa]** in the keto-enolic tautomeric form. As the carboxylic group of **[IId]** has a pKa of ~4 (Kees *et al.*, 1995) under physiological conditions, we used its deprotonated form for docking studies. These compounds were subsequently subjected to quantum mechanics calculations.

The results of clustered docking runs with the most favourable free binding energy for **[IId]** and **[VIa]** are given in Table 2. Graphical representations of top-ranking binding modes obtained for these ligands showing the important residues involved in binding are depicted in Figure 10. Compound **[IId]** was found to bind to D64 through the hydroxy group in both clusters, and it also established a coordination bond with Mg^{2+} by a combination of hydroxy and carboxylate groups. As shown in Figure 10, superimposition of the most energetically favourable conformers **[IId_{1-2}]** indicated the same bound conformation inside the active site. The triketo ester **[VIa]** formed H-bonds with D64 and established a coordination bond with Mg^{2+} ion through an hydroxy-keto system of the triketo moiety. Also of interest, the top and second conformers considerably overlap **[VId_{1-2}]** with regard to direction, but have the opposite orientation (Figure 10). However, they displayed the same mode of binding and adopted a similar position on the IN active site.

On the basis of these results, the inhibitory potency of compounds **[IId]** and **[VIa]** can be explained by their unique arrangement in the IN active site. In particular, the interactions with Mg^{2+} cation and D64 are important functions for the activity of IN (Ellison *et al.*, 1994; Kulkosky *et al.*, 1992; Hazuda *et al.*, 1997; Chiu *et al.*, 2004), represent the most important features.

Finally, the estimated free binding energy values (ΔG_{bind}) of the docked positions, expressed in kcal/mol, indicated favourable interactions and tight binding with key aminoacid residues on the active site of IN. In particular **[IId]** displayed better energy results than **[VIa],** which agree with their respective potency against IN.

Conclusions

The results of this study suggest that both the cyanoketo acid and the triketo ester moieties are important structural features required for the anti-HIV-1 IN activity. From the

Table 2. First (**[IId_1]** and **[VIa_1]**) and second (**[IId_2]** and **[VIa_2]**) docking results of 50 independent runs for compounds **[IId]** and **[VIa]**

Ligand	*N_{tot}	†f_{occ}	‡DG_{bind}	H-bonds
[IId_1]	8	15/10	–10.77	D64
[IId_2]	6	15/10	–10.08	D64, N155
[VIa_1]	16	10/6	–6.03	D64, C65, N120
[VIa_2]	10	10/6	–6.00	D64, C65, S119, N120

*Total number of clusters. †Number of distinct conformational clusters found out of 50 runs / number of multi-member conformational clusters. ‡Estimated free binding energy (kcal/mol).

structural point of view, the triketo ester fragment of compound **[VIa]** may be considered formally an homologue of the diketo acid functionality, while the cyanoketo acid side chain of compound **[IId]** constitutes a novel potential pharmacophore and a bioisostere of the diketo acid functionality. Chelation of metal co-factors have been implicated for diketo acids-based inhibitors (Grobler *et al.*, 2002; Pais *et al.*, 2002; Long *et al.*, 2004; Marchand *et al.*, 2003; Maurin *et al.*, 2003) and proposed for some other related classes of inhibitors (Neamati *et al.*, 1998, 2001, 2002; Zhao *et.*, 1997, Ouali *et al.*, 2000). In this context the lack of activity demonstrated by the isoxazole derivatives supports these assumptions.

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