Synthesis and evaluation of potential CT (computer tomography) contrast agents for bone structure and microdamage analysis†

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The design and synthesis of several novel X-ray contrast agents **1**–**3**, developed for targeting bone structures, and in particularly microcracks in bones, using CT (Computer Tomography) detection is described. These contrast agents are based on the use of the well known triiodobenzene platform, which was conjugated into one or more phenyliminodiacetate moieties, which can be used to 'lock' onto bone matrices. Compounds **1**–**3** were all tested for their ability to visualise cracks in bone structures (bovine bones) using μ-CT imaging.

Introduction

In medical diagnosis, contrast can be used to distinguish an organ or part of the body, *via* opacification or visualisation, thus providing important structural information. Despite the fact that Magnetic Resonance Imaging (MRI) has revolutionized medical imaging in recent times, X-ray based imaging is still one of the most employed medical diagnoses, with over 80% of all diagnostic imaging today being based on such radiation.[1] Contrast agents are essential parts of radiology and are routinely used in modern medicine.[2] As in the case of MRI,[3] contrast agents designed to target particular organs, tissues, *etc.*, can play a major role in X-ray based imaging.[4] Currently, two types of X-ray contrast agents are generally employed in medicine. These are based upon the use of barium sulfate suspensions (predominantly used for gastrointestinal tract imaging) and iodinated organic compounds.[1,5,6] At present, most available organic X-ray contrast agents are based on the use of single triiodobenzene derivatives or where two such aromatic rings have been connected to each other by a covalent spacer.[6,7] The introduction of an *N*-acetoxy or *N*-methylcarbamyl functionality into the triiodobenzene structure has been shown to greatly improve both the water solubility and biological safety of such molecules. Such modifications also allow for the introduction of other functionalities for achieving more targeted diagnostics, for instace, of particular organs, tissues, *etc*. Examples of such modifications have been carried out by Ranganathan and co-workers,[8] Böhle *et al.*[9] and Rongved *et al.*[10] who have developed water-soluble, biocompatible X-ray contrast agents for use in the vascular, gastrointestinal and nervous systems. However, to the best of our knowledge, no X-ray targeting contrast agents have been made specifically to characterize bone structures and bone damage, although recently such MRI contrast agents have been developed.[11]

Bone is continuously renewing and repairing itself from the ravages of time and injury. As bone is under constant stress due to the repetitive loading during normal day-to-day activity, this results in the generation of microcracks or microdamage. The problem with the imaging of bones is their inherent contrast due to their crystalline hydroxyapaptite and organic matrix.[12] Thus it is difficult to distinguish the contrast agent from the surrounding bone matrix. One way of approaching this problem is to increase the degree of opacity at the damaged site.[13] We are interested in the targeting of biologically important molecules using various spectroscopic methods[14] and we have recently achieved such bone targeting by using fluorescent molecules that have binding sites (chelators) for ions such as Ca(II) or Mg(II).[15] However, to date, the analysis of such bone structures has not been achieved using either iodine contrast agents or dual based iodine-fluorescent contrast agents. Consequently, we set out to develop such contrast agents (Fig. 1). Our objective was to synthesize organic-iodine based conjugates that could 'lock' onto bone cracks or microdamaged sites. To achieve this, such a structure would need to possess the specific ability to penetrate the bone matrix and incorporate into the free lattice, where the weak ionic interactions with the lattice can provide additional modes of binding. Hence, such contrast agents would need to have a receptor moiety conjugated into the iodine moiety with the aim of maximising the interactions with ions such as Ca(II) that are exposed on the surfaces on the damaged sites. Such structural designs could thus provide a non-invasive method of detecting and quantifying microdamage in patients. Herein, we describe the synthesis of three such first-generation ion-receptor based contrast agents **1**–**3** which are based on the use of one or two triiodobenzene moieties within a single structure and the analysis of these to detect microcracks in bovine bones using micro computer tomography (μCT) imaging.[16]

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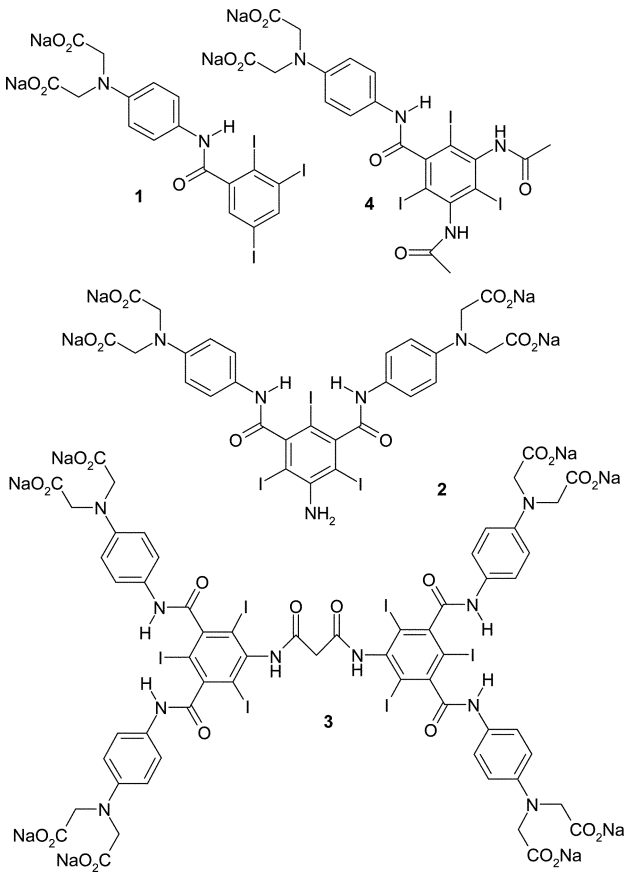


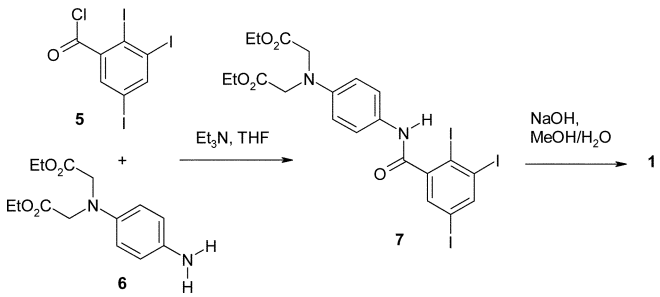
Fig. 1 Compounds **1**–**4**.

Results and discussion

Design and synthesis of 1–4

Compounds **1**–**4** shown in Fig. 1, are all based on the use of phenyliminodiacetate as an ion chelator linked *via* an amide linkage to a triiodo benzene skeleton.[17] We choose the phenyliminodiacetate moiety as a potential ion-receptor or chelator for Ca(II) and or Mg(II). Here the aim was that this simple receptor would lock or bind to these ions within the cracks of the bone structure, which would then allow the use of CT for the selective imaging of the ion-bound contrast agents.‡ As the objective of the current study was the 'proof of principle' of selective CT imaging of such bones, we chose to use bovine bones for our imaging studies (see later). This would then allow us to wash off any excess contrast agents (non-bound) prior to the CT imaging, allowing us to image only those agents that had bound to exposed damaged bone such as in scratches or microcracks.

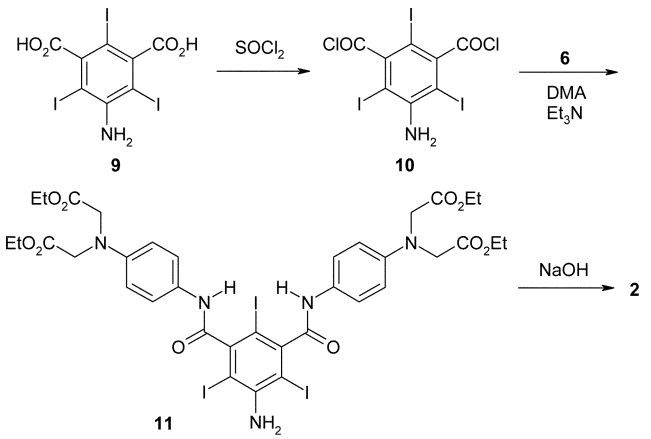
Several types of contrast agent were developed for this study, simply by varying the combination/numbers of both the ion-receptors and iodinated phenyl rings. In molecules **1** and **4**, a single phenyliminodiacetate unit was attached to the iodinated aromatic ring, *via* an amide spacer, while **2** has two such ion chelator units attached *meta* to each other. The dimer **3** contains, however, six iodine atoms and four chelator units in *meta* configuration, and as such would be expected to have the highest binding affinity as well as giving rise to the most efficient imaging. Moreover, as their sodium salts, these contrast agents would be expected to be highly water-soluble.[1] These compounds also have incorporated solubilising groups in the form of the amides that will render them safer and less viscous.[7b]



Scheme 1 Synthesis of **1** from **5** and **6**.

As discussed above, all of the four compounds are based on a triiodo moiety connected to the phenyliminodiacetate (made as the corresponding diethyl ester). Consequently, all of these were synthesized by making the phenyliminodiacetate moiety and connecting that into the relative carboxylic acid derivative of the triiodo compounds. As a starting point for this investigation, we synthesized **1** first as shown in Scheme 1. This involved the coupling of 2,3,5-triiodobenzoyl chloride **5**, which was synthesized from the commercially available carboxylic acid using thionyl chloride in 85% yield after recrystallization from a chloroform–hexane (1 : 1, v/v) mixture. The phenyliminodiacetate ethyl ester **6** was formed in four steps from aniline, involving the dialkylation using ethyl bromoacetate in the presence of K_2HPO_4 and in refluxing CH_3CN which gave the desired product in 90% yield. This was followed by nitration using 70% nitric acid (15 min) followed by recrystallization of the crude product from 2-propanol which gave **8** (not shown) in 76% yield. Hydrogenation on **8** using 10% Pd/C proceeded smoothly to give **6** in almost quantitative yields. Condensation of **5** with **6** was achieved in THF using Et_3N and resulted in the formation of the desired diester **7**, in 80% yield after purification by silica flash column chromatography (using 2 : 5 ethyl acetate–hexane as eluant). The alkaline hydrolysis (NaOH) of **7** to give **1** was achieved in MeOH–H_2O (10 : 1). The purification of **1** involved the evaporation of the solvent mixture to dryness, and the resulting residue was dissolved in a minimal amount of distilled water, followed by dilution with methanol. The resulting mixture was then heated at reflux in the presence activated charcoal, followed by cooling to room temperature. After standing at room temperature for a further two days, it was filtered and evaporated to dryness, diluted with a minimal amount of water and added dropwise to a stirred solution of ethanol to precipitate **1**. This purification method removed all of the coloured impurities and resulted in the isolation of **1** in 92% yield (see ^{1}H NMR of **1** in ESI†).

‡ For the future development of this technique, it is important to account for the fact that the concentrations of these ions within the bones would be significantly greater than found in both extra-and intra-cellular environments. Consequently, at the same time as the receptors should be Ca(II) selective, they would also need to have a low affinity for these ions to prevent any interference from background signals arising from the binding of these agents to free Ca(II) in blood or in cells. Because of this we chose to use the simple phenyliminodiaetate moiety as a receptor as it would hopefully fulfil these criteria.

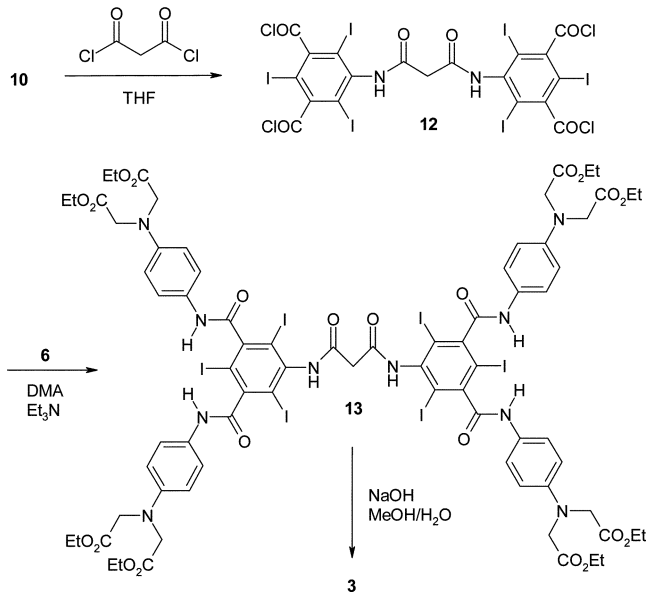


Scheme 2 Synthesis of **2** from **6** and **10**.

The contrast agent **2** was based on similar design strategy as developed for **1** and is outlined in Scheme 2. We predicted that by using two phenyliminodiacetate moieties in a *meta* configuration to each other, this could possibly result in an increased ionic interaction and stronger binding at the unsaturated lattice sites in damaged bone. Furthermore, the primary aromatic amine in **2** was introduced as an extra functionality with the aim of attaching a fluorescent tag such as fluorescein at this position. This would result in the formation of a dual-functionalised molecule consisting of an iodinated contrast agent and a histological stain.

The synthesis of **2** was achieved by firstly converting the commercially available 5-aminoisophthalic acid into the desired three iodine derivative **9** using a modified synthetic method developed by Larsen *et al.* using $KICl_2$–H_2O, generated *in situ* by combining potassium chloride (KCl) and iodine monochloride (ICl).[18] The purification of **9** was achieved by firstly converting the dicarboxylic acid to its potassium salt using 1 M KOH. The resulting solution was then decolourised with activated charcoal by heating at 70–80 °C for 30 min, followed by filtration. The solution was then acidified using conc. HCl to give **9** in 75% yield. The conversion of **9** to the corresponding bis-acid chloride **10** was accomplished in 85% yield using freshly distilled thionyl chloride.[19] The condensation of **10** and **6** in DMA, using Et_3N, resulted in the formation of **11** in 80%, after recrystallization from ethanol. Alkaline hydrolysis of **11** resulted in an almost quantitative yield of **2**, which was purified in a similar manner as described for **1** (see ESI for ^{1}H NMR of **2**†).

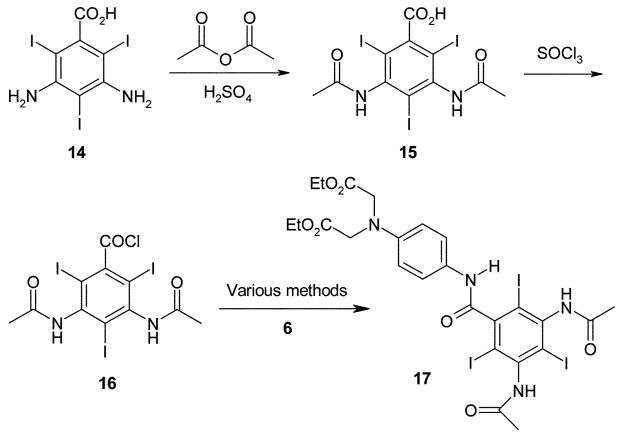
The synthesis of the hexa-iodo derivative **3** is illustrated in Scheme 3. The synthesis involved the use of the bis-acid chloride **10** synthesized above, which was first reacted with malonyl dichloride using an established literature method. This involved the refluxing of the two reagents in anhydrous THF, giving the desired product **12** in 75% yield.[19] The steric crowding of the bulky iodine atoms hindered the polymerisation of **10** and **12** was therefore obtained as the major single product. The condensation of **12** with 5 equiv. of **6** in DMA at 30 °C for 24 h in the presence of Et_3N resulted in the formation of the octa-ester **13** in 80% yield after recrystallization from ethanol. The subsequent basic (NaOH) hydrolysis of **13** in MeOH–H_2O afforded the target molecule **3** which was purified in a similar manner to that described for **1** above in 92% yield (see ESI for ^{1}H NMR of **3**†).



Scheme 3 Synthesis of compound **3**.

Hexa-substituted benzene compounds such as those synthesized above have been studied extensively for the phenomenon of conformational equilibrium analysis. Bradmante and Vittadini have carried out such comprehensive NMR studies on the steric effects of highly crowded molecules based on the 1,3,5-triiodobenzene platform, which were found to give rise to atropisomerism.[20] As in their cases, all of the positions in **1**–**3** are substituted and hence would thus also be expected to be sterically congested and lead to atropisomerism due to the lack of rotation around the Ar–CO, the Aryl–N and the CO–N bond of the isophthalimide moieties, and the N–CO bonds of the amide moieties. However, in **1**–**3** the ^{13}C NMR and ^{1}H NMR exhibited simple splitting patterns indicating that all the carbonyl functionalities were equivalent. Moreover, the aromatic and methylene protons of the phenyliminodiacetate chelators were found to be equivalent. The corresponding esters also exhibited similar spectral patterns, indicating that all these molecules existed as single isomers. The probable explanation for this could be the effect of stabilisation of one isomer induced by extended conjugation. Similar observations were made in natural products with extended conjugation such as biphyscion.[21]

The final compound in this study, **4** (see Fig. 1), was designed to represent a simple amide analogue of **1**, based on the substitution pattern employed in **2** and **3**. The attempted synthesis is shown in Scheme 4, which involved the iodination of 2,5-diaminobenzoic acid using $KICl_2$–H_2O followed by purification as described above for **9** to give **14** in 65% yield.[22] The acetylation of the **14** was achieved in 90% yield to give **15** using acetic anhydride in the presence of a small amount of conc. H_2SO_4.[23] The chlorination of **15** to give the acid chloride **16** was achieved in a yield of 71% by refluxing **15** in freshly distilled $SOCl_2$ for 3 h. The final step involved the formation of **17** from the condensation of **16** with **6**. As before, this condensation was first attempted in DMA and Et_3N, but the reaction was unsuccessful. Alternate reactions such as the use of HMPT and Et_3N; DMF and Et_3N, and THF and Et_3N were attempted but on all occasions the desired ester **17** was not formed. Because of this we did not achieve the synthesis of **4** as planned.



Scheme 4 Attempted synthesis of **17** from **6** and **16**.

Evaluation of contrast agents 1–3 in solid (powder) form and in solution using μ-CT

Upon computer tomography imaging using μ-CT, compounds **1**–**3** synthesized above were all able to provide good contrast from the surrounding bone matrix as powdered substances.[24] A two-dimensional sagittal reconstruction was performed in order to evaluate the contrast ability of the agents, in comparison with the surrounding bone matrix (see Experimental for further details). The first of those compounds to be tested was contrast agent **1**, which was applied to the right-hand side of the bone specimen as shown in the red box in the sagittal reconstruction in Fig. 2A. For comparison the left-hand side of the bone, within the blue box has no contrast agent applied. Both the bone and **1** gave an image that is predominantly white. However, areas where **1** has been applied appear to be more bright due to its higher X-ray absorption power, and hence it is distinct from the bone surface. A three-dimensional transparent section was also reconstructed to further illustrate the contrast ability of **1** (Fig. 2B). Here, it is clearly possible to distinguish the internal architecture of the bone in the transparent reconstructed section, as well as Haversian and Volkmann's canals and the resorption cavities due to the contrast provided by **1**. In a similar way, compounds **2** and **3** were tested under identical conditions. Similar results where obtained to those seen in Fig. 2. However, the reconstructed two-dimensional sagittal and transparent three-dimensional images clearly show that these contrast agents were able to absorb the X-rays more effectively than the bone, thus providing better contrast from the bone matrix. This is not unexpected as both **2** and **3** have a higher iodine content and as such should give rise to stronger imaging.

After performing qualitative studies with these contrast agents in powdered form, the next stage was to label bone scratches with 10^{-3} M aqueous solutions of the contrast agents **1**–**3**, using bovine tibiae, and image the resulting bone specimens using μ-CT. The preliminary μ-CT studies described below were performed in a similar manner as before. Even though the bone specimen labelled with compound **1** indicated that the scratch was visible, the background contrast for both the scratch depth and the surrounding bone matrix almost overlapped with that observed form **1** (Fig. 3). Consequently, it was difficult to differentiate between bone and contrast agent. Similar effects were observed for **2** and **3**. The most likely reason for this may be from the beam hardening effect, which masks the imaging from the agents.§ Nevertheless, these results clearly indicate that our agents can bind to bone cracks (microcracks) and as such the results obtained from **1**–**3** are important for the future development of bone-targeting CT imaging agents. We are currently working towards achieving that objective.

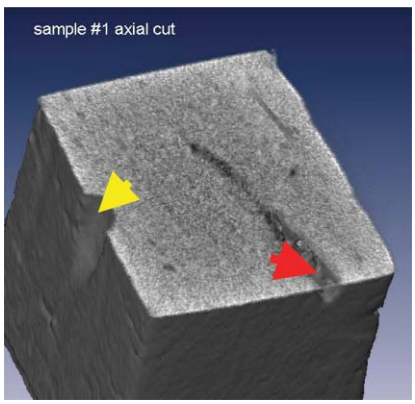


Fig. 3 Three-dimensional axial reconstruction of the bone immersed in an aqueous solution of **1** (1 mM). A scratch can be seen on the left-hand side (yellow arrow). The top of the axial plane shows a vessel canal (red arrow) within the bone tissue.[12a]

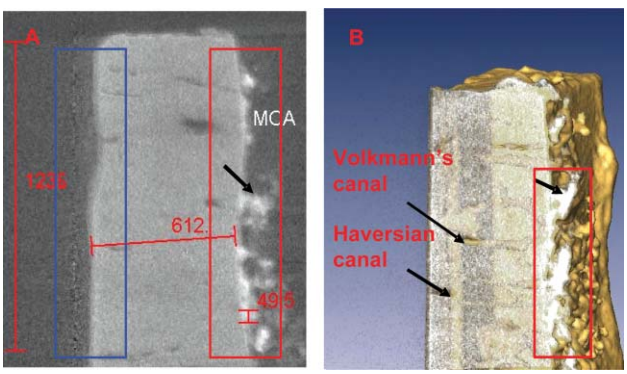


Fig. 2 Computer tomography images of **1**: (A) two-dimensional and (B) three-dimensional sagittal sections of a cortical bone specimen labelled with contrast agent **1**. The white powder on the bone edges is marked by a black arrow. Bars are in μm.

Conclusion

We have demonstrated the synthesis of several new bone-targeting contrast agents for CT imaging based on the use of the triiodobenzene framework, and we have carried out a preliminary evaluation of these to quantify microdamage in bone. All three contrast agents exhibited good water solubility and stability. Preliminary imaging studies of these contrast agents on bovine cortical bone specimens, using cone-beam μ-CT, demonstrated that agents in powder form are able to provide good contrast from the surrounding bone

§ Such beam hardening arises from X-ray photons striking the bone surface at different energy so that the photons of lower energy are absorbed with a higher probability than those of a higher energy. As a result, the attenuation of the X-ray is not homogenous and is dependent upon the energy of the radiation, the type of object used in the imaging and the direction of projection.

matrix. Solution phase studies of these contrast agents was, however, not as successful, possibly due to a beam hardening effect. We are currently working towards synthesising related structures of **1**–**3** with the aim of achieving such imaging in solution. We are currently modifying the structure of **2** with the aim of achieving dual CT and fluorescence analysis of bone structures.

Experimental

General

Reagents (obtained from Aldrich) and solvents were purified using standard techniques. Solvents were dried over the appropriate drying agent before use using standard procedures. Melting points were determined using a GallenKamp melting point apparatus. Infrared spectra were recorded on a Mattson Genesis II FTIR spectrophotometer equipped with a Gateway 2000 4DX2-66 workstation. Oils were analysed using NaCl plates; solid samples were dispersed in KBr and recorded as clear pressed discs. ^{1}H NMR spectra were recorded at 400 MHz using a Bruker Spectrospin DPX-400 instrument. ^{13}C NMR were recorded at 100 MHz using a Bruker Spectrospin DPX-400 instrument.

Materials and methods for CT imaging

The soft tissue was removed from all bones and they were stored at −20 °C until required. Longitudinal sections of cortical bones with dimensions of 2 × 2 mm from the mid-diaphysis were cut into 1 cm beams using a band saw and polished using emery paper (grade 400). All machining was carried out in wet conditions and the bones were not allowed to dry at any time. After machining the specimens were stored at −20 °C prior to CT imaging. The sample was mounted on the examination table and rotated 180° and a total of 360 projections were recorded. At a 5 s exposure time for each projection the entire scanning lasted 1 h. The data stored in the computer were analysed and reconstructed using the Amira software. The reconstruction lasted approximately 1.5 h. By manipulating 3D data-sets, it was possible to observe the canals, osteons and resorption cavities in the bones. The smallest volume element (voxel) achieved was 4 μm and the images were reconstructed using a Shep-Logan filtered modified Feldkamp algorithm. The X-ray absorption value for each and every point in space was reconstructed. The different absorption values (CT number or Hounsfield value) resulted in a two- or three-dimensional image of the scanned object. Reconstruction of images along sagittal (a two-dimensional plane going from the left to the right through the specimen), coronal (a two-dimensional plane going from the front to the back of the specimen) and transaxial planes (a two-dimensional plane going from the top to the bottom of the specimen) were made possible using Amira visualisation software. The two- and three-dimensional representation of the image is given in terms of pixel and voxel respectively.

Synthesis

[Ethoxycarbonylmethyl-(4-nitrophenyl)amino]acetic acid ethyl ester (8). [Ethoxycarbonylmethyl-(phenylamino)]acetic acid ethyl ester (6.00 g, 22.72 mmol) was taken into 50 ml of acetic acid and stirred at 0 °C. To this, 3 mL of HNO_3 was added. After 15 min, the reaction mixture was poured over ice, filtered and the resulting solid recrystallized from ethanol. This gave **8** as yellow brown needles in 76% yield. Mp = 160–162 °C MS (ES^+) m/z = 310 (M^+). Anal. calc. for $C_{14}H_{18}N_2O_6$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.12; H, 5.85; N, 8.98%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30 (t, 6H, J = 7.0 Hz, $NHCH_2CO_2CH_2CH_3$), 4.21 (s, 4H, $NHCH_2CO_2CH_2CH_3$), 4.27 (q, 4H, J = 7.0 Hz, $NHCH_2CO_2CH_2CH_3$), 6.6 (d, 2H, J = 9.5 Hz, Ar–*H*), 8.13 (d, 2H, J = 9.5 Hz, Ar–*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.74, 53.00, 61.29, 110.82, 125.55, 138.52, 152.23, 168.86. IR (ν_{max}, KBr, cm^{-1}): 3474, 3121, 3098, 2976, 2908, 2696, 2614, 2426, 2231, 1918, 1893, 1751, 1591, 1516, 1420, 1272, 1117, 1026, 961, 918, 871, 828, 757, 736, 696, 632, 586, 535, 559.72.

[(4-Aminophenyl)ethoxycarbonylmethylamino]acetic acid ethyl ester (6). A solution of **8** (1.0 g, 3.22 mmol) in methanol (50 mL) was hydrogenated at 1 atm for 15 min using Pd–C (10% w/w, 0.07 g). After completion the catalyst was filtered off and the solvent removed under reduced pressure to yield **9** (0.85 g, 95%) as a brown liquid. MS (ES^+) m/z = 281 $(MH)^+$. Anal. calc. for $C_{14}H_{20}N_2O_4$: C, 59.99; H, 7.19; N, 9.99. Found: C, 59.25; H, 7.03; N, 9.84%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (t, 6H, J = 6.8 Hz, $NCH_2CO_2CH_2CH_3$), 4.09 (s, 4H, $NCH_2CO_2CH_2CH_3$), 4.18–4.23 (q, 4H, J = 6.8 Hz, $NCH_2CO_2CH_2CH_3$), 6.57 (d, 2H, J = 8.9 Hz, Ar–*H*), 6.66 (d, 2H, J = 8.9 Hz, Ar–*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.88, 140.94, 138.02, 116.26, 114.54, 60.49, 53.68, 13.79. IR (ν_{max}, NaCl, cm^{-1}): 3330, 2981, 2930, 2355, 1733, 1616, 1519, 1448, 1412, 1347, 1255, 1188, 1097, 1025, 974, 918, 817, 729, 521.

Ethoxycarbonylmethyl-[4-(2,3,5-triiodobenzoylamino)phenyl]-amino}acetic acid ethyl ester (7). 2,3,5-Triodobenzoyl chloride, **5** (0.5 g, 0.96 mmol), was dissolved in anhydrous THF (20 mL). To this solution was added **6** (0.27 g, 0.96 mmol) in 10 mL of THF and freshly distilled Et_3N (0.15 g, 0.20 mL, 1.2 mmol). The resulting solution was kept at reflux for 24 h, after which the THF was removed under reduced pressure and the resulting residue extracted several times with chloroform. The combined organic layers were washed with HCl (1 M), $NaHCO_3$ (1 M) and brine, dried over $MgSO_4$ and the solvent removed under reduced pressure to give a white solid which was further purified using flash-column chromatography (chloroform). This gave **7** as a white solid in 90% yield (0.67 g). Mp = 174–176 °C. MS (ES^+) m/z = 762 $(MH)^+$. Anal. calc. for $C_{21}H_{21}I_3N_2O_5$: C, 33.10; H, 2.78; N, 3.68. Found: C, 33.40; H, 2.81; N, 3.51%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.20 (t, 6H, J = 7.0 Hz, $NHCH_2CO_2CH_2CH_3$), 4.12 (q, 4H, J = 7.0 Hz, $NHCH_2CO_2CH_2CH_3$), 4.18 (s, 4H, $NHCH_2CO_2CH_2CH_3$) 7.44 (d, 2H, J = 9.0 Hz, Ar–*H*), 7.69 (d, 2H, J = 9.0 Hz, Ar–*H*), 8.31 (d, 1H, J = 2.0 Hz, Ar–*H*), 10.19 (s, 1H, CON*H*). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.52, 165.73, 147.51, 146.06, 144.55, 134.50, 129.19, 121.02, 111.96, 113.30, 107.94, 95.51, 60.39, 52.81, 14.14. IR (ν_{max}, KBr, cm^{-1}): 3261, 2977, 1638, 1595, 1520, 1410, 1324, 1261, 1186, 1024, 974, 910, 866, 816, 765, 716, 567, 513.

Ethoxycarbonylmethyl-[4-(2,3,5-triiodobenzoylamino)phenyl]-amino}acetic acid sodium salt (1). Compound **7** (1.5 g, 1.97 mmol), was dissolved in methanol (20 mL). To this was added NaOH (2 mL, 3 M) and the resulting solution refluxed for 2 h. Addition of ethanol resulted in the precipitation of a white solid, which was collected by filtration and dried under vacuum to afford

1 (1.4 g, 95%) as a yellow solid. MS (ES^+) $m/z = 767$ ($M + H_2O$). Anal. calc. for $C_{17}H_{11}I_3N_2Na_2O_5 \cdot H_2O$: C, 26.59; H, 1.71; N, 3.65. Found: C, 26.67; H, 1.52; N, 3.48%. ^1H NMR (400 MHz, $CDCl_3$): δ 3.82 (s, 4H, NCH_2CO_2Na), 6.46 (d, 2H, $J = 8.9$ Hz, Ar–*H*), 7.2 (d, 2H, $J = 8.9$ Hz, Ar–*H*), 7.68 (s, 1H, Ar–*H*), 8.32 (s, 1H, CON*H*). ^{13}C NMR (100 MHz, D_2O): δ 178.89, 168.66, 147.35, 146.65, 144.43, 134.40, 124.36, 123.56, 118.86, 112.24, 105.92, 94.00, 55.22. IR (ν_{max}, KBr, cm^{-1}): 3587, 3379, 3099, 1690, 1520, 1477, 1418, 1319, 1269, 1247, 1171, 1125, 1098, 975, 894, 865, 819, 729, 703, 612, 525.

5-Amino-2,4,6-triiodoisophthalic acid (9). ICl (9.8 g, 100 mmol) was added to a solution of KCl (7.2 g 9.70 mmol) in H_2O (100 mL) and stirred at room temperature for 5 min. The mixture was filtered to remove any insoluble materials. The resulting orange solution (of $KICl_2$) was added dropwise over 1 h to a solution of 5-aminoisophthalic acid (3.32 g. 1.80 mmol) in H_2O (110 mL) at 55° C. After complete addition the mixture was stirred at 60 °C for a further 24 h and finally at 90 °C for 1 h. On cooling to room temperature, the resulting solid was dissolved in 20 ml of 1 M KOH and treated four times with activated charcoal at 40–50 °C for 30 min. The resulting product was then precipitated using conc. HCl, filtered and dried at 100 °C under vacuum. This gave **9** as a yellow solid in 75% yield (3.68 g). Mp = 260 °C (decomp.). MS (ES^+), $m/z = 559$ ($M + H^+$). Anal. calc. for $C_8H_4I_3NO_4 \cdot H_2O$: C, 16.66; H, 1.05; N, 2.43. Found: C, 16.86; H, 0.78; N, 2.16%. ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.11, 148.91, 147.78, 77.93, 70.62. IR (ν_{max}, KBr, cm^{-1}): 3629, 3442, 3378, 3305, 2894, 2600, 1785, 1600, 1367, 1290, 1009, 987, 867, 765.

4-Amino-*N*,*N*-bis(2,4,6-triiodo) bis-phenyliminoacetic acid diethyl ester (11). 5-Amino-2,4,6-triiodoisophthaloyl dichloride **10** (1.5 g, 2.5 mmol) was dissolved with stirring in anhydrous dimethylacetamide (35 mL) under argon. To this, **6** (1.43 g, 5.11 mmol) was added in 15 ml of dimethylacetamide, *via* a syringe, and freshly distilled Et_3N (0.51 g, 0.68 mL, 2.4 mmol). The resulting solution was stirred at room temperature for 24 h, after which the solvent was removed by vacuum distillation and the resulting residue extracted three times with chloroform. The combined organic layers were washed with HCl (1 M), $NaHCO_3$ (1 M) and brine, dried over $MgSO_4$ and finally the solvent was removed under reduced pressure to give a white solid which was further purified using flash-column chromatography (chloroform) and finally recrystallized from ethanol to give **11** as a white solid (2.3 g) in 85% yield. Mp = 187–189 °C. MS (ES^+) $m/z = 1083$ ($M + H$)$^+$. Anal. calc. for $C_{36}H_{40}I_3N_5O_{10} \cdot 2C_2H_5OH$: C, 40.87; H, 4.46; N, 5.96. Found: C, 40.83; H, 4.13; N, 6.46%. ^1H NMR (400 MHz, DMSO-d_6): δ 1.20 (t, 24H, $J = 6.5$ Hz), 4.14 (q, 16H, $J = 6.5$ Hz), 4.20 (s, 4H, 16H) 6.56 (d, 8H, $J = 7.5$ Hz), 7.69 (m, 8H), 10.27–10.43 (m, 6H, –NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.53, 165.73, 147.51, 146.06, 144.55, 14.14, 134.50, 129.19, 121.02, 113.30, 111.96, 107.94, 95.51, 60.39, 52.81, 14.14. IR (ν_{max}, KBr, cm^{-1}): 3233, 2962, 1745, 1640, 1590, 1066, 1024, 976, 815, 730, 645.

4-Amino-*N*,*N*-bis(2,4,6-triiodo) bis-phenyliminoacetic acid sodium salt (2). Compound **11** was dissolved in methanol (20 mL). To this solution was added NaOH (2 ml, 6 M) and the resulting solution refluxed for 3 h, after which the solution was concentrated and ethanol added. The resulting precipitate was collected by filtration and dried under vacuum to afford **2** as a white solid (2.27 g) in 93% yield. MS (ES^+) $m/z = 1106$ ($M + Na$)$^+$. Anal. calc. for $C_{28}H_{20}I_3N_5Na_4O_{10} \cdot 6H_2O$: C, 28.81; H, 2.76; N, 6.00. Found: C, 28.11; H, 2.22; N, 5.6%. ^1H NMR (400 MHz, D_2O): δ 3.83 (s, 8H, NCH_2CO_2Na), 6.5 (d, 2H, $J = 9.0$ Hz, Ar–*H*), 7.3 (d, 2H, $J = 9.0$ Hz, Ar–*H*). ^{13}C NMR (100 MHz, D_2O): δ 178.95, 170.83, 170.77, 147.96, 147.25, 147.21, 146.87, 123.72, 123.55, 111.17, 79.53, 72.56, 55.18. IR (ν_{max}, KBr, cm^{-1}): 3548, 3279, 3199, 1677, 1620, 1577, 1518, 1319, 1209, 1107, 1001, 975, 864, 805, 729, 713, 610, 565.

***N*,*N*-Bis(2,4,6-triiodo-3,5-benzenedichlorocarbonyl)malonamide tetraphenyliminodiethyl diester (13).** Compound **12** (1.09 g, 0.86 mmol) was dissolved under argon in anhydrous dimethylacetamide (20 mL). To this solution **6** (1.052 g, 3.75 mmol) was added in 15 mL of dimethylacetamide, *via* a syringe, and freshly distilled Et_3N (0.38 g, 0.51 mL, 3.75 mmol). The resulting solution was stirred at room temperature for 24 h, after which the solvent was removed by vacuum distillation. The resulting residue was extracted three times with chloroform, and the combined organic layer was washed with HCl (1 M), $NaHCO_3$ (1 M) and brine. The organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure to give a white solid which was further purified by recrystallization from ethanol. This gave **13** as a white solid (1.19 g) in 80% yield. MS (ES^+) m/z 1117 ($M^+/2$). Anal. calc. for $C_{75}H_{80}I_6N_{10}O_{22}$: C, 40.31; H, 3.61; N, 6.27. Found: C, 40.78; H, 3.98; N, 6.41%. ^1H NMR (400 MHz, DMSO-d_6): δ 1.20 (t, 24H, $J = 7.0$ Hz, $NHCH_2$–$CO_2CH_2CH_3$), 4.12 (q, 8H, $J = 7.0$ Hz, $NHCH_2CO_2CH_2CH_3$), 4.19 (s, 8H, $NHCH_2CO_2CH_2CH_3$), 6.55 (d, 4H, $J = 8.5$ Hz, Ar–*H*), 7.43–7.46 (m, 4H, Ar–*H*), 10.24 (brs, 2H, –CON*H*). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.55, 167.08, 150.06, 144.54, 142.75, 129.28, 129.24, 121.03, 121.06, 112.01, 111.98, 91.04, 60.40, 52.85, 14.15. IR (ν_{max}, KBr, cm^{-1}): 3243, 2982, 1735, 1650, 1530, 1186, 1024, 970, 815.

***N*,*N*-Bis(2,4,6-triiodo-3,5-benzenedichlorocarbonyl)malonamide tetraphenyliminoacetic acid sodium salt (3).** Compound **13** (2.7 g, 1.21 mmol) was dissolved in methanol (20 mL), and to this solution was added NaOH (3 mL, 6 M). The resulting solution was refluxed for 3 h, after which the solution was concentrated and ethanol added. The resulting precipitate was collected by filtration and dried under vacuum, which gave **3** as an off-white solid (2.43 g) in 92% yield. Mp = 400 °C (decomp.). Anal. calc. for $C_{59}H_{40}I_6N_{10}Na_8O_{22} \cdot 8H_2O$: C, 30.41; H, 2.42; N, 6.01. Found: C, 27.55; H, 2.05; N, 5.28. ^1H NMR (400 MHz, D_2O): δ 3.93 (s, 16H, $NHCH_2CO_2Na$), 3.82 (s, 2H, CH_2), 6.49 (d, 8H, $J = 8.5$ Hz, Ar–*H*). ^{13}C NMR (100 MHz, D_2O): δ 178.95, 170.38, 170.29, 147.96, 146.90, 123.81, 123.63, 111.22, 95.31, 56.96, 55.20, 53.94, 16.36. IR (ν_{max}, KBr, cm^{-1}): 3239, 1671, 1518, 1404, 1308, 1183, 976, 909, 820, 610.

2,4,6-Triiodo-3,5-diacetamidobenzoylchloride (15). Compound **14** (6 g, 9.78 mmol) was added to freshly distilled $SOCl_2$ (60 ml) and the mixture was refluxed for 3 h, after which the $SOCl_2$ was removed. The resulting residue was washed three times with hexane, which gave **15** as a yellow solid, which was then filtered to give the desired product in 71% yield (4.48 g). MS (ES^+) $m/z = 654$ (MNa)$^+$. Anal. calc. for $C_{11}H_8ClI_3N_2O_2$: C, 20.89;

H, 1.28; N, 4.43. Found: C, 20.69; H, 0.87; N, 3.80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.04 (s, 6H, NHCOCH_3), 10.09 (s, 1H, –NHCOCH$_3$), 10.16 (s, 1H, NHCOCH$_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.92, 167.70, 149.03, 145.02, 111.02, 95.13, 22.98, 22.9. IR (ν_{max}, KBr, cm^{-1}): 3242, 3007, 2058, 1889, 1675, 1498, 1345, 1299, 1007, 850, 782, 650.

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